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Phenotypic and genotypic characterisation of antimicrobial resistance in *Escherichia coli* indicator of animal, food and human origin.

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Abbreviations

(3-4-5)-GC	(3^{d}-4^{th}-5^{th})-Generation cephalosporin
ABC	ATP binding cassette efflux pump superfamily
AMP	Ampicillin
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
bp	Base pair
BSI	Bloodstream infection
C	Chloramphenicol
CAZ	Ceftazidime
CIA	Critically important antimicrobial
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
CS	Conserved sequence
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECOFF	Epidemiological cut-off
EFT	Ceftiofur
EHEC	Enterohemorrhagic *Escherichia coli*
EMA	European Medicine Agency
ENR	Enrofloxacin
ESBL	Extended spectrum beta-lactamase
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal Pathogenic *Escherichia coli*
FPA	Food-producing animal
HGT	Horizontal gene transfer
HP-CIA	Highest priority critically important antimicrobial
In	Integron
Inc	Incompatibility group

intI1^{+ve}	*intI1* positive strain
intI1^{-ve}	*intI1* negative strain
IPEC	Intestinal Pathogenic *Escherichia coli*
IR	Inverted repeat sequence
IS	Insertion Sequence
MATE	Multidrug and toxic compound extrusion efflux pump family
MDR	Multidrug resistance
MEM	Meropenem
MFS	Major facilitator efflux pump superfamily
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
MS	Member state
NA	Nalidixic acid
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
Pc	Promoter
PCR	Polymerase chain reaction
QAC	Quaternary ammonium compound
R	Resistant
RND	Resistance nodulation division efflux pump family
RTE	Ready-to-eat
S	Streptomycin
SMR	Small multidrug resistance efflux pump family
SNP	Single nucleotide polymorphism
ST	Sequence Type
ST	Sulfisoxazole
STEC	Shiga toxigenic *Escherichia coli*
SXT	Sulfamethozaxole/trimethoprim
TE	Tetracycline
Tn	Transposon
tnp	Transposease

TSA	Tryptone soya agar
UTI	Urinary tract infection
VAG	Virulence associated gene
VGT	Vertical gene transfer
WGS	Whole Genome Sequence/Sequencing
WT	Wild type

1. Introduction

1.1 Antimicrobial resistance: a global One Health Problem

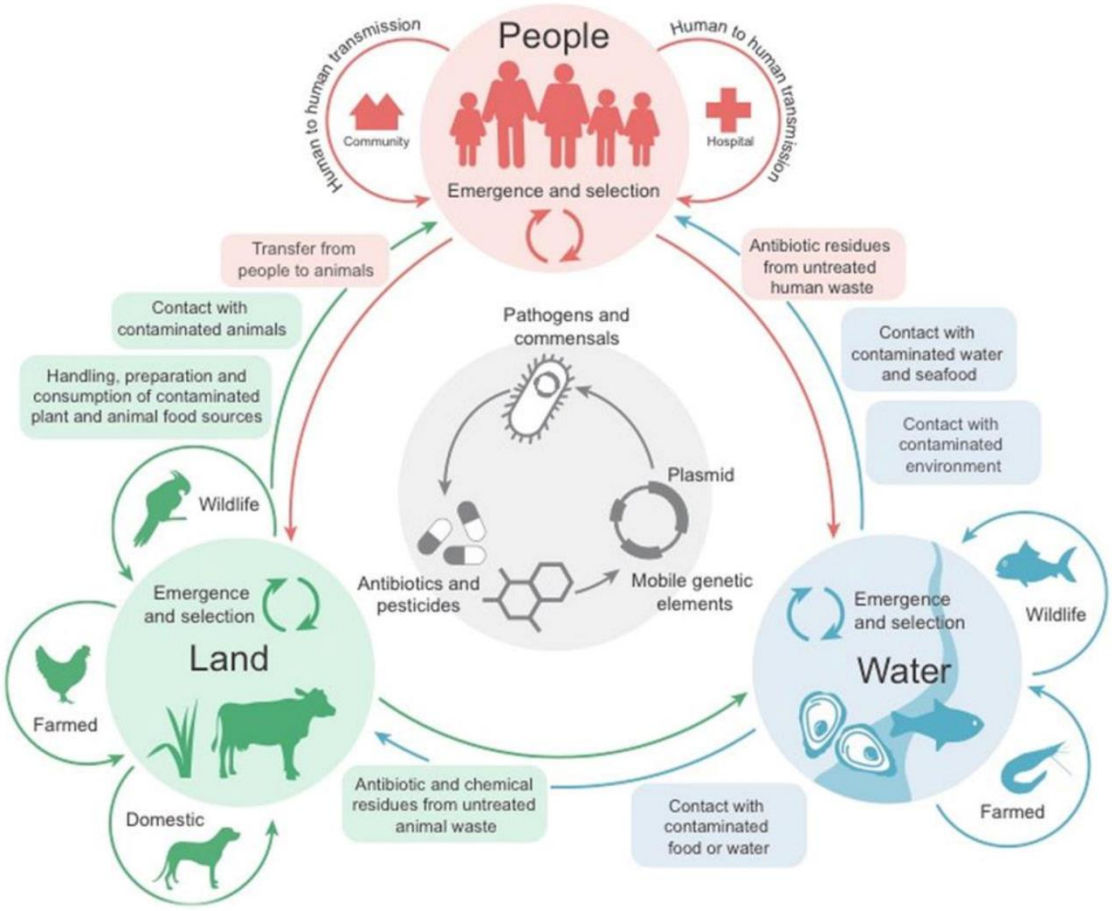
Antimicrobial resistance (AMR) is considered a critical and ever-growing threat to human and animal health (WHO, 2018a). In the last decades we have witnessed a dramatic spread and diffusion of multidrug-resistant (MDR) bacterial pathogens, unsusceptible to various antimicrobial classes. In particular, resistance to critically important antimicrobials (HP-CIAs) (including 3^{d}-4th-5th generation cephalosporins, quinolones, macrolides and polymyxins) and diffusion of extended spectrum beta-lactamases (ESBL) producers is of main concern in human medicine (Roca *et al.*, 2015). MDR bacterial infections are particularly worrisome, leading to an increase in treatment failure and hospitalization. AMR claims 700.000 victims globally every year (EFSA, 2018). Future prospects are even worse, attributing to AMR by 2050 an estimated 10 million deaths per year globally, with an economic cost of US $100 trillion (Tang *et al.*, 2017).

Enterobacteriaceae (especially *Escherichia coli* and *Klebsiella pneumoniae*) represent some of the most important organisms involved in serious human infections in both hospital and community settings (Bortolami *et al.*, 2019). Their frequent and increasing association with ESBL production (McNulty *et al.*, 2018) and carbapenemase resistance (Rodriguez-Bano *et al.*, 2018) is of remarkable concern, limiting therapeutic options and influencing patient outcomes.

1.2 AMR diffusion

Antimicrobial resistance arises from selective pressure induced by antimicrobial treatments in human and agricultural systems and environmental pollution with antimicrobial residues. Overall antimicrobial consumption in the European Union (EU) is mostly associated to food-producing animals (FPAs), playing an essential role in AMR emergence and diffusion (EFSA, 2017a). Antimicrobial use (and related selective pressure) is the primary driver allowing AMR spread. However, resistant bacteria and genes have also been identified in ecosystems with a rare or absent direct exposure to antimicrobials. In particular, AMR has been reported in wildlife in different studies (Guenther *et al.*, 2010a; Guenther *et al.*, 2011; Carroll *et al.*, 2015; Vittecoq *et al.*, 2016; Wang *et al.*,

2017). The geographical distribution of AMR bacteria and genes, also in remote areas, derived from the strict interconnection between different environments, sharing habitats and water sources. In this context, soil and water faecal contamination plays a primary role in AMR diffusion between ecosystems (Guenther *et al.*, 2011; Singer *et al.*, 2016). The link between human, animal and environment turns AMR into a global problem, which needs to be faced with a "One Health" approach.



(https://www.ausgem.net)

Figure 1.1 Schematic representation of environments and different potential pathways involved in AMR bacteria and genes diffusion.

1.3 The importance of AMR monitoring

AMR data collection from different environments is essential to perform epidemiological evaluations, understand the current state of the AMR problem, develop strategies to promote proper use of antimicrobials in order to reduce their consumption (Fuhrmeister and Jones, 2019). The result of these combined actions should lead to a progressive decrease of antimicrobial selective pressure, hence reducing AMR emergence. Subsequently, antimicrobial treatment efficacy should increase, reducing infection severity, therapeutic failures and health care costs.

Globally, different regional AMR surveillance programs and related databases have been implemented (ECDC - European Antimicrobial Resistance Surveillance Network, EARS-Net; Central Asian and Eastern European Surveillance of Antimicrobial Resistance, CAESAR; Rede Latinoamericana de Vigilancia de la Resistencia a los Antimicrobianos, ReLAVRA) (WHO, 2018a).

In Europe, constant AMR monitoring of different zoonotic and AMR indicator bacteria in food-producing animals and related food has been mandatory since 2014. Results are regularly published in the "European Union Summary on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food" by EFSA Authority (EFSA, 2018; EFSA, 2019).

1.4 *E. coli* as AMR barometer

Escherichia coli (*E. coli*) is a rod-shaped, Gram-negative, facultative anaerobic bacterium of the family *Enterobacteriaceae*. Usually, it is a commensal colonizer of gut (especially large intestine) in warm-blooded animals, representing the prevailing aerobic organism in the gastrointestinal microbiota (Tenaillon *et al.*, 2010). However, it could also arise as an important pathogen, showing an incredible variability in virulence traits. Pathogenic *E. coli* could be divided into 2 main groups: intestinal pathogenic *E. coli* (IPEC), responsible for intestinal infections, and extra-intestinal pathogenic *E. coli* (ExPEC), mainly associated to urinary tract infections (UTIs), meningitis and septicaemia (Kaper *et al.*, 2004). *E. coli* is one of the most common causes of bloodstream infections (BSIs) and community/ hospital associated UTIs worldwide (Ecdc, 2018; WHO, 2018b).

Commensal *E. coli* is considered an AMR indicator in Gram-negative bacteria, because of 2 important features, namely its ubiquity and genomic plasticity.

E. coli is a common inhabitant of animal and human intestine, but it also represents a widespread environmentally adapted bacteria, widespread in various ecosystems through faecal contamination (Jang *et al.*, 2017). Its genomic plasticity allows frequent and efficient exchange of different genes (including antimicrobial resistance and virulence ones) with other enteric bacteria and the environment. Hence, AMR data obtained from *E. coli* indicator are considered representative of the overall bacterial population and are important to understand the evolution in AMR phenotypic and genotypic pattern (EFSA, 2019). Commensal *E. coli* has been used as AMR indicator in the European AMR monitoring plan (EFSA, 2016; EFSA, 2017b; EFSA, 2018; EFSA, 2019) and in various other studies (Kaesbohrer *et al.*, 2012; Wasyl *et al.*, 2013; Hanon *et al.*, 2015; Osterberg *et al.*, 2016; Lambrecht *et al.*, 2018; Gay *et al.*, 2019).

1.5 AMR in *E. coli* indicator in Europe.

The rise of AMR is a public health concern that has led to increased interest in understanding the epidemiological aspects related to its emergence and diffusion. However, limited European AMR data in *E. coli* indicator are currently available.

AMR trend in EU-MSs are regularly published only for limited sources (cattle, poultry and pig), included in the European AMR monitoring plan. Instead, AMR knowledge in other niches is often fragmented and seldom. Moreover, most studies focus on AMR phenotypic evaluation. Furthermore, assessment of AMR genetic determinants is mainly performed with PCR and generally lacks the evaluation of the complex AMR genetic structures involved. This trend is particularly evident in Italian investigations.

Hereafter is a brief summary of the main phenotypic and genetic AMR profile identified in animals (livestock, companion animals, wild animals), food and human in *E. coli* indicator in Europe.

1.5.1 Food-producing animals and related food

Livestock represent an important driver of AMR diffusion, which could occur by direct/indirect contact with humans, related food and environmental (land and water) manure contamination (Seiffert *et al.*, 2013; Blaak *et al.*, 2014a; Dahms *et al.*, 2015; Economou and Gousia, 2015; Fletcher, 2015). Moreover, these sources could represent an important AMR reservoir, where foodborne pathogens could pick up new antimicrobial resistance genes (ARGs), hence acquiring new antimicrobial resistant traits (Verraes *et al.*, 2013).

High population size and high demand for meat coming from food-producing animals in the EU Member States (MSs) (http://www.fao.org/faostat/en/, last accessed October 2019) are driving European AMR monitoring, focused on limited AMR reservoirs (cattle, pig, turkey and chicken) for human (EFSA, 2014; EFSA, 2015; EFSA, 2016; EFSA, 2017b; EFSA, 2018; EFSA, 2019). Antimicrobial usage and selective pressure associated with these breeding species may play a primary role in AMR spread, if compared to those of other livestock categories, less represented in the European FPA system.

The last EFSA report shows variability in AMR occurrence in pigs and veal calves under one year in the EU-MSs. However, phenotypic AMR similarities have been identified. In both sources, overall resistance to tetracycline, sulfamethoxazole, ampicillin and trimethoprim was high. Chloramphenicol resistance was detected at a moderate level, meanwhile gentamicin, nalidixic acid, 3^{d} generation cephalosporins (3-GCs) (ceftazidime and cefuroxime), azithromycin and colistin resistance was overall low. Meropenem resistance was not observed. Ciprofloxacin resistance was detected at a low and moderate level in pig and veal calves under one year respectively (EFSA, 2019). A similar AMR profile has also been reported in pig and cattle meat, with the exception of chloramphenicol, whose resistance was rarely observed in both sources (EFSA, 2014).

Broiler and turkey also presented similar phenotypic AMR profile, despite variation have been observed in EU-MSs. Resistance to tetracycline, quinolones (nalidixic acid and enrofloxacin), ampicillin, sulfamethoxazole, trimethoprim has been frequently reported. Moderate chloramphenicol

resistance has been observed, meanwhile resistance to gentamicin, 3-GCs (ceftazidime and cefuroxime) and colistin was overall low. Meropenem resistance was observed only in one broiler isolates from Cyprus (EFSA, 2018). For these sources, AMR data from related meat were reported voluntarily from a limited number of EU-MSs (3) and were generally concordant with those identified in animals (EFSA, 2018).

AMR data observed in swine, calves and poultry animals were similar to those of the previous EFSA reports (EFSA, 2016; EFSA, 2017b), despite higher variability in chloramphenicol and quinolones resistance was reported in pig/poultry and pig/cattle respectively among EU-MSs.

Different European studies are generally in accordance with EFSA findings, reporting tetracycline, sulfamethoxazole, ampicillin and trimethoprim as common AMR resistant profiles in poultry, veal calves/beef cattle and pig (Bywater *et al.*, 2004; Szmolka *et al.*, 2011; Chantziaras *et al.*, 2014; Borjesson *et al.*, 2016; Osterberg *et al.*, 2016) and high quinolone resistance in poultry farms (de Jong *et al.*, 2012; Literak *et al.*, 2013; Hricova *et al.*, 2017). However, variations in AMR profile have been observed and probably derived from the different antimicrobial usage in livestock categories in each country.

Another important food-producing sector is represented by dairy farms. Europe is the first region for global milk production (http://www.fao.org/faostat/en/, last accessed October 2019). Most studies are focused on the pathogen *E. coli* and related AMR in dairy cattle (Orden *et al.*, 2000; Hendriksen *et al.*, 2008; Bengtsson *et al.*, 2009; Ruusunen *et al.*, 2013; de Jong *et al.*, 2018), given the high economic costs and the potential transmission of zoonotic pathogen to workers and surrounding environment.

The risk related to foodborne pathogen diffusion trough dairy products is rare. Dairy products usually undergo treatments such as heating, acidification and fermentation that reduce or remove bacterial contamination altogether (Little *et al.*, 2008; Dehkordi *et al.*, 2014). However, inadequate milk pasteurization or post-processing bacterial contamination could be responsible for pathogen recovery in milk and related product (Little *et al.*, 2008). Dietary habits have changed in the last few years, determining an increase in demand of raw milk and raw milk products, which potentially are important pathogen and AMR bacteria carriers (in particular Shiga toxin *Escherichia coli* - STEC)

(Baylis, 2009; Oliver *et al.*, 2009; Trevisani *et al.*, 2014). Raw dairy products may pose a risk to public health and need to be monitored. Nowadays limited AMR studies are available in *E. coli* indicator isolated from dairy animal and related products in Europe.

Beta-lactams (penicillin, ampicillin, amoxicillin, cephalothin), tetracycline, aminoglycosides (streptomycin) and sulfonamides (sulfamethoxazole, sulfisoxazole, sulfamethoxazole/trimethoprim) resistance is reported as the most common type despite variation in phenotypic antimicrobial profile. Resistance to quinolones varied from low to moderate among European countries (Miranda *et al.*, 2009; Dehkordi *et al.*, 2014; Duse *et al.*, 2015; Nobili *et al.*, 2016). Various studies associated dairy source to carriage of ESBL genes (Gonggrijp *et al.*, 2016; Ibrahim *et al.*, 2016; Odenthal *et al.*, 2016; Heuvelink *et al.*, 2019), suggesting its potential role in ESBL genes diffusion even though resistance to 3-GCs was rarely reported (Miranda *et al.*, 2009; Dehkordi *et al.*, 2014; Duse *et al.*, 2015; Nobili *et al.*, 2016).

New food-producing systems such as rabbit, fishery and mollusc productions have become increasingly important in UE-MSs in the last few decades.

Rabbit farms have drawn little attention despite Europe being the second region after Asia for rabbit and hares production (with Italy in the top 5 producer list in 2017) (http://www.fao.org/faostat; accessed October 2019). Indeed, rabbit production has been rarely associated to foodborne pathogens and therefore it has been perceived as a "safe" food-producing sector. Only fragmented European data about AMR in breeding rabbits and related meat are available, describing resistance to tetracyclines (tetra-doxycycline), penicillins/aminopenicillins (ampicillin, amoxicillin), aminoglycosides (streptomycin, gentamicin), quinolones (ciprofloxacin, nalidixic acid) and sulfonamides (sulphadiazine, sulfamethoxazole/trimethoprim) as common phenotypic AMR resistance profile (Blanco *et al.*, 1994; Dotto *et al.*, 2014; Medina *et al.*, 2015; Freitas-Silva *et al.*, 2018). Moreover, rabbit has been recently associated with colistin resistance gene *mcr1* carriage (Agnoletti *et al.*, 2018; Freitas-Silva *et al.*, 2018), suggesting its potential role in *mcr1* diffusion.

Aquaculture is a growing breeding system, whose production is estimated to reach 4 million tonnes by 2030 (FAO, 2016). Antimicrobials use in aquaculture is rare (FVE, 2016) and this could be the reason

behind the scarcity of data we have regarding the use of antimicrobials and AMR resistance in this production system.

Aquaculture usually undergoes a low direct antimicrobial selective pressure. Therefore, AMR identified in this ecosystem usually derives from AMR environmental pollution associated to other sources (human, animal) (Baquero *et al.*, 2008) and usually spreads through faecal contamination (Guenther *et al.*, 2011). Sewage and runoff from land have been hypothesised as the main carrier of AMR bacteria and genes (Martinez, 2009; Alves *et al.*, 2014; Baliere *et al.*, 2015).

To the best of our knowledge only limited phenotypic antimicrobial resistance studies in *E. coli* indicator have been performed in aquaculture in Europe (Sousa *et al.*, 2011; Grevskott *et al.*, 2017). These investigations reported beta-lactams (amoxicillin, ampicillin, 3d generation cephalosporins), aminoglycosides (streptomycin, gentamicin), tetracyclines (tetracycline, doxycycline) and sulfonamides (sulfamethoxazole/trimethoprim) resistance as the most common. Third-generation cephalosporin, amphenicol, nitrofuran and quinolone resistance were observed less frequently (Grevskott *et al.*, 2017).

1.5.2 Vegetables

Vegetables are essential for a healthy human diet. They are rich in micro/macro- nutrients that help us against a wide range of diseases (Dehghan *et al.*, 2013; Aliasgharzadeh *et al.*, 2015; Farhangi *et al.*, 2016). However, vegetables also represent an important source of AMR bacteria and genes, acquired from contaminated manure or irrigational water in primary production (Mercanoglu Taban and Halkman, 2011; Schwaiger *et al.*, 2011) or during food processing (Rasheed *et al.*, 2014).

In particular, ready-to-eat (RTE) products could pose a higher risk to public health compared to traditional vegetables. Producers normally guarantee RTE product standards, therefore no additional washing step is recommended before their consumption (Rodriguez-Caturla *et al.*, 2012). Unfortunately previous food processing steps could make RTE-vegetables a highly risky carrier of AMR and pathogenic bacteria.

Vegetables have been rarely investigated in Europe despite the growing demand for vegetables and their potential role in human exposure to AMR (Campos *et al.*, 2013).

Generally, resistance to ampicillin, tetracycline, aminoglycosides, sulfonamides has been widely reported in vegetables (Ruimy *et al.*, 2010; Campos *et al.*, 2013; Holvoet *et al.*, 2013; Jensen *et al.*, 2013). Chloramphenicol, 3-GC and quinolone resistance was reported at low levels (Campos *et al.*, 2013; Holvoet *et al.*, 2013). Moreover, different studies evaluated the role of vegetables as a possible reservoir of ESBL producers, identifying in many cases ESBL strains and genes associated to these food products (Egea *et al.*, 2011; Holvoet *et al.*, 2013; Reuland *et al.*, 2014; van Hoek *et al.*, 2019).

1.5.3 Companion animal

AMR data of commensal *E. coli* in companion animals is limited in Europe. Generally, low levels of resistance have been identified, with ampicillin, streptomycin, tetracycline and sulfonamide resistance as the most common phenotypic AMR profile. Phenotypic resistance to fluoroquinolones and 3-GCs was usually not observed or identified at a low level (Costa *et al.*, 2008a; Damborg *et al.*, 2008).

However, different studies identified companion animals as carriers of ESBL and AmpC genes, conferring potential resistance to most beta-lactams antimicrobials (Costa *et al.*, 2004; Carattoli *et al.*, 2005; Ewers *et al.*, 2012; Belas *et al.*, 2014). These data are particularly worrisome considering that nowadays cats and dogs live in strict relationships with human, with frequent physical contacts (by touching, petting and licking) and sharing the same household (Guardabassi *et al.*, 2004a). This could allow transmission of AMR (but also pathogen) bacteria between pets and human (Guardabassi *et al.*, 2004a; Reeves *et al.*, 2011; Martins *et al.*, 2013) .

1.5.4 Wild animal

Wild animals have received increasing attention in the last few years because of their potential role as AMR reservoir, carriers and bioindicators (Dolejska *et al.*, 2007; Literak *et al.*, 2010). Wildlife is considered as a “free-antimicrobials” environment, where the emergence of resistance is strictly related to AMR pollution from human-related environments. The remoteness of the area, livestock and

human density, and association degree with human activity (Skurnik *et al.*, 2006; Allen *et al.*, 2010) represent the most important parameters, influencing bacterial resistance observed in wild animals. Streptomycin, ampicillin/amoxicillin, tetracycline and sulfonamides represented the most common phenotypic resistant profile in *E. coli* indicator and *Enterobacteriaceae* (Costa *et al.*, 2008b; Guenther *et al.*, 2010b; Literak *et al.*, 2010; Giacopello *et al.*, 2016), despite variations in AMR profile in wildlife were observed in different studies. HP-CIAs (3-GCs, quinolones, meropenem and colistin) resistance was overall low or absent (Costa *et al.*, 2008b; Guenther *et al.*, 2010b; Literak *et al.*, 2010; Giacopello *et al.*, 2016), except in one study where resistance to 3-GC was observed at a moderate level (Costa, Poeta *et al.* 2008). Notably, various studies reported ESBL (Simões *et al.*, 2010; Garmyn *et al.*, 2011; Wallensten *et al.*, 2011; Stedt *et al.*, 2015; Alcala *et al.*, 2016; Atterby *et al.*, 2017; Oteo *et al.*, 2018) and carbapenem (Fischer *et al.*, 2013; Dolejska *et al.*, 2015; Giacopello *et al.*, 2016; Vergara *et al.*, 2017; Vittecoq *et al.*, 2017; Bouaziz *et al.*, 2018) genetic determinants in *Enterobacteriaceae* isolated from wild birds worldwide, suggesting their potential role as reservoir and carriers of HP-CIAs resistant bacteria. Presumably, wild birds acquire these AMR profiles from human and agricultural environments, which they attend for better living conditions (availability of food source and refuges, absence of natural predators). However, their migratory movements could determine dissemination of the acquired AMR profile in other human-related environments or remote areas (Bonnedahl and Järhult, 2014), allowing an alarming spread of AMR to HP-CIAs.

1.5.5 Human

Most human studies focus on *E. coli* pathogens and their AMR profile (Bielaszewska *et al.*, 2011; Frank *et al.*, 2011; Kappeli *et al.*, 2011; Beutin and Martin, 2012; Fruth *et al.*, 2015; Januszkiewicz *et al.*, 2015), attempting to obtain valuable epidemiological AMR data associated to clinical settings. However, gut microbiota could represent a perfect environment for emergence and diffusion of AMR, due to the high bacterial population and the important selective pressure associated to antimicrobial treatments. Moreover, it could represent an important AMR source of human pathogens (Penders *et al.*, 2013; Brinkac *et al.*, 2017).

Actual data report ampicillin, tetracycline and sulfonamides resistance as the most common AMR phenotypic profile in human commensal *E. coli.* Resistance rate to chloramphenicol and streptomycin was variable (Pallecchi *et al.*, 2007; Skurnik *et al.*, 2008; Bailey *et al.*, 2010; Johnson *et al.*, 2012; Tadesse *et al.*, 2012), meanwhile 3-GCs and quinolone resistance was generally not observed (Skurnik *et al.*, 2008; Bailey *et al.*, 2010; Johnson *et al.*, 2012; Tadesse *et al.*, 2012). To the best of our knowledge, phenotypic colistin and carbapenem resistance were never reported in commensal *E. coli* from human origin.

Notably, different studies observed ESBL genes carriage in human commensal *E. coli* (Rodríguez-Baño *et al.*, 2008; Guimaraes *et al.*, 2009; Vinué *et al.*, 2009; Hammerum *et al.*, 2011; Karanika *et al.*, 2016; van Duijkeren *et al.*, 2017), suggesting humans as potential reservoir of ESBL producers.

1.6 The genetic basis of AMR

Bacteria represent the "micro-environment" where genetic AMR mechanisms develop in response to selective pressure and diffuse through horizontal gene transfer (HGT) in the microbial population.

AMR is mediated by specific genes conferring resistance by the following mechanisms: antimicrobial enzymatic degradation, antimicrobial target alteration, bacterial cell wall modification and alternative metabolic pathways to elude antimicrobial action (Tenover, 2006). The acquisition of these genes by a microbe is the result of de novo chromosomal mutation (transmitted to daughter cells via vertical gene transfer (VGT) or transfer from other bacteria via HGT (Tenover, 2006).

Antimicrobial class	Antimicrobial resistance genes	Source	Reference
Aminoglycoside	*aadA*	human	Guimaraes *et al.*, 2009
	aac(3')-II	rabbit	Medina *et al.*, 2015
	aac(3)-IVa	swine	Carattoli *et al.*, 2017
	aac(3)-IVa	swine	Carattoli *et al.*, 2017
	aac(3')-IV	rabbit	Medina *et al.*, 2015
		cattle	Dehkordi *et al.*, 2014
	aadA1	rabbit	Dotto *et al.*, 2014
		swine	Carattoli *et al.*, 2017
		swine	Szmolka *et al.*, 2011
		cattle	Dehkordi *et al.*, 2014
		poultry	Literak *et al.*, 2013
		seabream	Sousa *et al.*, 2011
		willife	Literak *et al.*, 2010
	aadA5	rabbit	Dotto *et al.*, 2014
	aadA1, aadA5	swine	Guenther *et al.*, 2017
	aph(3')-1c	swine	Carattoli *et al.*, 2017
	aph(4)-1a	swine	Carattoli *et al.*, 2017
	aadA, strA-strB	carp farms	Piotrowska *et al.*, 2017
	strA-B, aac(3')-IId, aph(3')-Ia; aadA5	bivalve mollusc	Grevskott et al, 2017
	aadA, strA-B	vegetable	Campos *et al.*, 2013
	strA	willife	Literak *et al.*, 2010
	strA/B	swine	Guenther *et al.*, 2017
		swine	Carattoli *et al.*, 2017
		swine	Szmolka *et al.*, 2011
		poultry	Literak *et al.*, 2013
Amphenicol	*catA*	vegetable	Campos *et al.*, 2013
	catA1	swine	Carattoli *et al.*, 2017
		cattle	Dehkordi *et al.*, 2014
		bivalve mollusc	Grevskott *et al.*, 2017
	cmlA	cattle	Dehkordi *et al.*, 2014
		seabream	Sousa *et al.*, 2011
		human	Guimaraes *et al.*, 2009
		willife	Alcala *et al.*, 2016
	cmlA1	swine	Guenther *et al.*, 2017
		human	Bailey *et al.*, 2010
	catI, cmlA6	human	Pallecchi *et al.*, 2007
	floR	swine	Carattoli *et al.*, 2017
Beta-lactam	*bla*$_{\text{TEM}}$	rabbit	Medina *et al.*, 2015
		vegetable	Campos *et al.*, 2013
		cattle	Ibrahim *et al.*, 2016
		cattle	Odenthal *et al.*, 2016
		cattle	Heuvelink *et al.*, 2019

Table 1.1 Representative antimicrobial resistance genetic determinants identified in animal, food and human sources

Antimicrobial class	Antimicrobial resistance genes	Source	Reference
Beta-lactam	*bla*$_{TEM}$	poultry	Ghodousi *et al.*, 2015
		human	Pallecchi *et al.*, 2007
		human	Bailey *et al.*, 2010
		comapnion animal	Carattoli *et al.*, 2005
		wildlife	Literak *et al.*, 2010
	bla$_{TEM-1b}$	swine	Carattoli *et al.*, 2017
		swine	Guenther *et al.*, 2017
		swine	Szmolka *et al.*, 2011
		poultry	Literak *et al.*, 2013
		wildlife	Alcala *et al.*, 2016
	bla$_{TEM-52}$	cattle	Gonggrijp *et al.*, 2016
		swine	Overdevest *et al.*, 2011
		human	Vinué *et al.*, 2009
		comapnion animal	Costa *et al.*, 2004
	bla$_{TEM-52}$, *bla*$_{SHV-12}$	seabream	Sousa *et al.*, 2011
	bla$_{CTX-M}$	cattle	Ibrahim *et al.*, 2016
	*bla*CTX-M-1	swine	Guenther *et al.*, 2017
		cattle	Dolejska *et al.*, 2011
		comapnion animal	Costa *et al.*, 2004
		comapnion animal	Carattoli *et al.*, 2005
	bla$_{CTX-M14}$	poultry	Borjesson *et al.*, 2016
		human	Hammerum *et al.*, 2011
	bla$_{CTX-M9}$	rabbit	Medina *et al.*, 2015
	bla$_{CTX-M1,2}$	swine	Overdevest *et al.*, 2011
	bla$_{CTX-M1,14}$	wildlife	Alcala *et al.*, 2016
	bla$_{CTX-M1,52}$	wildlife	Literak *et al.*, 2010
	bla$_{CTX-M1,2,9}$	poultry	Ghodousi *et al.*, 2015
	bla$_{CTX-M1-14-15}$	vegetable	Reuland et l., 2014*
	bla$_{CTX-M15,1,2,3}$	swine	Borjesson *et al.*, 2016
		cattle	Borjesson *et al.*, 2016
	bla$_{CTX-M1,2,9}$	cattle	Heuvelink *et al.*, 2019
	bla$_{CTX-M1,8,14,32}$	human	Vinué *et al.*, 2009
	bla$_{CTX-M1,2,8,9}$	cattle	Odenthal *et al.*, 2016
	bla$_{CTX-M1,9,15,32}$	comapnion animal	Belas *et al.*, 2014
	bla$_{CTX-M1,2,14,15,32,55}$	cattle	Gonggrijp *et al.*, 2016
	bla$_{CTX-M1,14,15,32,55}$	wildlife	Atterby *et al.*, 2017
	bla$_{CTX-M1}$, *bla*$_{TEM52}$, and *bla*$_{SHV12}$	human	Guimaraes *et al.*, 2009
	bla$_{TEM1b-1c}$, *bla*$_{CTX-M14-15}$	marine bivalve mollusc	Grevskott *et al.*, 2017
	bla$_{OXA}$	cattle	Ibrahim *et al.*, 2016
	bla$_{CMY}$	cattle	Heuvelink *et al.*, 2019
	bla$_{CMY-2}$	swine	Borjesson *et al.*, 2016
		cattle	Borjesson *et al.*, 2016
	bla$_{CMY-2}$	poultry	Borjesson *et al.*, 2016

Table 1.1 ..\..

Antimicrobial class	Antimicrobial resistance genes	Source	Reference
	bla$_{CMY-2}$	cattle	Gonggrijp *et al.*, 2016
		human	Hammerum *et al.*, 2011
		comapnion animal	Carattoli *et al.*, 2005
		comapnion animal	Belas *et al.*, 2014
		willife	Alcala *et al.*, 2016
	bla$_{SHV}$	cattle	Dehkordi *et al.*, 2014
		cattle	Odenthal *et al.*, 2016
		poultry	Ghodousi *et al.*, 2015
	bla$_{SHV12}$	vegetable	Egea *et al.*, 2011
		vegetable	van Hoek *et al.*, 2015
		vegetable	Reuland *et al.*, 2014
		swine	Overdevest *et al.*, 2011
		willife	Alcala *et al.*, 2016
		comapnion animal	Carattoli *et al.*, 2005
		willife	Atterby *et al.*, 2017
colistin	*mcr1*	rabbit	Agnoletti *et al.*, 2018
		rabbit	Freitas-Silva *et al.*, 2018
		turkey	Perrin-Guyomard *et al.*, 2016
		broyler	Perrin-Guyomard *et al.*, 2016
		swine	Guenther *et al.*, 2017
	mcr1-3	cattle	Hernandez *et al.*, 2017
	mcr4	swine	Carattoli *et al.*, 2017
quinolone	*qnr*	cattle	Dehkordi *et al.*, 2014
	qnrA	poultry	Ghodousi *et al.*, 2015
	qnrS1	swine	Szmolka *et al.*, 2011
		bivalve mollusc	Grevskott et al, 2017
	qnrB19, qnrS1	poultry	Literak *et al.*, 2013
	gyrA, parC	rabbit	Medina *et al.*, 2015
		human	Guimaraes *et al.*, 2009
	oqxA/B	rabbit	Dotto *et al.*, 2014
sulfonamides	*sul1*	cattle	Dehkordi *et al.*, 2014
	sul2	swine	Szmolka *et al.*, 2011
	sul3	swine	Guenther *et al.*, 2017
	sul1,2	vegetable	Campos *et al.*, 2013
		swine	Guenther *et al.*, 2017
		swine	Carattoli *et al.*, 2017
		human	Pallecchi *et al.*, 2007
		willife	Literak *et al.*, 2010
		bivalve mollusc	Grevskott et al, 2017
	sul1, sul3	human	Guimaraes *et al.*, 2009
	sul1,sul2,sul3	poultry	Literak *et al.*, 2013
		seabream	Sousa *et al.*, 2011

Table 1.1 ..\..

Antimicrobial class	Antimicrobial resistance genes	Source	Reference
tetracycline	*sul1, sul2, sul3*	human	Bailey *et al.*, 2010
		willife	Alcala *et al.*, 2016
	tetA	swine	Szmolka *et al.*, 2011
		marine aquaculture	Rodriguez-Blanco *et al.*, 2012
		human	Guimaraes *et al.*, 2009
	tetA,tetB	rabbit	Medina *et al.*, 2015
		vegetable	Campos *et al.*, 2013
		swine	Guenther *et al.*, 2017
		dairy cattle	Dehkordi *et al.*, 2014
		swine	Carattoli *et al.*, 2017
		poultry	Literak *et al.*, 2013
		human	Pallecchi *et al.*, 2007
		wildlife	Alcala *et al.*, 2016
		wildlife	Literak *et al.*, 2010
	tetA-B-D	bivalve mollusc	Grevskott *et al.*, 2017
	oqxA/B	rabbit	Dotto *et al.*, 2014
trimethoprim	*dfrA1*	rabbit	Dotto *et al.*, 2014
		swine	Carattoli *et al.*, 2017
		dairy cattle	Dehkordi *et al.*, 2014
		poultry	Literak *et al.*, 2013
	dfrA12	swine	Szmolka *et al.*, 2011
	dfrA1-dfrA12	vegetable	Campos *et al.*, 2013
	dfrA1-dfrA17	swine	Guenther *et al.*, 2017
	dfrA5-A14-A17	bivalve mollusc	Grevskott *et al.*, 2017
	dfrA1, dfrA7, dfrA8, dfrA17	human	Pallecchi *et al.*, 2007
	dfrA1, dfrA5, dfrA7, dfrA12 or dfrA17	human	Bailey *et al.*, 2010

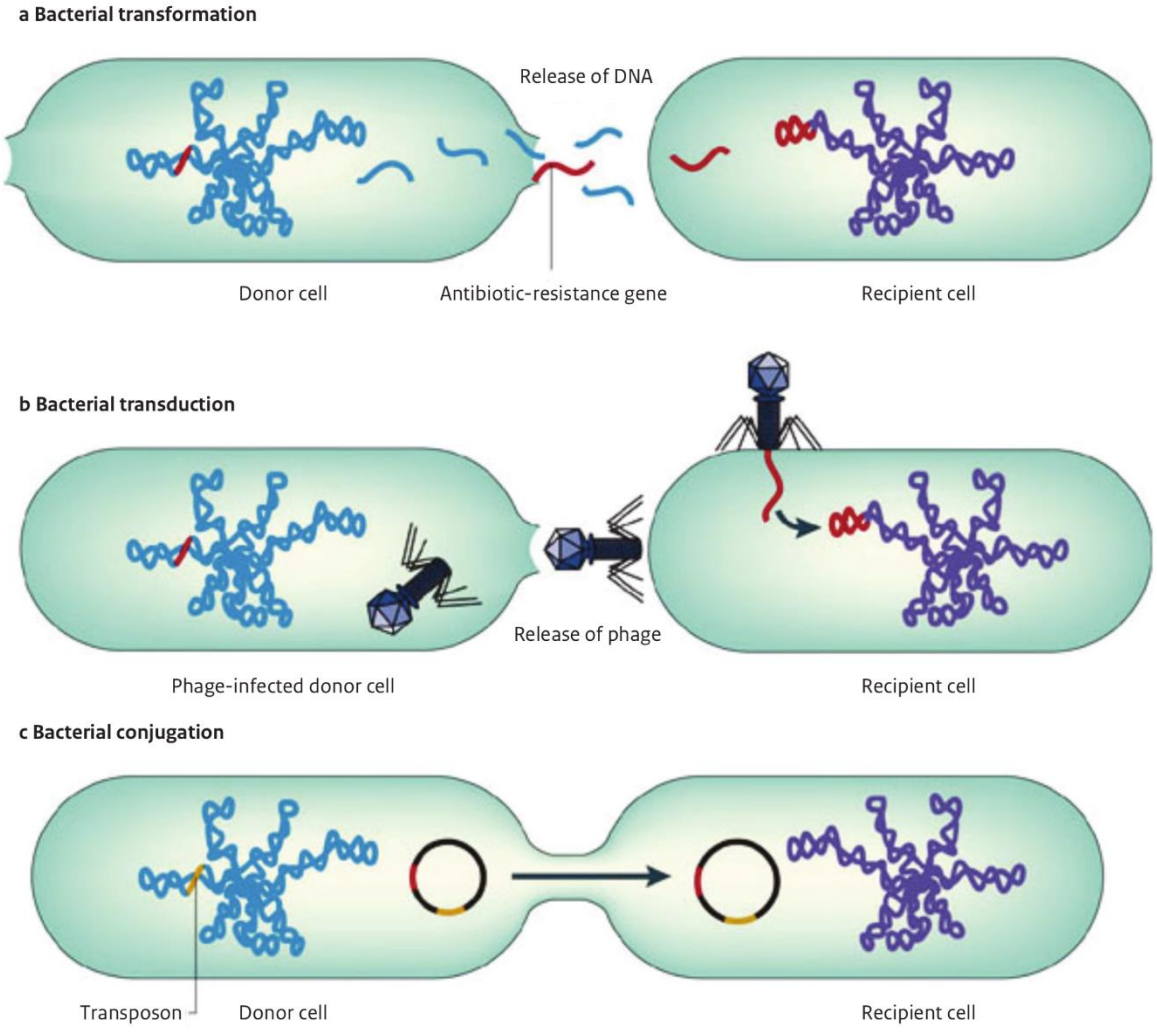
Table 1.1 ..\..

HGT represents an extremely efficient mechanism to exchange a great variety of genes, including ARGs between bacteria of the same or different species. The enrichment of bacterial genetic pool drives and promotes microbial AMR evolution.

The three main HGT mechanisms in the bacterial population are:

- conjugation: DNA transfer between bacterial cell through direct contact between donor and recipient cell;
- transformation: uptake of environmental DNA by bacterial cell;
- transduction: bacteriophage-mediated DNA transfer in a bacterial cell (Soucy *et al.*, 2015).

In particular, conjugation is recognised to play a primary role in ARGs diffusion among bacterial population (Mathur and Singh, 2005). This mechanism (but also the others described) relies on mobile genetic elements (MGEs) like plasmids and transposons (Tns), which facilitate the transfer and incorporation of exogenous DNA (including ARGs) in the host cell. These MGEs could also be arranged/gathered into insertion sequences (ISs) or integrons (Ins) in complex assemblages (Verraes *et al.*, 2013).



(Geenen *et al.*, 2011)

Figure 1.2 Main mechanisms of horizontal gene transfer in bacteria.
a. Transformation: naked DNA is taken up from the surrounding environment; b. Transduction: transfer of genetic material from a donor to a recipient cell through a bacteriophage intermediate; c. Conjugation: DNA is transferred from the donor to the recipient cell via a conjugation pilus. (Geenen *et al.*, 2011)

1.6.1 Plasmids

Plasmids are circular, extra-chromosomal DNA molecules with extremely variable size (less than a kilobase to several megabases), able to replicate independently. Their structure normally includes a backbone of housekeeping genes encoding replicative functions, and accessory regions. The latter are usually made up of numerous genes conferring resistance to antimicrobials, heavy metals and virulence genes. Also gene-associated MGEs (IS, Tn, In) could be present (Frost *et al.*, 2005; Fernández-Alarcón *et al.*, 2011; Shintani *et al.*, 2015).

A common plasmid classification is based on the "incompatibility phenomenon", whereby closely related plasmids sharing the same replication mechanism could not coexist and be propagated in the same cell. (Shintani *et al.*, 2015). Plasmids with the identical replication control are included in the same incompatibility (Inc) group. Inc groups in *Enterobacteriaceae* are divided in A/C, B/O, D, F, G, H, I, K, J, L/M, N, P, Q, R, T, U, W, X, Y,Z Col and related variants (Rozwandowicz *et al.*, 2018).

Plasmids represent an important vehicle for AMR. Their AMR genetic region often results from recombination events between transposons, insertions sequences and other plasmids (Szmolka and Nagy, 2013). Multi-resistant plasmids usually encode resistance to different antimicrobial classes, including aminoglycosides, β-lactams, phenicols, quinolones, tetracyclines, and sulfonamides (Carattoli, 2013). In particular, IncA/C, IncF, IncI1, IncHI1 and IncN seem to play an important role in multiple antimicrobial determinants dissemination (Szczepanowski *et al.*, 2005; Carattoli and chemotherapy, 2009; Cain *et al.*, 2010; Venturini *et al.*, 2010; Fernandez-Alarcon *et al.*, 2011; Cain and Hall, 2012; Wibberg *et al.*, 2013).

Different HP-CIAs ARGs have been identified on different major plasmid families, summarised hereafter:

Inc group	Resistance genes
A/C	AmpC beta-lactamases (*bla*$_{CMY2-4}$); ESBL (*bla*$_{CTX-M-2-3-14-15-56}$); Metallo beta-lactamases (*bla*$_{IMP-4-8-13}$, *bla*$_{VIM-4}$)
F	ESBL (*bla*$_{CTX-M1-2-3-9-14-15-24-27}$); AmpC beta-lactamases (*bla*$_{CMY2}$); Quinolones (*qnrA1*, *qnrB2*, *qnrB4*, *qnrB6*, *qnrB19*, *qnrS1*)
HI2	ESBL (*bla*$_{CTX-M-2-3-9-14}$); Metallo beta-lactamases (*bla*$_{IMP-4}$, *bla*$_{VIM}$); Quinolones (*qnrA1, qnrS1*)
I1	AmpC beta-lactamases (*bla*$_{CMY-2-7-21}$); ESBL (*bla*$_{CTX-M-1-2-3-9-14-15-24}$); Metallo beta-lactamases (*bla*$_{VIM-1}$); Quinolones (*qnrA1*)
L/M	ESBL (*bla*$_{CTX-M-1-3-15-42}$); Metallo beta-lactamases (*bla*$_{IMP-4-8}$); Quinolones (*qnrA1, qnrB1, qnrB2, qnrB4, qnrS1*)
N	Carpanemeases (*bla*$_{KPC-2}$) ; ESBL (*bla*$_{CTX-M-1-3-15-32-40}$); Metallo beta-lactamases (*bla*$_{VIM-1}$) Quinolones (*qnrA3, qnrB2, qnrB19, qnrS1*)

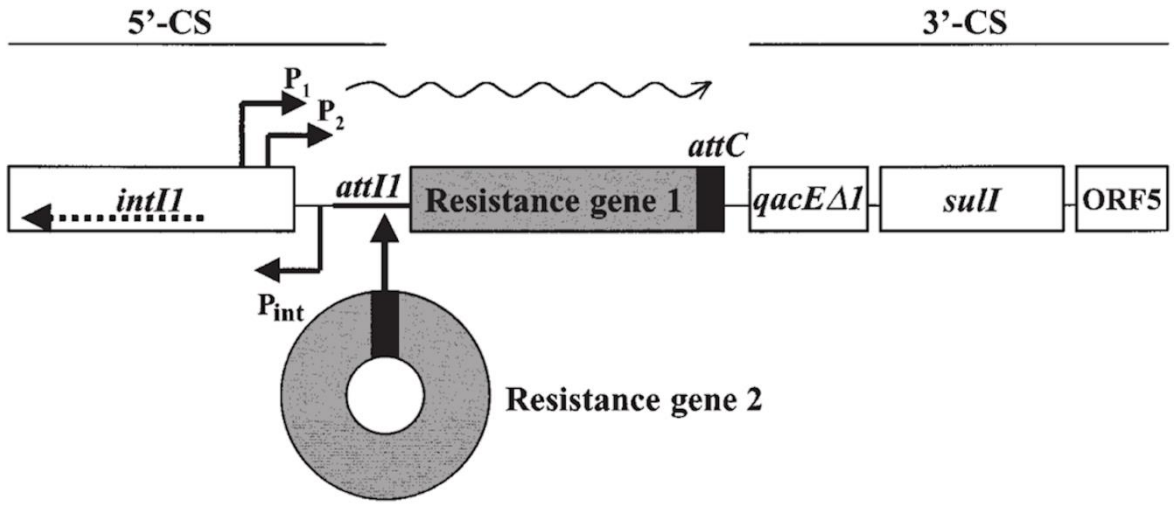
(Carattoli *et al.*, 2009; modified)

Table 1.2 Major plasmid families and associated HP-CIA ARGs identified in Enterobacteriaceae from animal and human origin worldwide.

1.6.2 Class 1 integron

Various classes of integrons have been described but only 3 (class 1, 2, 3 integron) have been associated to AMR (Kaushik *et al.*, 2018). Among the latter, class 1 integron is the most diffused and clinically important type among enteric bacteria (Labbate *et al.*, 2009; Deng *et al.*, 2015). It is recognised as a primary source of ARGs (Kheiri and Akhtari, 2016) and plays an essential role in the dissemination and evolution of MDR among Gram-negative bacteria (Prapasawat *et al.*, 2017). A typical class 1 integron structure is characterised by:

- 2 conserved sequences:
    - 5'-CS composed by
        - a gene (*intI1*) encoding for a site-specific integrase (intI1), responsible for the insertion of free gene cassettes into the variable region at the attI site;
        - an attachment site (attI), where gene cassettes are inserted;
        - a promoter (Pc), which express gene cassettes inserted in the variable region;
    - 3'-CS composed by
        - *sul1* gene, conferring resistance to sulfonamides;
        - a truncated copy of *qacE* gene (*qacEΔ1*), conferring resistance to quaternary ammonium compounds;
        - ORF5, encoding a protein of unknown function:
- 1 variable region, characterised by a variable number of gene cassettes;
- 25 bp inverted repeat sequences (IRi, IRt), bounding integron structure (Carattoli, 2001).

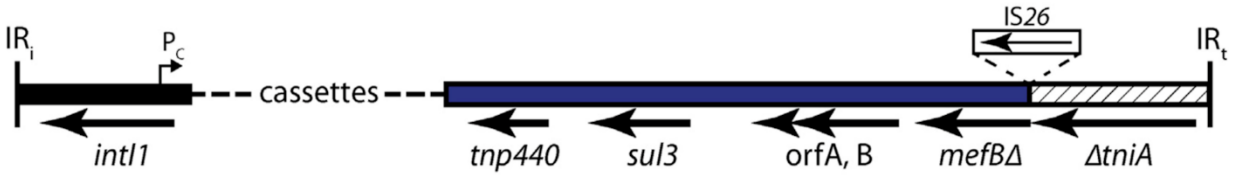


(Carattoli, 2001)

Figure 1.3 Schematic representation of a class 1 integron showing 5` and 3` conserved segments, attI and attC recombination sites and gene cassettes.

Gene cassettes consist of an open reading frame (ORF) and a recombination site (attC). An integrase-mediated reaction between attI and attC allows gene cassettes recombination into the variable region of the integron (Labbate *et al.*, 2009). Approximately 130 different gene cassettes have been identified conferring resistance to numerous antimicrobials (β-lactams, all aminoglycosides, chloramphenicol, streptomycin, trimethoprim, rifampin, erythromycin, quinolones, fosfomycin and lincomycin) (Deng *et al.*, 2015).

Atypical class 1 integrons do not exhibit the classic structure. They are the result of recombination events or interruption of IS elements. One example is the *sul3* module, replacing the 3'-CS in *sul3*-associated integrons. It comprises a putative transposase of IS*440* (*tnp440)*, a sulfonamide resistance gene *(sul3)*, two hypothetical proteins (*orfA* and *orfB*) and the macrolide efflux gene (*mefB)* truncated by IS*26* (Moran *et al.*, 2016).



(Moran *et al.,* 2016)

Figure 1.4 *sul3*-associated class 1 integron showing the *sul3*-module (blue) and IS26 insertion.

sul3 associated class1 integron has been observed in commensal and pathogen *E. coli* from animal and human origin worldwide (Bischoff *et al.*, 2005; Sunde *et al.*, 2008; Sáenz *et al.*, 2010; Moran *et al.*, 2016).

Class 1 integrons are not independently mobile, therefore their diffusion depends on plasmid and transposon (especially those belonging to Tn*3* family) availability (Labbate *et al.*, 2009). In particular, the Tn*3* family member Tn*21* transposon, in association to class 1 integron, has played a primary role in AMR diffusion (Liebert *et al.*, 1999).

1.6.3 Insertion sequences

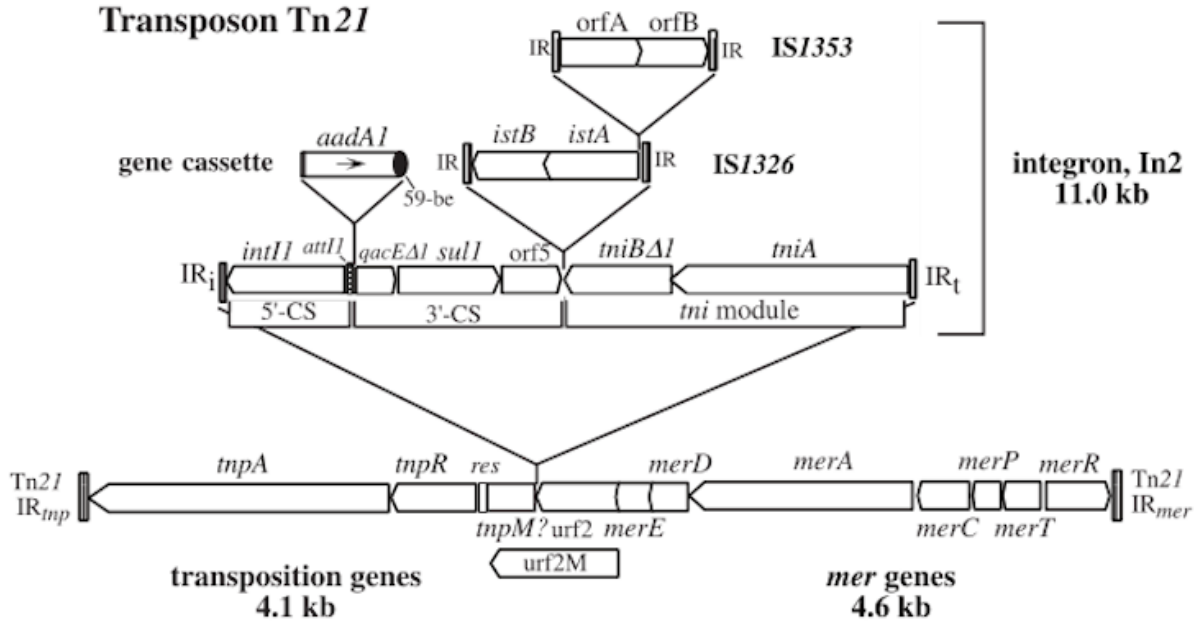
Insertion sequences are small (0.7 - 2.5 kb) mobile genetic elements, characterised by 1 (sometimes 2) transposase gene (*tnp*) and bonded by inverted repeat sequences (IRs). They are able to insert themselves in different sites in a genome (Siguier *et al.*, 2015). Usually, their ability to move ARGs is associated to composite transposon formation. This structure is assembled by a central region, flanked by 2 copies of the same or related IS elements. IS families are numerous and associated to a wide variety of ARGs (Partridge *et al.*, 2018).

IS26 is one of the most widespread members and it has been frequently associated with different antimicrobial regions in important clinical plasmids (Loli *et al.*, 2006; Doublet *et al.*, 2008; Cullik *et al.*, 2010; Zienkiewicz *et al.*, 2013).

1.6.4 Transposons

Transposons are MGEs characterised by a *tnp* gene and delimited by IRs. They are able to self-excise and insert in non-homologous DNA molecules. They often carry different genes, including ARGs, and could be associated to ISs or integrons or inserted in plasmids (Partridge *et al.*, 2018). (Partridge *et al.*, 2018). Within *E. coli* common transposons include derivatives of Tn*3,* Tn*5*, Tn*7* associated to ampicillin, kanamycin and trimethoprim and streptomycin resistance respectively (Iyer *et al.*, 2013).

In particular, Tn*21* is a widely diffused member of the Tn*3* family with a complex and variable structure, including a *mer* resistant region (conferring mercury resistance) (Figure 1.5.). It is important in AMR diffusion because it is usually associated with integron platform and various ARGs (Siguier *et al.*, 2015). The co-association between class 1 integron and Tn*21* family members/related variants is reported as common in clinical isolates (Betteridge *et al.*, 2011).



(Liebert *et al.*, 1999)

Figure 1.5 Schematic representation of Tn*21* structure.

In conclusion, AMR is an ever-growing phenomenon in rapid evolution. Current knowledge is focused on AMR in limited niches. Food-producing animals have drawn most attention (in particular poultry, cattle, swine) for the potential transmission of AMR bacteria through related foodstuff and the AMR genetic pool made available for foodborne pathogens. The need for comprehensive AMR knowledge in all the ecosystems is impellent, considering the rapid spread and exchange of ARGs between commensal, environmental and pathogen bacteria, allowed by the strict interconnection between environments and HGT.

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2. Aim of the study

Antimicrobial resistance has become a serious public health issue in the last few years, associated with a large number of human deaths and economic costs.

The analysis of the literature highlighted the increasing interest in AMR epidemiological monitoring, providing essential information for the development of effective strategic plans to face the AMR phenomenon, such as the AMR spatial and temporal trend and identification of potential AMR sources. Despite recent efforts, AMR still remains a problematic issue in Europe, with limited knowledge about association with various environments and its genetic mechanisms.

The European AMR monitoring plan currently evaluates phenotypic data of limited sources such as pig, cattle, chicken and turkey. These FPAs are also the niches mainly investigated in AMR genomic studies. The latter are often performed by PCR, which does not allow the identification of the comprehensive antimicrobial genetic structures involved. This issue seems to be particularly problematic in Italian studies.

This study attempts to reduce the knowledge gap in the current Italian AMR situation and to contribute to the global understanding of the European AMR dynamics.

The specifics aims of this study are:

- Understand and collect data regarding the phenotypic AMR trend in *E. coli* indicator in Italy, particularly focusing on resistance to HP-CIAs;
- Determine the population structure, multi-locus sequence types, antimicrobial resistance, virulence and disinfectant resistance genetic traits and associated mobile genetic elements (integrons, transposons, plasmids, ISs);
- Delve into the epidemiological role played by animals, food and humans in AMR maintenance and diffusion, establishing potential risks to public health.

3. AMR phenotypic characterisation

3.1 Materials and Methods

In the period between November 2016 and July 2018 a total of 288 commensal *E. coli* isolates of animal, food and human origin were collected in the Emilia Romagna region of Italy. The specific sources were narrowed down to dairy, beef, wild boar, companion animal, rabbit, poultry, swine, vegetable, fishery, mollusc, wild animal and human. Each source was characterised by 25 *E. coli* isolates with the exception of companion animals, consisting of 13 strains only.

3.1.1 Sampling

A total of 433 samples were collected and processed from food, animal and human samples (beef, 34; wild boar, 34; fresh vegetable, 165; fishery, 95; human, 27; companion animal, 13; swine, 4; poultry, 33; rabbit, 16). Moreover, 124 *E. coli* strains provided by the Department of Veterinary Science – University of Bologna – Service of Food Safety and Istituto Zooprofilattico Sperimentale of Emilia Romagna, were included in the study.

Food Samples

Chicken, rabbit, swine, fish and vegetable products were sampled from major supermarkets located in Bologna Province, Emilia Romagna region, Italy.

Unwrapped items were individually placed in clean plastic bags using disposable gloves.

Sponges of beef carcass were collected from the educational abattoir of the Veterinary Sciences Department, University of Bologna, during slaughtering procedures. Sponges were poured with sterile Buttersfield tampon (Oxoid, Basington, UK) before use and put in a sterile stomacher bag after sampling.

Animal Samples

Only healthy animals that had not had antimicrobials in the last month were included in the study. Cat and dog faeces were provided from volunteers, meanwhile poultry cloacal swabs were collected from the animal facility of the Veterinary Sciences Department, University of Bologna.

Wild boar diaphragm pillars were supplied from Istituto Zooprofilattico Sperimentale of Bologna.

Human Samples

Human faeces were collected from healthy volunteers that had not had antimicrobial treatment in the last month. Sterile containers were provided to individuals for storage of the samples. All participants were properly apprised of the specifics of the study and signed the designated informed consent.

All food, animal and human samples were carried to the laboratory in aseptic conditions in a cold box within 2 hours from the time of purchase and refrigerated until processing. The analysis was then performed within 2 hours after their arrival into the laboratory.

Strains

E. coli strains isolated from milk, cheese and milking system filters (25) were included in the current study. They were provided by the Service of Food Security, Department of Veterinary Science, University of Bologna.

As part of a collaboration, *E. coli* strains isolated from rabbits (12 intestines, 1 liver), beef (2 faeces, 1 minced meat, 1 muscle), companion animals (13 faeces) and cultured bivalve molluscs (25) were supplied by Istituto Zooprofilattico Sperimentale of Bologna. *E. coli* collected from wild animals (25) and from swine faeces (19) were provided by Istituto Zooprofilattico Sperimentale of Forlì and Istituto Zooprofilattico Sperimentale of Reggio Emilia, respectively.

More details about strain collection are available in the Supplementary Material, Table S1.

3.1.2 Strain Isolation

Meat, vegetable, fish, beef skin sponge

A sharp sterile knife was used to cut food samples in pieces (except for carcass sponges). Sections more likely to be contaminated with *E. coli* were selected from vegetables (roots and external leaves) and fish (guts, gills, fins and skin).

Twenty grams of each sample (in case of skin sponge, each one separately) were placed into a sterile blender bag, diluted with 180 ml of sterile EC-Broth (Oxoid, Basington, UK) and macerated in a stomacher for 1 minute. Samples were incubated overnight at 37 ± 1°C. Ten microliters of overnight culture were streaked onto MacConkey's (Oxoid, Basington, UK) and Levine's (Oxoid, Basington, UK) agar plates and incubated for 18 – 24 h at 37 ± 1°C.

Faeces

Suspension of 1 g of faeces and 9 ml of peptone water (Oxoid, Basington, UK) were prepared. Bacterial isolation was performed by plating 10 µl of each suspension (or directly in the case of the cloacal swabs) on MacConkey's (Oxoid, Basington, UK) and Levine's (Oxoid, Basington, UK) agar plates, followed by incubation for 18 – 24 h at 37 ± 1°C.

For all the samples, lactose fermenting colonies were picked and identified by Gram stain and standard biochemical test (indole probe). *E. coli* ATCC 25922 was used as a control strain.

3.1.3 Strain molecular characterization

Bacteria were grown on Tryptone Soya Agar (TSA) (Oxoid, Basington, UK) plate overnight at 37 ± 1°C. Genomic DNA was extracted using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen), following the manufacturer's instruction.

A multiplex PCR targeting 4 genes (*lacY, lacZ, uidA, cyd*) was used for *E. coli* identification, following the method described by Horakova *et al.* (2008).

The PCR amplification was performed in a reaction volume of 10 µl containing 5 µl REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, St Louis, MO, USA), 0.25 µl primers (10 pmol) and 1.5 µl DNA.
The following amplification parameters were applied: initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 25 seconds, elongation at 72°C for 30 seconds and a final extension at 72°C for 3 minutes.

Primer ID	Target	Primer sequence	PCR product (bp)	Reference
lacY-f	*lacY*	5'-ACCAGACCCAGCACCAGATAAG-3'	463	Horakova *et al.,* 2008
lacY-r		5'-GCACCTACGATGTTTTTGACCA-3'		
lacZ-f	*lacZ*	5'-ATGAAAGCTGGCTACAGGAAGGCC-3'	264	Bej *et al.,* 1991
lacZ-r		5'-GGTTTATGCAGCAACGAGACGTCA-3'		
uidA-f	*uidA*	5'-AAAACGGCAAGAAAAAGCAG-3'	319	Horakova *et al.,* 2006
uidA-r		5'-ACGCGTGGTTACAGTCTTGCG-3'		
cyd-f	*cyd*	5'-CCGTATCATGGTGGCGTGTGG-3'	393	Horakova *et al.,* 2006
cyd-r		5'-GCCGGCTGAGTAGTCGTGGAAG-3'		

Table 3.1 PCR primers used in *E. coli* identification.

The amplified products were loaded onto a 2% agarose gel containing Syber Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and ran in 1X TBE buffer at 100 V for 1 h.

PCR fragments were visualized with a UV transilluminator. A pUC19 DNA/MspI (Hpall) Marker (Thermo Fisher Scientific, Waltham, MA, USA) was loaded on each gel as a DNA size standard. *E. coli* ATCC 25922 was present in every load as control strain. Strains showing PCR products of 463 bp, 393 bp, 319 bp and 264 bp as described in Table 3.1 were considered *E. coli*.

3.1.4 Phylotyping

Phylogroup evaluation was performed using a multiplex PCR designed by Clermont *et al.* (2013), targeting 5 different genes or DNA fragments (*chuA, YiaA,* TspE4.C2*, arpA, trpA*). Clermont method allowed us to discriminate up to 8 different phylogroups (A, B1, B2, C, D, E, F, clade I).

All PCR reactions were carried out in a 10 µl volume containing 5 µl REDExtract-N-Amp PCR ReadyMix, 0.25 µl primers and 1.5 µl DNA. The amounts of primer used are 20 pmol, except for AceK.f (40 pmol), ArpA1.r (40 pmol), trpBA.f (12 pmol) and trpBA.r (12 pmol).

PCR reactions were performed under the following conditions: denaturation 4 min at 94°C, 30 cycles of 5 s at 94°C and 20 s at 59°C, and a final extension step of 5 min at 72°C.

Primer ID	Target	Primer sequence	PCR product (bp)	Reference
chuA.1b	*chuA*	5'-ATGGTACCGGACGAACCAAC-3'	288	Clermont *et al.,* 2000
chuA.2		5'-TGCCGCCAGTACCAAAGACA-3'		Clermont *et al.,* 2013
yjaA.1b	*YiaA*	5'-CAAACGTGAAGTGTCAGGAG-3'	211	Clermont *et al.,* 2013
yjaA.2b		5'-AATGCGTTCCTCAACCTGTG-3'		Clermont *et al.,* 2013
TspE4C2.1b	TspE4.C2	5'-CACTATTCGTAAGGTCATCC-3'	152	Clermont *et al.,* 2013
TspE4C2.2b		5'-AGTTTATCGCTGCGGGTCGC-3'		Clermont *et al.,* 2013
AceK.f	*arpA*	5'-AACGCTATTCGCCAGCTTGC-3'	400	Clermont *et al.,* 2013
ArpA1.r		5'-TCTCCCCATACCGTACGCTA-3'		Clermont *et al.,* 2004
trpBA.f	*trpA*	5'-CGGCGATAAAGACATCTTCAC-3'	489	Clermont *et al.,* 2008
trpBA.r		5'-GCAACGCGGCCTGGCGGAAG-3'		Clermont *et al.,* 2008

Table 3.2 PCR primers used in phylogroup identification.

arpA	***chuA***	***yiaA***	**TspE4.C2**	**Phylogroup**
+	-	-	-	A
+	-	-	+	B1
-	+	-	-	F
-	+	+	-	B2
-	+	+	+	B2
-	+	-	+	B2
+	-	+	-	A or C
+	+	-	-	D or E
+	+	-	+	D or E
+	+	+	-	E or clade I
-	-	+	-	Clade I or II
-	-	-	-	Clade III, IV or V
-	-	-	+	Unknown
-	-	+	+	Unknown
+	-	+	+	Unknown
+	+	+	+	Unknown
-	-	-	-	Unknown

Table 3.3 Pattern observed after phylogroup PCR performing. Combination of different genes/DNA fragment is phylogroup specific. +: presence of the gene/DNA fragment; -: absence of the gene/DNA fragment.

PCR products were loaded onto a 2% agarose gel containing Syber Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and pUC19 DNA/MspI (Hpall) (Thermo Fisher Scientific, Waltham, USA) was used as a Marker. Gel was then run in 1X TBE buffer at 100 V for 1 h.

Strains showing A or C phylogroup pattern were screened using C specific primers. PCR reactions were performed as described above.

Primer ID	Target	Primer sequence	PCR product (bp)	Reference
trpAgpC.1	*trpA*	5'-AGTTTTATGCCCAGTGCGAG-3'	219	Lescat *et al.,* 2013
trpAgpC.2		5'-TCTGCGCCGGTCACGCCC-3'		Lescat *et al.,* 2013

Table 3.4 PCR primers designated for phylogroup C identification.

Strains showing D or E and E or clade I pattern were screened using E specific primers and PCR reaction was performed as described above, except for annealing time and temperature (20 s at 57°C).

Primer ID	Target	Primer sequence	PCR product (bp)	Reference
ArpAgpE.f	*arpA*	5'-GATTCCATCTTGTCAAAATATGCC-3'	301	Lescat *et al.,* 2013
ArpAgpE.r		5'-GAAAAGAAAAAGAATTCCCAAGAG-3'		Lescat *et al.,* 2013

Table 3.5 PCR primers designated for phylogroup E identification.

Strains designated clade I-clade II and clade III -clade IV-clade V were tested with multiplex PCR for cryptic *E. coli* lineages (Clermont *et al.*, 2011).

PCR reaction was carried out in a 10 µl volume containing 5 µl REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, St Louis, MO, USA), 0.25 µl of primers (20 pmol each) and 1.5 µl of DNA.

PCR reaction was performed under the following conditions: denaturation 4 min at 94°C, 30 cycles of 5 s at 94°C, 30 s at 63°C, 30 s at 72°C and a final extension step of 5 min at 72°C.

PCR products were loaded onto a 2% agarose gel stained containing pUC19 DNA/MspI (Hpall) Marker (Thermo Fisher Scientific, Waltham, USA) and run in 1X TBE buffer at 100 V for 1 h.

Primer ID	Target	Primer sequence	PCR product (bp)	Reference	clade
aesI.1	*aesI*	5'-ATGGTACCGGACGAACCAAC-3'	315	Clermont *et al.,* 2011	1
aesI.2		5'-TGCCGCCAGTACCAAAGACA-3'		Clermont *et al.,* 2011	
aesII.1	*aesII*	5'-CAAACGTGAAGTGTCAGGAG-3'	125	Clermont *et al.,* 2011	2
aesII.2		5'-AATGCGTTCCTCAACCTGTG-3'		Clermont *et al.,* 2011	
chuIII.1	*aesIII*	5'-CACTATTCGTAAGGTCATCC-3'	183	Clermont *et al.,* 2011	3
chuIII.2		5'-AGTTTATCGCTGCGGGTCGC-3'		Clermont *et al.,* 2011	
chuIV.1	*aesIV*	5'-AACGCTATTCGCCAGCTTGC-3'	461	Clermont *et al.,* 2011	4
chuIV.2		5'-TCTCCCCATACCGTACGCTA-3'		Clermont *et al.,* 2011	
chuV.1	*aesV*	5'-CGGCGATAAAGACATCTTCAC-3'	600	Clermont *et al.,* 2011	5
chuV.2		5'-GCAACGCGGCCTGGCGGAAG-3'		Clermont *et al.,* 2011	

Table 3.6 PCR primers designated for cryptic lineages identification.

3.1.5 Antimicrobial susceptibility testing

3.1.5.1 Agar disk diffusion method

Susceptibility to 12 different antimicrobials was evaluated with Kirby-Bauer disk agar diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (CLSI) (CLSI, 2018). The antimicrobial panel was chosen considering the importance of antimicrobial classes in the treatment of human and animal infections and the intrinsic resistance of *E. coli* (EUCAST, 2016).

E. coli strains were streaked on TSA (Oxoid, Basington, UK) and incubated 18 – 24 h at 37 ± 1°C. Colonies were collected and suspended in a physiological solution to a density of 0.5 McFarland turbidity standard. Sterile cotton swabs were used to streak the suspension onto Mueller - Hinton (Oxoid, Basington, UK) agar plates. Commercially prepared antimicrobial agent disks were placed on the inoculated plates which were then incubated aerobically for 16 – 18 h at 35 ± 2°C. The diameters of the growth inhibition zones were measured by using a precision caliper. *E. coli* ATCC 25922 was used as positive control.

Results were interpreted referring to the epidemiological cut-off values (ECOFFs) for *E. coli* proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org) and, when not present, to the EUCAST clinical breakpoints for

Enterobacteriaceae (EUCAST, 2019) or finally to the CLSI clinical breakpoints for *Enterobacteriaceae* (CLSI, 2004; CLSI, 2017; CLSI, 2018).

Antimicrobial agent	Disc drug concentration (µg)	Diffusion zone breakpoint/ECOFF	Note
Nalidixic acid (NA)	30	≥19	c.b.; CLSI, 2017
Ampicillin (AMP)	10	≥14	ECOFF (02/10/2019)
Ceftazidime (CAZ)	10	≥22	ECOFF (02/10/2019)
Ceftiofur (EFT)	30	≥21	c.b.; CLSI, 2004
Chloramphenicol (C)	30	≥17	ECOFF (02/10/2019)
Enrofloxacin (ENR)	5	≥21	c.b.; CLSI, 2004
Gentamicin (CN)	10	≥16	ECOFF (02/10/2019)
Meropenem (MEM)	10	≥25	ECOFF (02/10/2019)
Streptomycin (S)	10	≥15	c.b.; CLSI, 2018
Sulfisoxazole (ST)	300	≥17	c.b.; CLSI, 2018
Tetracycline (TE)	30	≥15	c.b.; CLSI, 2018
Trimethoprim-sulfisoxazolo (SXT)	25	≥16	ECOFF (02/10/2019)

Table 3.7 Antimicrobial panel used in the study.
c.b.: clinical breakpoint. Associated to the ECOFFs is shown the data of the last EUCAST website consultation.

3.1.5.2 ESBL producers

The combination disk test (EUCAST, 2017) was performed for the evaluation of ESBL producing *E. coli*. Briefly, the isolates were tested for cephalosporins (ceftazidime and cefuroxime) alone and in combination with clavulanic acid using Kirby Bauer disk agar diffusion method.

The inhibition zones of the cephalosporins alone and combined with clavulanic acid were compared. The test was considered positive when an increase ≥ 5 mm in zone diameter was observed in the presence of clavulanic acid, compared with the cephalosporin alone.

Antimicrobial agent	ESBL screening	Antimicrobial agent	ESBL confirmation
ceftazidime (10µg)	inhibition zone ≤21	ceftazidime (30µg) + clavulanic acid (10µg)	≥5 mm increase in inhibition zone respect antimicrobial
cefurozime (5µg)	inhibition zone ≤20	cefuroxime (30µg) + clavulanic acid (10µg)	≥5 mm increase in inhibition zone respect antimicrobial

Table 3.8 Screening and confirmation methods for phenotypic detection of ESBLs.

3.1.5.3 Colistin minimum inhibitory concentration (MIC)

Colistin MIC was evaluated through customized Sensititre™ 96-well microtitre plates (Trek Diagnostic Systems, East Grinstead, UK) manufactured to follow CLSI-EUCAST recommendations (EUCAST, 2016b).

Bacteria were grown on TSA (Oxoid, Basington, UK) for 18-24 h at 37 ± 1°C. Colonies were collected and suspended in deionized water to a density of 0.5 McFarland. Bacterial suspensions (2.3 µl) were placed in 2.5 ml Mueller - Hinton Broth cation adjusted (Oxoid, Basington, UK) and mixed by vortexing. Fifty microliters of the final suspension were put into all wells of the same stripe within 30 minutes after its preparation. Plates were incubated for 18 – 24 h at 35 °C. *E. coli* ATCC 25922 was used as positive control.

For each sample the first well without a visible bacterial grown was considered as MIC. Results were interpreted referring to the epidemiological cut-off value (ECOFF= 2 mg/L) for *E. coli* proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org; last accessed October 2019)

3.2 Results

3.2.1 Antimicrobial resistance phenotypes

Resistance to at least one antimicrobial agent was observed in 229/288 (79.5%) strains, of which 107/229 (46.7%) were considered MDR, according to the definition of Magiorakos *et al.* (2012). (Magiorakos *et al.*, 2012)

The most common phenotypic AMR pattern included resistance to streptomycin (148/288; 51.4%), sulfisoxazole (124/288; 43.1%), gentamicin (103/288; 35.8%), ampicillin (86/288; 29.9%) and tetracycline (85/288; 29.5%). Important resistance rate were observed to trimethoprim/sulfamethoxazole (59/288; 20.5%), nalidixic acid (56/288; 19.4%), enrofloxacin (44/288; 15.3%), chloramphenicol and ceftazidime (24/288; 8.3% both) and ceftiofur (19/288; 6.6%). Lastly, meropenem resistance was reported in 2 (0.7%) strains from swine (1) and human (1) origin.

Twenty four isolates out of 288 strains were positive in the ESBL screening and therefore suitable for ESBL confirmation testing according to EUCAST guidelines (EUCAST, 2017). Twenty-tree presumptive ESBL strains were confirmed, mostly associated to food-producing animal/food (5 beef, 4 poultry, 3 vegetable, 2 fishery, 2 dairy, 1 swine isolates) and, to a lesser extent, to humans (5 isolates) and companion animal (1 isolate). Notably, 18/23 (78.3%) ESBL producers were MDR (from 3 up to 8 different antimicrobial classes). Colistin resistance was observed in 4/288 (1.4%) strains (2 swine, 1 rabbit and 1 poultry isolates).

Considering the different sources investigated, the mean number resistance phenotypes was: rabbit, 6; poultry, 5; dairy and human, 3; beef, vegetable, fishery, companion animal, swine, wild animal, 2; wild boar and mollusc, 1.

Interestingly, resistance to aminoglycosides, sulfonamides, ampicillin and tetracycline was widely diffused among all sources investigated.

Notably, rabbit and poultry were the niches displaying the most extensive AMR, with all isolates (100%) resistant to at least one antimicrobial agent. Moreover, most of the strains (25/25, 100% in

Source	R ≥ 1	MDR	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
dairy	21 (84%)	9 (36%)	5 (20%)	3 (12%)	3 (12%)	2 (8%)	3 (12%)	3 (12%)	16 (64%)	15 (60%)	0 (0%)	12 (48%)	5 (20%)	8 (32%)	0 (0%)	2 (8%)
beef	21 (84%)	9 (36%)	8 (32%)	1 (4%)	1 (4%)	5 (20%)	2 (8%)	3 (12%)	15 (60%)	12 (48%)	0 (0%)	7 (28%)	3 (12%)	4 (16%)	0 (0%)	5 (20%)
wild boar	14 (56%)	3 (12%)	2 (8%)	2 (8%)	0 (0%)	1 (4%)	0 (0%)	2 (8%)	13 (52%)	5 (20%)	0 (0%)	3 (12%)	1 (4%)	2 (8%)	0 (0%)	0 (0%)
vegetable	16 (64%)	5 (20%)	3 (12%)	0 (0%)	4 (16%)	3 (12%)	0 (0%)	0 (0%)	12 (48%)	9 (36%)	0 (0%)	8 (32%)	1 (4%)	1 (4%)	0 (0%)	3 (12%)
fishery	17 (68%)	5 (20%)	4 (16%)	0 (0%)	1 (4%)	2 (8%)	2 (8%)	0 (0%)	10 (40%)	11 (44%)	0 (0%)	10 (40%)	2 (8%)	5 (20%)	0 (0%)	2 (8%)
companion animal	5 (38.5%)	4 (30.8%)	5 (38.4%)	1 (7.7%)	1 (7.7%)	1 (7.7%)	1 (7.7%)	1 (7.7%)	2 (15.4%)	3 (23.1%)	0 (0%)	4 (30.8%)	3 (23.1%)	4 (30.8%)	0 (0%)	1 (7.7%)
swine	20 (80%)	8 (32%)	6 (24%)	3 (12%)	2 (8%)	1 (4%)	2 (8%)	2 (8%)	7 (28%)	11 (44%)	1 (4%)	7 (28%)	4 (16%)	11 (44%)	2 (8%)	1 (4%)
poultry	25 (100%)	20 (80%)	21 (84%)	15 (60%)	1 (4%)	4 (16%)	8 (32%)	13 (52%)	15 (60%)	17 (68%)	0 (0%)	17 (68%)	12 (48%)	13 (52%)	1 (4%)	4 (16%)
rabbit	25 (100%)	25 (100%)	10 (40%)	19 (76%)	0 (0%)	0 (0%)	2 (8%)	11 (44%)	11 (44%)	18 (72%)	0 (0%)	24 (96%)	22 (88%)	24 (96%)	1 (4%)	0 (0%)
mollusc	19 (76%)	3 (12%)	12 (48%)	1 (4%)	1 (4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (16%)	0 (0%)	12 (48%)	0 (0%)	2 (8%)	0 (0%)	0 (0%)
human	24 (96%)	9 (36%)	6 (24%)	7 (28%)	4 (16%)	5 (20%)	3 (12%)	5 (20%)	2 (8%)	23 (92%)	1 (4%)	10 (40%)	5 (20%)	6 (24%)	0 (0%)	5 (20%)
wild animal	22 (88%)	7 (28%)	4 (16%)	4 (16%)	1 (4%)	0 (0%)	1 (4%)	4 (16%)	0 (0%)	20 (80%)	0 (0%)	10 (40%)	1 (4%)	5 (20%)	0 (0%)	0 (0%)
Total	229 (79.5%)	107 (37.2%)	86 (29.9%)	56 (19.4%)	19 (6.6%)	24 (8.3%)	24 (8.3%)	44 (15.3%)	103 (35.8%)	148 (51.4%)	2 (0.7%)	124 (43.1%)	59 (20.5%)	85 (29.5%)	4 (1.4%)	23 (8%)

Table 3.9 Resistance rate to the antimicrobial panel in the sources investigated.

R≥1: percentage of strain resistant to at least one antimicrobial; MDR: multiresistant isolate; AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; SXT: trimethoprim/sulfamethoxazole, TE: tetracycline; COL: colistin; ESBL: extendes pectrum beta-lactamase profile.

rabbit; 20/25, 80% in poultry) were MDR from 3 up to 8 different antimicrobial classes. In these sources, nalidixic acid (15/25, 60%, poultry; 19/25, 76%, rabbit) and enrofloxacin (13/25, 52%, poultry; 11/25, 44%, rabbit) resistance was the highest.

Beef (6/25, 24%), human (5/25, 20%), poultry (5/25 strains, 20%) and vegetable (5/25, 20%) were the sources with the highest resistance to 3rd generation cephalosporins (3-GCs). Moreover, they carried the highest number of ESBL producers (see above).

Notably, swine source showed the highest number of strains (2) resistant to colistin. Also resistance to other HP-CIAs (3-GCs, 2/25, 8%; quinolone/fluoroquinolone, 6/25, 24%; meropenem, 1/25, 4%; ESBL producers, 1/25, 4%) was observed.

Wild animal, vegetable, fishery and companion animal niches generally displayed low levels of AMR if compared to the other sources. Considering their phenotypic antimicrobial profile, resistance to aminoglycosides, tetracycline, ampicillin and sulfonamides was the most identified. In these niches, low numbers of MDR strains were detected (companion animal, 4/13, 30.8%; wild animal, 7/25, 28%; vegetable, 5/25, 20%; fishery, 5/25, 20%;), though resistance to different HP-CIAs (quinolone/fluoroquinolone, 3-GCs, ESBL profile) was observed in some cases. Wild boars and molluscs exhibited the lowest antimicrobial resistance rate among the collection. Despite the number of isolates showing resistance to at least one antimicrobial agent (14/25, 56%, wild boar; 19/25, 76%, mollusc), only 3 strains (12%) were MDR in both niches. Resistance to HP-CIAs (quinolone/fluoroquinolone, 3-GCs) was detected in 4/25 (16%) wild boar and 2/25 (8%) mollusc strains.

Details of the phenotypic antimicrobial profile in the single niches are present in the Supplementary Material, Table S1-12.

3.2.2 Phylogroups

Eight different phylogroups/clades (A, B1, B2, C, D, E, F and clade I-II) were identified in the study collection. Phylogroup B1 was the most common (131/288, 45.5%), followed by C (42/288, 14.6%), A

(39/288, 13.5%), E (26/288, 9%), B2 (21/288, 7.3%), F (12/288, 4.2%), D (9/288, 3.1%) and clade I-II (2/288, 0.7%). We were not able to assign a phylogroup for 6 of the strains.

Phylogroup B1 was mainly associated to beef (23/25, 92%), rabbit (22/25, 88%), wild animal (18/25, 72%), mollusc (15/25, 60%), vegetable (14/25, 56%), companion animal (6/13, 46.2%) and dairy (9/25, 36%) sources, meanwhile phylogroup A was most common in fishery (10/25, 40%) and human (7/25, 28%) niches. Swine strains were equally associated to phylogroup A (8/25, 32%), B1 (8/25, 32%) and C (8/25, 32%). Phylogroup C was mostly represented in poultry (8/25, 32%), meanwhile phylogroup E was mainly observed in wild boar (11/25, 44%) isolates.

More details are present in the Supplementary Material, Table S1-12.

	Phylogroup								
Source	**A**	**B1**	**B2**	**C**	**D**	**E**	**F**	**clade I-II**	**unknown**
dairy	8	9	2	4	1	1	0	0	0
beef	0	23	0	1	1	0	0	0	0
wild boar	0	3	6	0	0	11	0	2	3
vegetable	1	14	1	6	0	1	0	0	2
fishery	10	5	2	5	0	3	0	0	0
companion animal	0	6	3	2	0	2	0	0	0
swine	8	8	0	8	0	1	0	0	0
poultry	2	6	1	8	0	2	5	0	1
rabbit	0	22	1	0	2	0	0	0	0
mollusc	2	15	0	4	3	1	0	0	0
human	7	2	2	2	2	4	6	0	0
wild animal	1	18	3	2	0	0	1	0	0
Total	39 (13.5%)	131 (45.5%)	21 (7.3%)	42 (14.6%)	9 (3.1%)	26 (9%)	12 (4.2%)	2 (0.7%)	6 (2.1%)

Table 3.10 Representation of the phylogroup distribution in the sources investigated.

3.3 Discussion

Multiple sources (animals, agriculture, human, food) have been investigated as potential reservoir of AMR in the last decades (Anaissie *et al.*, 2002; Ewers *et al.*, 2012; Carroll *et al.*, 2015; Economou and Gousia, 2015; van Schaik, 2015; Madec and Haenni, 2018). Within these niches, resistant bacteria and genes can be gathered, maintained, transmitted to other environments or evolve new genotypic and phenotypic AMR profiles. Understanding the role of different sources in the maintenance, diffusion and evolution of AMR is an essential step for AMR preventive measure implementation and epidemiological evaluation. Commensal *E. coli* are considered AMR indicator among Gram-negative bacteria. They are ubiquitous, living in intestine of warm-blooded animals but also in the environment (Jang *et al.*, 2017). Genomic plasticity represents one of their most important features, allowing acquisition of genetic determinants from other bacteria and the environment. For these reasons, AMR data obtained from commensal *E. coli* are considered emblematic of the overall bacterial population (EFSA, 2019).

This study aims to determine AMR phenotypes in commensal *E. coli* isolated from different environments that may pose a threat to public health. We included sources frequently associated with AMR detection (livestock, food, companion animal, human) as well as sources playing an emerging role in AMR dynamics (wildlife), in order to provide a holistic cross-section of AMR circulating in Italy. (Blaak *et al.*, 2014b)

In this study particular attention was focused on food-producing animals and related food, following the European trend, which since 2014 has included food-producing animals and related food in their monitoring program. Livestock are widely considered to play an important epidemiological role in AMR transmission through direct/indirect contact with humans (Dierikx *et al.*, 2013; Pletinckx *et al.*, 2013), related foodstuff (Davis *et al.*, 2015; Manges, 2016) and environmental manure contamination (Blaak *et al.*, 2014b; van Hoek *et al.*, 2015). In general the role of the food chain in the diffusion of AMR bacteria has been widely investigated in the last years (Wang *et al.*, 2012; Rasheed *et al.*, 2014; Economou and Gousia, 2015; Hudson *et al.*, 2017; Florez-Cuadrado *et al.*, 2018; Yazdankhah *et al.*,

2018). All studies have concluded that resistant bacteria in FPA could increase the AMR gene pool available for foodborne pathogens, influencing the development of new antimicrobial resistance patterns (Verraes *et al.*, 2013).

ECOFFs were used to differentiate wild type (WT) and non-WT isolates since our aim was to determine the epidemiology of AMR in *E. coli* indicator. Non-WT strains related to acquired or mutational resistance to antimicrobials were addressed as "resistant". However, in some cases, clinical breakpoints were adopted, due to the lack of ECOFFs for all molecules tested. Clinical breakpoints represented predictors of clinical success of antimicrobial treatments and could not allow evaluation related to emergence and change in bacterial resistance profile (Bywater *et al.*, 2006; Simjee *et al.*, 2008). However, they could give useful information about consolidated resistance in bacterial population. Moreover, changing in common clinical resistant profiles could indirectly suggest the emergence of new resistance patterns occurring among bacteria.

3.3.1 Collective findings

Generally, sulfonamides, ampicillin, tetracycline and aminoglycosides represented the most common phenotypic resistance among FPAs and food, companion animals, human and wildlife.

In food-producing animals and related food, sulfonamide-ampicillin-tetracycline resistance profile was congruent with the Italian antimicrobial stewardship practice in the food-producing sector. Indeed, these molecules characterised the most sold antimicrobials for livestock in Italy, according to the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) data (EMA, 2019). It should be noted that the ESVAC didn't include all FPA categories (i.e. fishery, mollusc), nor the stratification of antimicrobial consumption data by FPA species. However, the European Medicine Agency (EMA) launched a project in 2018 in order to bridge the lack of knowledge in this field.

High frequency of resistance to sulfonamide-ampicillin-tetracycline in swine, poultry, beef, rabbit and dairy isolates was generally congruent with AMR profiles previously reported in Europe (de Verdier *et al.*, 2012; Dotto *et al.*, 2014; Skockova *et al.*, 2015; EFSA, 2016; EFSA, 2017b; EFSA, 2018; EFSA, 2019).

In human and companion animal sources ampicillin resistance was widely diffused. This finding was not surprising, considering that penicillins represented the most widely used antimicrobial treatments in these niches. Phenotypic resistance to tetracycline and sulfonamides was well represented although these antibiotics are not commonly employed in these categories (ECDC, 2018a; EMA, 2019).

Sulfonamide-ampicillin-tetracycline pattern was also observed in fishery, mollusc and vegetable sources, usually exposed to low or no direct antimicrobial selective pressure (FVE, 2016). The consistency of AMR profile identified in these niches with those observed in the remaining collection (livestock, human, companion animals) suggested an important AMR transmission from settings highly associated to antimicrobial use. In particular, faecal contamination is considered the major path for AMR bacteria, genes and antimicrobial residues diffusion (Guenther *et al.*, 2011; Singer *et al.*, 2016). Sewage and manure might contaminate groundwater and aquatic systems. Therefore, irrigation water and manure (representing a common fertilizer in agriculture) could act as carriers of AMR bacteria and genes, polluting agricultural production (Walczak *et al.*, 2011; Holvoet *et al.*, 2013). In the same way, sewage and runoff from land could be responsible for AMR observed in aquaculture systems (Martinez, 2009; Alves *et al.*, 2014; Baliere *et al.*, 2015). The AMR profile in aquaculture and vegetable was generally in accordance with the ones reported in previous studies in Europe (Campos *et al.*, 2013; Holvoet *et al.*, 2013; Grevskott *et al.*, 2017).

The same considerations related to aquaculture and vegetable could be also extended to wild animal and wild boar sources, showing similar resistance to the remaining collection. Wild animals resistome is strictly associated to AMR diffusion from other environments and, therefore, wildlife has been often considered an AMR indicator (Dolejska *et al.*, 2007; Literak *et al.*, 2010).

Notably, aminoglycosides represented the most common phenotypic resistance in all livestock/food, pets, wildlife and human, despite their seldom use in disease treatments (ECDC, 2018a; EMA, 2019). Our findings are generally consistent with previous studies (Costa *et al.*, 2008b; Skurnik *et al.*, 2008; Guenther *et al.*, 2011; Holvoet *et al.*, 2013; Dotto *et al.*, 2014; Nobili *et al.*, 2016; Grevskott *et al.*, 2017), despite low level of aminoglycoside resistance have been usually reported in pig, poultry and beef (EFSA, 2016; EFSA, 2017b; EFSA, 2018; EFSA, 2019).

The wide diffusion of resistance to antimicrobials not commonly (tetracycline and sulfonamides in human and companion animals) or rarely (aminoglycoside in animals and human) used (ECDC, 2018a; EMA, 2019) in different environments could suggest important environmental pollution of resistant bacteria and genes related to these molecules.

In particular, tetracycline and sulfonamides represented the most sold antimicrobial classes in food-producing animal systems (EMA, 2019), associated to the major amount of antimicrobials overall used (EFSA, 2017a). The high selective pressure deriving from the agricultural environment could have led to the diffusion of sulfonamide and tetracycline resistant bacteria and genes to other ecosystems (human, companion animals, wildlife).

High aminoglycoside resistance is not explainable considering the seldom employment of these antimicrobials. The wide resistance is probably referable to an efficient and active circulation of related ARGs in the bacterial population. In particular, aminoglycosides resistance genes *strA/B* and *aadA* are commonly identified in *Enterobacteriaceae* of human and animal origin (Pezzella *et al.*, 2004) and might be responsible for phenotypic aminoglycoside resistance identified in our collection. However, the genetic mechanisms involved in aminoglycosides resistance are multiple and the events leading to the predominance and spread of specific ARGs are complex. The understanding of aminoglycoside resistance epidemiology needs to be investigated with molecular/sequencing approaches.

HP-CIAs (including 3-4-5 GCs, quinolones, polymyxins and carbapenems) represent the last line therapeutic options for serious human infection disease (WHO, 2019). Concerningly, resistance to these antimicrobials was observed in the collection.

Among livestock and food, beef, poultry and vegetable were the sources showing the highest number of 3-GCs resistant strains and ESBL producers. The identification of this phenotypic AMR profile in these sources has already been observed in different studies, despite in a low number of isolates (Egea *et al.*, 2011; Reuland *et al.*, 2014; EFSA, 2016; Araujo *et al.*, 2017; EFSA, 2017b; EFSA, 2018; EFSA, 2019).

Similar findings were also observed in human sources, with 5 isolates showing 3-GCs resistance and ESBL profile. These data are particularly worrisome, considering the increasing trend in 3-GCs resistance in EU, with Italy as one of the countries with the highest resistance rate in clinical *E. coli* of human origin (ECDC, 2018b).

Notably, one swine and one human strain were resistant to meropenem. Although carbapenem resistance is rarely observed in Europe (EFSA, 2017b; ECDC, 2018b; EFSA, 2019), its discovery is particularly worrisome. Indeed, carbapenemases may confer resistance to most beta-lactams (Nordmann *et al.*, 2012). Moreover, carbapenemase producing *Enterobacteriaceae* infections have been associated with high mortality rates (Bratu *et al.*, 2005; Marchaim *et al.*, 2008; Souli *et al.*, 2010).

Resistance to quinolone/fluoroquinolone was mostly observed in rabbit (19) and poultry (15) strains and was notably lower in the other sources investigated. Our findings are in accordance with those reported by other authors, underlying the frequent identification of quinolone/fluoroquinolone resistance among broiler/related meat and rabbit in Europe and Italy (Dotto *et al.*, 2014; EFSA, 2016; EFSA, 2017b; EFSA, 2018). These data could be explained considering the common antimicrobial treatments employed in these producing sectors (FVE, 2016).

Polymyxins represent one of the few treatment options to cure infections deriving from carbapenemase-producing Gram-negative bacteria (Poirel *et al.*, 2017). Colistin resistance was exclusively observed in livestock and related foodstuff (2 swine faeces, 1 rabbit intestine, 1 poultry meat) and its identification was seldom, in accordance with the data reported in *E. coli* of food-producing animal/food origin in Europe (Dotto *et al.*, 2014; EFSA, 2016; EFSA, 2017b; EFSA, 2018; EFSA, 2019).

As expected, phylogroup B1, C and A were the most common among the collection, characterising 212/288 (73.6%) strains. Indeed, phylogroup A and B1 are usually associated to environmental and commensal *E. coli*, meanwhile phylogroup C is closely related and shares the same ecological niches of phylogroup B1 (Gordon *et al.*, 2008; Clermont *et al.*, 2013). Interesting, 30 strains of different origin were associated to phylogroup B2 and D, usually identified in pathogens (Coque *et al.*, 2008).

Hereafter, we present important findings observed in some of the sources investigated.

3.3.2 Rabbit

Notably, rabbit was the source mostly associated to AMR. According to the common AMR profile (Dotto *et al.*, 2014) and antimicrobial stewardship (SSR-ER, 2006; FVE, 2016) reported in rabbit farms in Italy, resistance to tetracycline, aminoglycosides, quinolones and sulfamethoxazole/trimethoprim was widely diffused. Concerningly, one rabbit strain was colistin resistant. Despite polymyxins represented seldom antimicrobial treatments in rabbit farms (SSR-ER, 2006; FVE, 2016), a recent Italian study (Agnoletti *et al.*, 2018) underlined their wide use in rabbit producing sector. Interestingly, different recent investigations (Agnoletti *et al.*, 2018; Freitas-Silva *et al.*, 2018) associated colistin resistance to breeding rabbit, suggesting its possible role in colistin resistance diffusion.

In the past, little attention was focused on breeding rabbit system because of its rare association with foodborne pathogens (Dalle Zotte, 2002; Rodriguez-Calleja *et al.*, 2006). Therefore, limited European microbiological studies from healthy rabbits and related foodstuff are available (Garcia and Fox, 2003; Rodriguez-Calleja *et al.*, 2004; Kohler *et al.*, 2008; Marín *et al.*, 2016). Similarly, AMR investigations are lacking, especially in *E. coli* indicator (Dotto *et al.*, 2014; Medina *et al.*, 2015; Agnoletti *et al.*, 2018; Freitas-Silva *et al.*, 2018).

In our study, the important AMR associated to this niche suggests rabbit as en emerging reservoir of AMR (also to HP-CIAs) and underlines the need to deepen and clarify its epidemiological role in AMR diffusion. (Johnson *et al.*, 2007; Leverstein-van Hall *et al.*, 2011)

3.3.3 Poultry

Poultry was the second source showing the highest antimicrobial resistance. The phenotypic resistance profile reflected common European and Italian antimicrobial treatments in the poultry production system (SSR-ER, 2006; FVE, 2016). AMR pattern was generally in accordance with those

identified in poultry and related meat in Europe (EFSA, 2016; EFSA, 2018), despite higher 3GCs resistance and ESBL profile were observed in our isolates.

Concerningly, poultry was the niche mostly associated to HP-CIAs resistance (especially to quinolone/fluoroquinolones,3-GCs, ESBL profile) among the collection.

These data are particularly worrisome, considering that the potential transmission of AMR *E. coli* from poultry/related foodstuff to human represented a feasible event. Indeed, different authors (Johnson *et al.*, 2007; Jakobsen *et al.*, 2010; Leverstein-van Hall *et al.*, 2011) observed similarities in AMR isolates of human and poultry origin, suggesting the latter as a source of AMR. The potential role of poultry meat (widely included in our study) in the transmission of AMR (also to HP-CIAs) has been described by different authors (Mesa *et al.*, 2006; Doi *et al.*, 2010; Overdevest *et al.*, 2011; Ghodousi *et al.*, 2015; Hasman *et al.*, 2015). Faecal contamination of carcasses during slaughtering procedures has been entailed in bacterial poultry meat contamination (Alonso *et al.*, 2011), including potential pathogens (Mor-Mur and Yuste, 2010; Lyhs *et al.*, 2012; Noori and Alwan, 2016).

Our findings highlighted an association between poultry source (particularly meat) and HP-CIAs resistant bacteria, which may pose a potential AMR risk to human health.

3.3.4 Beef

Considering livestock and related food, beef was the source mostly associated with 3-GCs resistance and ESBL producers (5 isolates), despite lower trend has usually been described in Europe (EFSA, 2017b; EFSA, 2019). Our data could be explained, considering that 3/4-GCs represented some of the most used antimicrobials in the cattle breeding system (FVE, 2016).

Beef ESBL strains derived from carcass samples obtained during slaughtering procedures. Different studies identified ESBL producer *E. coli* in cattle at abattoirs (Geser *et al.*, 2011; Reist *et al.*, 2013; Haenni *et al.*, 2014), but also from related meat in stores (Jensen *et al.*, 2006). Slaughtering procedures represented an important risk factor for carcass contamination, determining the diffusion of AMR and virulent bacteria in the food chain. In particular faeces and hides have been associated to carcasses

bacterial pollution (Koutsoumanis and Sofos, 2004; Mather *et al.*, 2008), including with important human pathogens (Elder *et al.*, 2000).

Our findings suggest possible contamination of beef meat with faecal bacteria at the abattoir and imply the role of beef meat in the diffusion of 3-GC resistant and ESBL bacteria. The observation of stringent hygiene measures during slaughtering procedures is essential to ensure microbiological beef meat quality and public health protection, avoiding diffusion of AMR bacteria and potential pathogens through the food chain.

3.3.5 RTE vegetable

Despite vegetables are not exposed to a direct antimicrobial selective pressure, important AMR has been observed. Concerningly, all ESBL and most MDR strains of vegetable origin were isolated from ready-to-eat (RTE) products. RTE- vegetables have already been associated to ESBL producers/genes, and therefore suggested as potential ESBL reservoir (Egea *et al.*, 2011; Campos *et al.*, 2013; Reuland *et al.*, 2014; Said *et al.*, 2015). These products represented one of the most diffused food commodities, due to their convenience and popularity among consumers. Indeed RTE vegetables are not intended to undergo a terminal heating or washing step prior to consumption (Rodriguez-Caturla *et al.*, 2012) and, for this reason, could represent an important source of AMR bacteria and genes. Bacterial recovery in vegetables derived mostly from insufficiently treated water and fertilizers in primary production (Mercanoglu Taban and Halkman, 2011; Schwaiger *et al.*, 2011) or from improper hygienic measures during handling and processing in the food chain (Rasheed *et al.*, 2014).

In particular, RTE products are processed in numerous steps (including a selection from the fresh product, peeling, cutting, washing, drying and packaging) (De Oliveira *et al.*, 2011), all representing a possible risk for bacterial contamination.

The involvement in human outbreaks (Mercanoglu Taban and Halkman, 2011) and the frequent association to ESBL profile, underline the potential risk posed by RTE products and the importance of additional washing steps before their consumption.

3.3.6 Wildlife

According to different studies (Costa *et al.*, 2008b; Guenther *et al.*, 2010b; Guenther *et al.*, 2011; Marinho *et al.*, 2014; Giacopello *et al.*, 2016), aminoglycosides, tetracycline, sulfonamides and ampicillin resistance was widely identified among our wildlife isolates.

HP-CIAs resistance was observed, in particular to quinolone/fluoroquinolone (wild boar, 3; wild birds, 4), as reported by other authors (Costa *et al.*, 2008b; Giacopello *et al.*, 2016). Instead, only two isolates showed resistance to 3-GCs (1 wild boar and 1 wild animal strains).

Wildlife AMR profile was generally concordant to those observed in human/livestock/pet. Wildlife ecosystem is not exposed to direct antimicrobial selective pressure. Remoteness of the area, livestock and human density (Allen *et al.*, 2010) and association degree with human activity (Skurnik *et al.*, 2006; Allen *et al.*, 2010) represented essential elements conditioning bacteria antimicrobial resistance observed in wild animals. Our findings, as reported by other authors (Dolejska *et al.*, 2007; Literak *et al.*, 2007), suggest wildlife as an important indicator of AMR pollution, giving useful information about AMR circulating in human related environments.

3.3.7 Companion animals

AMR was observed in different strains (5/13; 38.5%) of companion animal origin. Most of these isolates (4/5) were MDR from 3 up to 9 different antimicrobials. Concording with previous studies (Costa *et al.*, 2008a; Damborg *et al.*, 2008), low HP-CIAs (quinolones and 3-GCs) resistance was observed. Only one ESBL isolate was identified, showing an important AMR profile (resistance to 9 different antimicrobials).

The resistance rate observed in pets is worrisome. Cat and dog usually share the same household and are in strict contact with humans. This could possibly allow transmission of bacteria (Guardabassi *et al.*, 2004b; Costa *et al.*, 2008b). Pets have been already recognised as a possible source of pathogens for humans (Johnson *et al.*, 2001; Damborg *et al.*, 2008).

The possibility to be a simultaneous reservoir of AMR bacteria and genes is a feasible event and needs to be investigated with further studies. Despite the potential threat represented by pets, this animal

category is not included in European AMR surveillance. In light of the evidence suggesting pet involvement in AMR maintenance and diffusion, further studies and constant monitoring of this niche should to be implemented, in order to elucidate its involvement in AMR transmission to humans.

3.3.8 Human

Concerningly, human represented the source (together with beef) associated to the highest number of ESBL producers. Different studies reported carriage of ESBL *E. coli* in healthy human, with an increasing trend during the years (Valverde *et al.*, 2004; Rodríguez-Baño *et al.*, 2008; Guimaraes *et al.*, 2009; Vinué *et al.*, 2009; Hammerum *et al.*, 2011; Karanika *et al.*, 2016; van Duijkeren *et al.*, 2018).

These data suggest healthy humans as an emergent reservoir of ESBL producers, probably depending on the wide use of beta-lactams in both community and hospital settings (ECDC, 2018a).

Notably, human ESBL strains showed also resistance to other HP-CIAs (2 strains to quinolone/fluoroquinolone; 1 isolate to meropenem), underlying the possible difficulties in antimicrobial treatment in case of infection.

The potential threat represented by human as ESBL reservoir is concerning, considering that infections are often preceded by asymptomatic carriage (Ny *et al.*, 2017). Moreover, these bacteria could easily be diffused in the community or be transmitted to human related animals. It has been shown that ESBL strains could persist at least for 8 months in healthy humans, increasing the possibility of diffusion (van Duijkeren *et al.*, 2017).

The last ECDC report underlined the high detection of cephalosporin resistance (mainly associated to ESBL producers) in clinical *E. coli* isolated from humans in Europe, with Italy as one of the countries showing the highest resistance rate (ECDC, 2018b). The increasing growth of ESBL problem suggests the need for constant monitoring in *E. coli* indicator isolated from healthy humans, which nowadays is not performed.

In conclusion, our findings provided a general overview of phenotypic AMR circulating in different environments in Italy. We are aware that unfortunately the low sample size of each niche (13 strains

for companion animals, 25 isolates for all the other sources) reduces the representativeness of our data and prevents the assessment of possible AMR transmission paths between different niches.

Briefly, the role of food-producing animal and food chain in AMR diffusion has been reiterated. Notably, poultry and rabbit niches have been associated to the highest antimicrobial resistance rate in our collection, including to HP-CIAs. Beef, vegetable and human isolates have been suggested as possible sources of ESBL producers. One of the main concern was the involvement of healthy human in ESBL transmission, considering the increasing resistance to 3-GCs in human clinical isolates in Europe. Also the role of companion animals in AMR maintenance and diffusion has been hypothesised. AMR observed in wildlife, aquaculture and vegetable suggests an important antimicrobial environmental pollution, spreading also in ecosystems not directly related to antimicrobial use. In particular, our findings reiterated the role of wildlife as AMR indicator, supplying important information about AMR circulating in human and agricultural settings.

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4. AMR genotypic characterisation

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4.1 Paper: *Escherichia coli* from companion animals, livestock, wildlife and food as potential sources of antimicrobial resistance and virulence associated gene

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4.1.1 Abstract

Animals, human and food are all interconnected sources of antimicrobial resistance, allowing extensive and rapid exchange of AMR bacteria and genes. AMR monitoring in these niches represents an essential step for preventive measure implementation and epidemiological evaluation.

Here, we report whole-genome sequence analysis of 279 *Escherichia coli* indicators isolated from animals (livestock, companion animals, wildlife), food and human in Italy.

Strain collection was predominantly associated to phylogroup B1 (46.6%) and A (29%). One hundred and thirty-six STs were observed, including different pandemic (ST69, ST95, ST131) and emerging (ST10, ST23, ST58, ST117, ST405, ST648) ExPEC lineages. Eight ARGs and 4 chromosomal mutations conferring resistance to HP-CIAs were identified (*qnrS1*, *qnrB19*, *mcr1*, $bla_{\text{CTX-M1,15,55}}$, $bla_{\text{CMY-2}}$, *gyrA/parC/parE* and *pmrB*). Twenty-two class 1 integron arrangements in 34 strains were characterised and 11 ARGs were designated as *intI1* related gene cassettes (*aadA1*, *aadA2*, *aadA5*, *aad23*, *ant2_Ia*, *dfrA1*, *dfrA7*, *dfrA14*, *dfrA12*, *dfrA17*, *cmlA1*).

Most *intI1* positive strains belonged to rabbit (38%) and poultry (24%) sources. Virulence-associated genes were usually typical of ExPEC pathotype.

Three rabbit samples carried the *mcr1* colistin resistance gene in association with IS*6* family insertion elements. Poultry meat harboured some of the most prominent ExPEC STs, including ST131, ST69, ST10, ST23, and ST117. Wildlife showed a high average number of VAGs (mean=10), mostly associated to ExPEC pathotype, and some predominant ExPEC lineages (ST23, ST117, ST648).

Our study reiterated the role of food-producing animals as a reservoir of potential zoonotic pathogens, with variable antimicrobial resistance and virulence traits among the sources investigated. Moreover, our findings suggest the potential role of wildlife in the maintenance and diffusion of virulence determinants.

Keywords: commensal *E. coli*, One Health, antimicrobial resistance, virulence, microbial genomic epidemiology.

4.1.2 Introduction

Antimicrobial resistance has been recognised as one of the world's most pressing public health problems, affecting both human and veterinary medicine. In recent decades we have witnessed a dramatic spread and diffusion of MDR pathogens. In the context of human medicine, the decrease of

bacterial susceptibility to critically important antimicrobials (CIAs) and the quick diffusion of ESBL producers are of main concern (Roca *et al.*, 2015).

The consequences of MDR are particularly severe and characterised by an increase in infection severity, treatment failure, hospitalisations and mortality, with growing costs for health care. It has been estimated that AMR causes 700,000 deaths every year globally, with 25,000 victims and over €1.5 billion in healthcare costs only in the European Union (EFSA, 2017a).

The 3 predominant processes involved in AMR maintenance and diffusion are the emergence, invasion and occupation by ARGs of significant environments for human health (Baquero *et al.*, 2015). In this context, HGT plays a primary role in the exchange of genetic material amongst bacteria and drives rapid evolution, particularly under selective pressure. HGT of ARGs is mediated by mobile genetic elements and facilitated by the interconnection between bacteria, their hosts and environments.

Animal (livestock, companion animals, wildlife), community, hospital and industrial settings (including food-production) have been investigated in the past as possible sources of AMR (Anaissie *et al.*, 2002; Ewers *et al.*, 2012; Economou and Gousia, 2015; van Schaik, 2015; Madec and Haenni, 2018). The interaction between these environments is very complex and understanding vectors of AMR transfer is an ongoing area of investigation. Municipal, agricultural and industrial wastewater, aquaculture, animal manure and sewage sludge have been recognized as major pathways of AMR transmission between different ecosystems (Singer *et al.*, 2016).

Data describing AMR spatial/temporal trends and related sources are essential for epidemiological evaluation and preventive measure implementation. In this context, the EU has defined AMR as a special health issue that has to be covered by epidemiological surveillance (European Commission, 2018).

Commensal *E. coli* is recognised as a good indicator of AMR in Gram-negative bacterial population. The reasons derive from its wide diffusion in animal and human feces and their high genetic plasticity, allowing the frequent acquisition of antimicrobial genetic determinants from other enteric bacteria and the environment. Moreover, *E. coli* also represents important pathogens, usually associated with

enteric and extraintestinal infections (e.g. urinary tract infections, meningitis and septicaemia) (Kaper *et al.*, 2004).

In Europe, extensive genomic evaluation of ARGs, VAGs and their association with MGEs in non-clinical *E. coli* is generally lacking. Most studies focus on phenotypic AMR analysis in limited sources (poultry, pig and cattle), as opposed to genomic characterisation (Bywater *et al.*, 2004; Kaesbohrer *et al.*, 2012; Hanon *et al.*, 2015; Hesp *et al.*, 2019). When implemented, genotypic evaluation is mainly performed by PCR, without considering the comprehensive mobile antimicrobial genetic structures involved (Domínguez *et al.*, 2002; Österberg *et al.*, 2016; Perrin-Guyomard *et al.*, 2016; El Garch *et al.*, 2018; Ceccarelli *et al.*, 2019). This is particularly evident in Italian studies (Bortolaia *et al.*, 2010; Dotto *et al.*, 2014; Ghodousi *et al.*, 2015; El Garch *et al.*, 2018).

Further investigations are needed to address the lack of knowledge in AMR dissemination and epidemiology in *E. coli* indicators from different environments in Europe and in particular in Italy. Here, we presented WGS of 288 *E. coli*, isolated from different potential AMR sources (including livestock, companion animals, wildlife, food and human) in Italy. We provide data related to their phylogenetic diversity, carriage of ARGs, VAGs, biocide resistance genes and their association with mobile genetic elements.

4.1.3 Materials and methods

4.1.3.1 Sampling

In the period between November 2016 and July 2018 a total of 288 commensal *E. coli* were collected from 12 different food, animal and human sources (dairy, beef, wild boar, rabbit, poultry, swine, vegetable, fishery, mollusc, wild animal and human), mainly in the Emilia Romagna region of Italy.

Food samples (chicken-rabbit-swine meat products, vegetable and fishery) were collected from major supermarkets located in the province of Bologna and from the educational abattoir of the Veterinary Sciences Department (University of Bologna), during slaughtering procedures (sponge of beef carcasses).

Among animal samples, faeces (cat and dog) and cloacal swabs (poultry) were collected from healthy individuals, that had not taken antimicrobials in the month prior to the collection. Wild boar diaphragm samples were supplied by Istituto Zooprofilattico Sperimentale of Bologna.

Human faeces were collected from sound volunteers that were approached for recruitment in person. These participants had not had any antimicrobial treatments in the month prior the collection.

All food, animal and human samples were carried to the laboratory in aseptic conditions and analysed within 2 hours from the time of purchase.

Twenty-five *E. coli* isolated from milk, cheese and milking system filter were provided by the Service of Food Security, Department of Veterinary Science, University of Bologna. Istituto Zooprofilattico Sperimentale of Bologna, Forlì and Reggio Emilia contributed to the strain collection with 99 hypothetical *E. coli*, isolated from different animal sources (mollusc, wild animal, rabbit, beef, companion animal, swine). Each source was characterised by 25 *E. coli* isolates with the exception of companion animals, consisting of 13 strains only.

More details about strain collection are provided in Supplementary Material, Table S13.

4.1.3.2 Bacterial isolation

20 g of food sample (in the case of carcass sponge, each one separately) were placed into sterile blender bags, diluted in 180 ml of sterile EC-Broth (Oxoid, Basington, UK) and macerated in a stomacher for 1 minute. Samples were incubated overnight at 37 ± 1°C. Faecal samples (1 g each) were diluted (1:10) in peptone water (Oxoid, Basington, UK) and homogenated by vortexing.

Ten µl of overnight culture in EC-Broth (Oxoid, Basington, UK) and 10 µl of faeces solution (or directly in the case of the cloacal swabs) were streaked onto MacConkey (Oxoid, Basington, UK) and Levine (Oxoid, Basington, UK) agar plates and incubated for 18 – 24 h at 37 ± 1°C. For all the samples, lactose fermenting colonies were collected and assessed for Gram stain and standard biochemical test (indole probe). *E. coli* ATCC 25922 was used as a control strain.

4.1.3.3 Storage

All strains were freshly cultured on TSA (Oxoid, Basington, UK) for 18-24h at 37 ± 1°C and stored at -80°C in cryoprotective medium, composed of Tryptone Soya Broth (Oxoid, Basington, UK) and 20% glycerol.

4.1.3.4 DNA extraction and isolate identification

Bacteria were grown on TSA (Oxoid, Basington, UK) for 18-24h at 37 ± 1°C. Genomic DNA was extracted using the commercial kit DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer instruction.

E. coli identification was performed through a multiplex PCR targeting 4 different genes (cytochrome bd complex, lactose permease, b-d-glucuronidase, and b-d-galactosidase) using primers and following suggestions of Horakova *et al.* (2008). The amplified products were loaded onto a 2% agarose gel containing Syber Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and run in 1X TBE buffer at 100 V for 1 h. PCR fragments were visualized with a UV transilluminator. A pUC19 DNA/MspI (Hpall) Marker (Thermo Fisher Scientific, Waltham, MA, USA) was loaded on each gel as a DNA size standard. *E. coli* ATCC 25922 was used as positive control.

4.1.3.5 WGS and assembly

Library preparation was performed using the Nextera Flex library preparation kit (Illumina, San Diego, CA, USA). Briefly, genomic DNA was quantitatively assessed using Quant-iT picogreen dsDNA assay kit (Invitrogen, USA). The sample was then normalised to the concentration of 1ng/µl and 10 ng of DNA was used for library preparation. After the tagmentation step, DNA was amplified using the facility's custom designed i7 and i5 barcodes, with 12 cycles of PCR.

Due to the number of samples, the quality control for the samples was done by sequencing a pool of samples using MiSeq V2 nano kit – 300 cycles (Illumina, San Diego, CA, USA). Briefly, 3 µl of each library was pooled into a library pool, cleaned up using SPRI beads following the Nextera Flex clean up

and size selection protocol. The pool was then sequenced using MiSeq V2 nano kit (Illumina, San Diego, CA, USA). Based on the sequencing data generated, the read count for each sample was used to pool libraries at a different amount to ensure equal representation in the final pool and to discard failed libraries (i.e. libraries with less than 100 reads). The final pool was then sequenced on Illumina NextSeq 500, 2x150 bp at Ramaciotti Centre for Genomics (University of New South Wales, Australia).

Sequence read quality was assessed using FastQC version 0.11.5 (http://www.bioinformatics.abraham.ac.uk/projects/fastqc/). Illumina raw reads passing quality control were assembled into draft genome sequences using Shovill v1.0.4 with default settings, and trimming options (https://github.com/tseemann/shovill).

Genome sequences have been deposited in the National Center for Biotechnology Information (NCBI) database with study accession number PRJNA528851. Accession numbers for each sample are listed in Supplementary Material, Table S1.

4.1.3.6 Gene identification, serotyping, phylogrouping and multilocus sequence typing

All gene screening was performed using ARIBA (Hunt *et al.*, 2017) as well as reference sequences from a variety of publicly available databases. Resistance, virulence, plasmid-associated genes and OH antigen genes were obtained from ResFinder (Zankari *et al.*, 2012), PlasmidFinder (Carattoli *et al.*, 2014), VirulenceFinder (Joensen *et al.*, 2014) and SerotypeFinder (Joensen *et al.*, 2015).
Other sequences of interest (insertion sequence elements, AMR and virulence associated gene sequences), available at https://github.com/maxlcummins/E_coli_customDB and not present within the previous databases were also screened. Moreover, different biocide resistance gene sequences collected from GenBank were considered. Pointfinder (Zankari *et al.*, 2017) was used to establish chromosomal mutation in *gyrA/B* – *parA/C/E* and *pmrA/B*, predicting phenotypic resistance to quinolones and colistin respectively.

E. coli phylogroups were determined using the Clermont scheme (Clermont *et al.*, 2000), meanwhile the Achtman scheme was used to evaluate *E. coli* multilocus sequence types (MLST)

(http://mlst.warwick.ac.uk/mlst/). ARIBA results were then processed and summarized with ARIBAlord (https://github.com/maxlcummins/ARIBAlord)

4.1.3.7 Phylogenetic analysis

Maximum-likelihood phylogenetic distances between genomes were analysed using the PhyloSift pipeline (Darling *et al.*, 2014), and a tree was generated using FastTree2 (Price *et al.*, 2010). The tree was constructed using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and iTOL (https://itol.embl.de/)

4.1.4 Results

The original collection consisted of 288 *E. coli* strains. Nine of these (EM1_Be20, EM1_Ca14, EM1_Fs21, EM1_Rb15, EM1_Rb25, EM1_Ve16, WM1_Wb2, EM1_Wb24, EM1_Wb25) were excluded due to PCR misidentification or inadequate DNA quality. Therefore the final study collection consisted of 279 *E. coli* (25, poultry; 25 swine; 25, dairy; 25 human; 25, mollusc; 25, wild animal; 24, beef; 24, vegetable; 24, fishery; 23, rabbit; 22, wild boar; 12, companion animal).

We identified a total of 136 sequence types, with ST10 (16; 5.7%), ST155, ST847 (9; 3.2% both), ST69 (8; 2.9%), ST20 (7; 2.5%), ST23, ST117, ST216 (6; 2.2% all) being the most common. Ninty-five sequence types were represented by a single isolate. Among all sources STs variability was extremely high. Exceptions were represented by beef, swine poultry with eight ST847, six ST10 and five ST117 respectively. Sixteen strains, with a single SNP in at least one reference allele, were assigned putative sequence types (denotated by an asterisk). Moreover, 14 strains, characterised by SNPs or new combinations of known alleles, were designated as novel.

One hundred and fifty-eight different serotypes were predicted for the 279 strains analysed. Forty-four strains were O non typable with 20 different H types, meanwhile for one O107 isolate we could not determine the H type. Furthermore, 22 strains were assigned to putative serotype (denotated by an asterisk). Serotype variability was pronounced among source, except for human sources with six O1:H7 strains (4, ST59; 2, ST95).

The most common phylogroup identified among the collection was B1 (130; 46.6%), followed by A (81, 29%) D (47; 16.8%) and B2 (21; 7.5%). Most beef, companion animal, mollusc, rabbit, vegetable and wild animal strains (≥50%) belonged to phylogroup B1, meanwhile phylogroup A was the most representative (≥40% in each source) in dairy, fishery, poultry and swine sources. Phylogroup D was prevalent in human (12/25; 48%) and wild boar (11/22; 50%) isolates. Interestingly, wild boar and wild animal sources were associated to the highest number of B2 strains (6 and 3 respectively), which are usually associated with pathogens.

Phylosift analysis produced a maximum-likelihood tree with two main clades (Figure 4.1.). Tree topology was highly congruent with Achtman MLST, in silico serotyping and generally congruent with phylogroup distribution.

Clade 1 was composed by 2 subclades, one mostly associated to phylogroup B2 and one to phylogroup D. ST69 (ST69*) was the prevalent sequence types of clade 1, characterising 10 different D strains.
Clade 2 was assembled into 4 subclades, the first mostly associated to phylogroup D, the second and the third to phylogroup A and the fourth to phylogroup B1. The most common lineage of clade 2 was ST10 (ST10*), identified in 19 A isolates, all belonging to subclade 2. Several exceptions with regard to phylogroup-clade grouping were identified.
Tree topology showed congruence with sources distribution. Human (13) and wild boar (11) isolates were frequently associated to clade 1 and distributed in both subclades, meanwhile fishery (13) and swine (13) niches belonged mostly to clade 2, subclade 2.

Beef (23), rabbit (20), wild animal (17), vegetable (15), mollusc (15), dairy (12) and companion animal (7) were mainly identified in clade 2, subclade 4. Poultry strains were mostly assigned to clade 2, subclade 2 (7). Six poultry isolates were also identified in both clade 1, subclade 2 and clade 2, subclade 4.

4.1.4.1 ARGs

One hundred and seventy-one strains (61.3%) were not carrying any ARGs. The remaining 108 (38.7%) isolates carried between 1 and 18 ARGs, with 77 (71.3%) strains containing at least 3. Among the latter, 43/77 (55.8%) strains belonged to phylogroup B1, 20/77 (26%) to phylogroup A, 12/77 (15.6%) to phylogroup D and 2/77 (26%) to phylogroup B2. Interestingly, most strains carrying ≥10 ARGs belonged to phylogroups B1 (8/14) and A (4/14).

The mean number of ARGs present in each source was: rabbit, 8; poultry, 4; dairy, 3; swine and human, 2 each; wild animal, beef, companion animal, fishery and mollusc, 1 each. The sources carrying the lowest number of ARGs were wild boar and vegetable, with only 1 and 2 strains respectively.

Fifty-three different ARGs were identified in our collection, with tetracycline resistance gene *tetA* (57/279; 20.4%), sulfonamide resistance gene *sul2* (45/279; 16.1%), penicillin resistance gene $bla_{\text{TEM-1b}}$ (43/279; 15.4%) and streptomycin resistance genes *strA*/*B* (42/279; 15.1%) being the most common. Colistin resistance gene *mcr1* was discovered in 6 (2.2%) strains, namely 3 rabbit, 2 swine and 1 dairy strains. Genes encoding extended spectrum beta-lactamase ($bla_{\text{CTX-M1}}$, $bla_{\text{CTX-M15}}$, $bla_{\text{CTX-M55}}$) were identified in 4 (1.4%) isolates, namely 2 dairies, 1 wild animal and 1 human. All ESBL strains carried a high number of ARGs (mean=9) and VAGs (mean = 16). AmpC beta lactamase gene $bla_{\text{CMY-2}}$ was identified in 1 human isolate. Fluoroquinolone resistance genes (*qnrS1* and *qnrB19*) were discovered in 2 dairy, 1 human and 1 rabbit strains.

Mutations in *gyrA*, *parC* and *parE* genes, responsible for fluoroquinolone resistance, were identified in 61 strains, mostly of rabbit (17/54), poultry (13/54) and human (9/54) origin. Only one swine isolate was associated with SNPs in *pmrB*, conferring presumptive resistance to colistin. The complete virulence profile of the strain collection is shown in Figure 4.2.

4.1.4.2 VAGs

The whole collection was screened for VAGs associated with ExPEC and IPEC pathotypes. One hundred and eleven different VAGs were identified (Figure 4.3.). All strains carried between 1 and 37 VAGs. A high number (≥10) of virulence determinants were identified in 102 (36.6%) isolates, most of them

belonging to phylogroup B1 (41, 40,2%), followed by D (27; 26.5%), A (21, 20.6%) and B2 (13; 12.7). Most VAGs were typical of ExPEC pathotype, including different genes encoding for adhesins (*fimH*, *pap*, *iha*, *bmaE*, *sfaS*), invasins (*ibeA*), iron acquisition systems (*iucD*, *iutA*, *fyuA*, *irp2*, *iroN*, *ireA*, *tsh*, *sitA*), toxins (*hlyE*, *cnf1*, *cdtB*, *usp*, *sat*, *picU*, *vat*) and protectins (*kpsMT-II*, *traT*, *ompT*, *iss*, *cvaC*).

The presence of both ExPEC and IPEC VAGs in the same strains suggested the rise of potential hybrid pathogens, mainly associated to rabbit source (four ST20 and four ST40 strains).

The mean number of VAGs for each source was: poultry and human, 15; rabbit, 14; wild animal and companion animal, 11; swine, 10; dairy, mollusc and wild boar, 8; beef and vegetable, 6; fishery, 5. Three strains (1.1%) carried ≥30 VAGs, including two from poultry (1, ST117; 1, ST131) and one ST648 from a wild animal (stork).

4.1.4.3 Class 1 integron structures

Short read screening identified the *intI1* class 1 integrase gene in 50/279 (17.9%) strains, mostly associated to phylogroup B1 (31/50; 62%), followed by A and D (9/50; 18% each) and B2 (1/50; 2%).

Following *de novo* assembly, we identified 44 strains with scaffolds carrying a complete *intI1* gene. Thiry-four out of 44 starins carried cassette array genes and were annotated in order to characterise the different integron structures present in the collection. Twenty-two arrangements were characterised and designated letters (A-V). Derivatives of (A-V) were named with the letter of the principal structure followed by a number (Figure 4.4). Eleven ARGs were identified as *intI1* related gene cassettes, namely the aminoglycoside adenyltransferase genes *aadA1*, *aadA2*, *aadA5*, *aad23*, the aminoglycoside O-nucleotidylyltransferase gene *ant2_Ia*, the dihydrofolate reductase genes *dfrA1*, *dfrA7*, *dfrA12, dfrA14*, *dfrA17* and the chloramphenicol efflux protein precursor gene *cmlA1*. The most common cassette array in class 1 integron structure was *aadA1-dfrA1*, present in 14/35 (40%) different strains. Both *sul1* and *sul3* were associated with integrons. Two isolates (1 human and 1 poultry strains) harboured both *intI1* and *intI2* integrase genes.

4.1.4.4 intI1 positive and negative strains in comparison

Short read sequencing indicated that 50/279 (17.9%) strains carried *intI1* gene, most of them isolated from food-producing animals (19/50; 38%) and related food (24/50; 48%). In particular, rabbit (19/50; 38%) and poultry (12/50; 24%) sources were the most represented, followed by 4 human (8%), 2 wild animal (4%), 1 companion animal (2%) and 1 fishery (2%) isolates. *intI1* negative *(intI1*$^{-ve}$) group numbered 229/279 (82.1%) strains, isolated from food (124, 54,1%), wildlife (45, 19.7%), food-producing animals (28, 12.2%), human (21, 9.2%) and companion animals (11, 4.8%). Among food, mollusc (25), vegetable (24), fishery (23), beef (21) were the sources mostly represented.

A higher number of ARGs was harboured by *intI1* positive (*intI1*$^{+ve}$) strains compared to *intI1*$^{-ve}$ ones, with a mean of 8 to 1 respectively.

ARGs which are usually not associated to class 1 integron structure, as *tetA* (*intI1*$^{+ve}$, 42/50, 84%; *intI1*$^{-ve}$ 34/229, 14.8%), *bla*$_{\text{TEM}}$ (*intI1*$^{+ve}$, 33/50, 66%; *intI1*$^{-ve}$ 30/229, 13.1%) and *strA/B* (*intI1*$^{+ve}$, 22/50, 44%; *intI*$^{-ve}$ 22/229, 9.6%), were present in both groups.

As expected, aminoglycoside (*aadA*) and trimethoprim (*dfr*) resistant determinants, widely observed as gene cassettes ,were widespread among *intI1*$^{+ve}$ *strains* (43/50, 86% and 46/50, 92% respectively) and rare in *intI1*$^{-ve}$ isolates (3/229, 1.3% and 5/229, 2.2% respectively).

ESBL, polymyxin, and quinolone resistance genes were rarely identified among *intI1*$^{+ve}$ (2/50, 4%; 4/50, 8%; 2/50, 4% respectively) and *intI1*$^{-ve}$ (2/229, 0.9%; 2/229, 0.9%; 2/229, 0.9% respectively) isolates. *bla*$_{\text{CMY-2}}$ gene was harboured by an *intI1*$^{+ve}$ isolate.

VAGs appeared to be more common in *intI1*$^{+ve}$ strains when compared to the *intI1*$^{-ve}$ group, with means of 14 to 9 respectively. Most VAGs were typical of ExPEC pathotype. The IncF plasmid replicon was the most frequent in both *intI1*$^{+ve}$ (47/50, 94%) and *intI1*$^{-ve}$ (135/229, 59%) strains.

Notably, pandemic (ST69, ST95, ST131) and emerging (ST10, ST23 ST58, ST117, ST405, ST648) ExPEC lineages were present in both groups (*intI1*$^{+ve}$,8/50, 16%; *intI*$^{-ve}$, 37/229, 16.2%).

4.1.4.5 Plasmid incompatibility group.

Thirty-three different plasmid replicons were identified in the collection and IncF was the most common (182/279; 65.2%). IncI (57/279; 20.4%) and IncX (45/279; 16.1%) types were also frequently observed. These replicons were present across multiple sequence types.

4.1.4.6 Biocide resistance genes

Our collection was screened for different efflux pump genes and related regulators, extruding a wide variety of molecules (including biocides and antimicrobials). Most genetic determinants, known to be chromosomally encoded, were widely diffused among the collection. Interestingly, various genes involved in clinically significant MDR (*acrA*, 279/2791 100%; *acrB*, 279/279, 100%; *acrD*, 279/279, 100%; *acrE*, 267/279, 95.7%; *acrF*, 269/279, 96.4%; *acrR*, 279/279, 100%; *acrS*, 273/279, 97.8%; *TolC*, 279/279, 100%) and in quaternary ammonium compound resistance (*mdfA*, 279/279; *qacE*, 34/279; *qacI,* 15/279) have been identified. Plasmid encoding *qacE* and *qacI* genes were mostly observed in rabbit (17) and poultry (10) sources, alone or in combination.

4.1.5 DISCUSSION

The antimicrobial genetic pattern identified in our collection was generally consistent with phenotypic resistance of commensal animal and human *E. coli* previously reported in Europe (Bailey *et al.*, 2010; Guenther *et al.*, 2011; de Verdier *et al.*, 2012; Dotto *et al.*, 2014; Carvalho *et al.*, 2016; Grevskott *et al.*, 2017; EFSA, 2018; EFSA, 2019). A notable exception was the wide diffusion of aminoglycoside ARGs in livestock (cattle, pig, poultry), despite the low phenotypic aminoglycoside resistance reported in the niches by the European data (EFSA, 2018; EFSA, 2019).

The most common ARGs in all the categories investigated were *tet* (mostly *tetA*, 57/279), *sul* (mostly *sul2,* 45/279) and bla_{TEM} (mostly $bla_{\text{TEM-1b}}$, 43/279).

The *tet–sul–bla*$_{TEM}$ genetic pattern in livestock (cattle, swine, poultry, rabbit) reflected the actual Italian antimicrobial stewardship practice in food-producing animal systems where tetracycline, sulfonamides and penicillins are the most commonly used compounds (EMA, 2019).

Penicillins are the most used antimicrobials in both companion animals and humans (ECDC, 2018a), explaining the wide diffusion of *bla*$_{TEM}$ genes in these sources. Surprisingly, tetracycline and sulfonamide ARGs were commonly identified in these niches despite these two antimicrobial classes being sporadically used as a treatment in companion animals and humans (EMA, 2019).

The same AMR genetic profile (*tet-sul-bla*$_{TEM}$) was also identified in environments not commonly associated to direct antimicrobial selective pressure, like aquaculture, vegetables and wildlife.

Resistance to tetracycline, sulfonamides and beta-lactams has already been reported in aquaculture (Sousa *et al.*, 2011; Grevskott *et al.*, 2017) and vegetable (Campos *et al.*, 2013; Holvoet *et al.*, 2013; Jensen *et al.*, 2013) in Europe. However, European and Italian data about AMR are lacking, probably because of the rare (aquaculture) or absent (vegetables) antimicrobial use in these food producing sectors (FVE, 2016). Hence, a proper comparison between our findings and previous phenotypic reports in these sources is difficult to perform. As expected, tetracycline, sulfonamides and beta-lactams resistance were also observed in wildlife, as reported by previous studies (Literak *et al.*, 2010; Giacopello *et al.*, 2016). Indeed, wildlife is considered an AMR bioindicator (Dolejska *et al.*, 2007; Literak *et al.*, 2010; Furness *et al.*, 2017) primarily because AMR observed in wild animals is influenced by livestock and human density/activity (Skurnik *et al.*, 2006; Allen *et al.*, 2010), where high antimicrobial selective pressure is more likely to be present.

These findings could suggest an important universal environmental pollution by penicillins, tetracycline and sulfonamides genetic determinants. Indeed, these molecules represented some of the oldest antimicrobial classes that have been used for decades and are still active (Eliopoulos *et al.*, 2003; Loudon, 2008; Petri, 2011). Moreover, overall antimicrobial consumption is mostly associated with food-producing animal systems (EFSA, 2017a), for which tetracycline and sulfonamides represent the most utilised antimicrobial compounds (EMA, 2019). The consequent high selective

pressure of these antimicrobials in the agricultural environment could have led to a widespread diffusion of related ARGs, spreading to other ecosystems mainly through faecal contamination (Radhouani *et al.*, 2014).

Streptomycin resistance genes were also frequently observed (42/279; 15%) among the collection. Currently, streptomycin has limited therapeutic usage in both humans and animals in Europe (ECDC, 2018a; EMA, 2019). Similar to sulfonamides and tetracycline, the seldom use of this antimicrobial could not explain the wide diffusion of related genetic determinants. However, *strA/B* are common streptomycin determinants among *Enterobacteriaceae* isolated from human and animals (Pezzella et al., 2004). *strA/B* are usually genetically linked and have been frequently associated with Tn5393 (Pezzella et al., 2004) on different multiresistant plasmids (including IncH1, IncH2, IncHII, IncN, IncQ, IncU) (Rozwandowicz et al., 2018), circulating in bacterial populations. Moreover, these genes are often clustered on different plasmids with the *sul2* gene (Sherley et al., 2004; Enne et al., 2004; Daly et al., 2005; Szczepanowski et al., 2005), encoding resistance to some of the most frequently sold antimicrobials for livestock. A possible co-selection and co-diffusion of *sul2-strA/B* complex could be hypothesised.

Cephalosporins (3rd, 4th and 5th generation), polymyxins and quinolones are considered Highest Priority Critically Important Antimicrobials (HP-CIAs). These molecules represent the last defense treatment for serious human infectious diseases, whose resistant bacteria are suspected to be generating from non-human sources (WHO, 2019).

ESBL and polymixin ARGs/*pmr* chromosomal mutations were detected in low frequency among the collection and were mostly identified in food-producing animals and related food. Our findings are in accordance with low cephalosporin and colistin phenotypic resistance identified in *E. coli* from livestock and related meat in Europe (Dotto *et al.*, 2014; EFSA, 2016; EFSA, 2017b; Grevskott *et al.*, 2017; EFSA, 2018; EFSA, 2019).

Notably, most ESBL genes (2 $bla_{\text{CTX-M1}}$ genes in wild animal and dairy strains; 1 $bla_{\text{CTX-M15}}$ in a dairy strain; 1 $bla_{\text{CTX-M55}}$ in a human strain) were found in proximity to IS*Ecp1.* IS*Ecp1* is a member of the

IS*1380* family and has previously been associated with ESBL genes (Lartigue *et al.*, 2006; Dhanji *et al.*, 2011). Notably, a single copy of this IS element is able to mobilize a downstream gene through transposition (Poirel *et al.*, 2003; Poirel *et al.*, 2008).

De novo assemblies revealed that *mcr1* was flanked by IS*6* family members in five out of seven carriers. IS*26* was identified in short read screening of these strains (3 rabbits and 2 swine), despite scaffold breaks preventing a definitive identification. IS*26* plays an important role in the evolution and mobilisation of ARGs worldwide. Here we report for the first time the *mcr1* gene flanked by IS*6* family elements in isolates of swine (2) and rabbit (2) origin. These findings suggest a possible involvement of IS*26* in the spread of *mcr1*. Further studies are necessary to establish the precise IS elements involved and their effective ability to transmit *mcr1* gene.

Fluoroquinolones/quinolones are important antimicrobials used in both hospital and community settings, with a minor role in companion and food-producing animals (ECDC, 2018a; EMA, 2019). In recent years, there has been a significant increase in fluoroquinolone resistance in human clinical *E. coli* in Europe, with the highest resistance rates observed in Italy (ECDC, 2018b). On the contrary, low resistance to these antimicrobials has been usually observed among livestock in EU Member States, with the exception of poultry source (EFSA, 2018; EFSA, 2019).

Interestingly, quinolone/fluoroquinolone resistance mostly derived from chromosomal mutations in *gyrA/parC/parE* genes, mainly identified in poultry (13) and rabbit (15) sources. Our findings are in accordance with these reported in poultry (EFSA, 2018) and rabbit (Dotto *et al.*, 2014) by other authors, showing high level of phenotypic quinolone/fluoroquinolone resistance. Dotto *et al*. (2014) attributed phenotypic resistance in Italian rabbit farms exclusively to *oqxA* and *oqxB* genes presence, which were not identified in our collection. However, the latter authors did not include chromosomal mutations in *gyrA*/*parC*/*parE* in AMR genetic screening. Fluoroquinolones have been reported as some of the most used antimicrobials in both poultry and rabbit production systems in Europe (FVE, 2016) despite the European Medicines Agency (EMA) not supplying stratified sales data of veterinary antimicrobials by food-producing animal species (EMA, 2019).

Our collection was associated to a high number of VAGs (in particular in human, poultry and rabbit niches) despite the fact that our study focused on commensal/environmental *E. coli*. Furthermore, genetic virulence patterns indicated the presence of potential extraintestinal pathogenic *E. coli* (ExPEC) pathotypes. This is rather concerning since *E. coli* is one of the most common causative agents of UTI and BSI globally (Riley, 2014). This assumption was strengthened by the identification of different sequence types, recognised as pandemic (ST69, ST95, ST131) (Riley, 2014) or emergent ExPEC lineages (ST10, ST23 ST58, ST117, ST405, ST648) (Manges *et al.*, 2019). The most potential pathogen sources were human and food-producing animals/related food (poultry and rabbit sources).

Notably, different strains harboured plasmid encoded *qacE* and *qacI* genes, known to be associated with integron structure (Gaze *et al.*, 2005; Soufi *et al.*, 2009; Wannaprasat *et al.*, 2011; Romero *et al.*, 2017). *qacE* and *qacI* genes coded for SMR family efflux pump proteins, conferring resistance to quaternary ammonium compounds (QACs). QACs are membrane-active biocide agents commonly used as disinfectants and antiseptics in different environments (hospital, healthcare facilities and food industry) (Gerba, 2015). Moreover, different genes encoding RND family efflux pumps (AcrAB, AcrEF, AcrD) were widely identified in the collection. RND family efflux pumps are involved in clinically significant MDR (Piddock, 2006) and may target a wide variety of molecules (Rosenberg *et al.*, 2000; Nishino and Yamaguchi, 2001; Nagakubo *et al.*, 2002; Piddock, 2006). The identification of efflux pump genes could suggest potential phenotypic resistance, where antimicrobial determinants are not identified. However, this event is strictly related to efflux pump overexpression genotypes (Piddock, 2006).

4.1.5.1 intI1 positive strains are more resistant and virulent

Class 1 integrons are recognised as a major source of ARGs (Kheiri and Akhtari, 2016) and play an essential role in the dissemination and evolution of MDR Gram-negative bacteria (Prapasawat *et al.*, 2017).

As expected, *intI1*$^{+ve}$ strains carried more ARGs (mean = 8) than *intI1*$^{-ve}$ isolates (mean=1). The main reason is associated to the ability of *intI1* site specific recombination site (attI) to recognise numerous

gene cassette attC sites (Labbate *et al.*, 2009). Indeed, a wide variety of antimicrobial genetic determinants have been identified as *intI1* related gene cassettes (Partridge *et al.*, 2009). In particular, *aadA1-dfrA1*, the most widespread cassette array detected in both commensal/clinical strains isolated from animal and food sources (Kaushik *et al.*, 2018), was identified in numerous isolates (15/34; 44.1%). Another important feature of class 1 integron is the ability of its transposition system to target resolution sites of different plasmids and transposons (in particular of Tn3 family) as site of insertion (Labbate *et al.*, 2009; Partridge *et al.*, 2009). These MGEs could harbour numerous antimicrobial determinants, allowing the co-carriage and co-transmission of class 1 integrons and numerous ARGs. In particular, the most frequent MGEs associated with class 1 integron in our collection were Tn*21* and close relatives, identified in 26 of 35 (74.3%) *intI1*$^{+ve}$ strains. Tn*21* transposon and related variants (belonging to Tn3 family) are globally disseminated and frequently involved in multiple ARGs and class 1 integron carriage in *Enterobacteriaceae*, despite numerous variations in their structure have been observed (Liebert *et al.*, 1999; Partridge *et al.*, 2018).

Interestingly, *intI1*$^{+ve}$ strains also carried a higher number of VAGs (mean =14; *intI1*$^{-ve}$ isolates, mean=9), and often harboured F plasmid replicons. F incompatibility group members are recognised as the majority of virulence associated plasmids in *E. coli*. They are known to carry different antimicrobial resistance determinants (Johnson and Nolan, 2009; da Silva and Mendonça, 2012) and class 1 integrons (Moura *et al.*, 2012), creating a concerning combination of virulence and antimicrobial resistance traits. Our findings suggest that ARGs and VAGs may be co-localised on F plasmids in our collection. Long read sequencing data and analysis are required to confirm this hypothesis.

Notably, *intI1*$^{+ve}$ strains were mostly associated to ExPEG VAGs and to different ExPEC lineages (ST10, 2; ST58, 1; ST69, 2; ST117, 2; ST131, 1). These findings are particularly worrisome given *E. coli* represents one of the most common causes of human UTI and BSI (Riley, 2014) and given the frequent identification of class 1 integron in multiresistant *Enterobacteriaceae* in hospital environment (Martinez-Freijo *et al.*, 1998; Leverstein-van Hall *et al.*, 2003) and in *E. coli* responsible for UTI (Gündoğdu *et al.*, 2011; Fallah *et al.*, 2012).

Our findings suggested that *intI* related strains could represent a serious threat to human health, as a consequence of the high number of ARGs (also conferring resistance to CIA) and VAGs they carry. Moreover, important ExPEC genetic traits and pandemic lineages have been associated with *intI1*$^{+ve}$ strains, suggesting their potential role as extraintestinal pathogens.

4.1.5.2 Concerning sources

Rabbit, poultry and wildlife sources were notable for their concerning antimicrobial resistance and/or virulence profiles.

Rabbit represented the niche carrying the highest number of *mcr1* gene (3 strains from 1 animal and 2 meat samples) among our collection. It was always associated, and in two cases flanked, with IS*6* family members. A recent Italian investigation (Agnoletti *et al.*, 2018) reported polymyxin (colistin) as a widely used antimicrobial in rabbit farms even though two reports indicated colistin was not a common treatment in rabbit breeding systems (SSR-ER, 2006; FVE, 2016). Resistance to colistin has been previously reported in breeding rabbits in Europe, supporting the data from the third report (Freitas-Silva *et al.*, 2018). In our knowledge, this is the first report of *mcr1* identified in rabbit meat as food. This finding suggests rabbit farms and meat should be investigated as a potential reservoir of *mcr1* and as a vector for its transmission. Concerningly, different rabbit strains belonged to hybrid ST20 (4/23) and ST40 (4/23). Their virulence profile, characterised by the co-presence of both ExPEC (i.e *fimH, cva*, *cvi, iss*, *traT, iroN*) and IPEC (i.e. *eae*, *efa1, cif*, *esp*, *nle*, *tir)* VAGs, did not correspond to the usual pathotype observed in these lineages. Indeed, ST20 and ST40 have been usually reported as human diarrheagenic pathogens (Yu *et al.*, 2018), often identified as shiga toxin producing *E. coli* (STEC) (Prager *et al.*, 2009; Fierz *et al.*, 2017; van Hoek *et al.*, 2019). The emergence of new hybrid pathotype is worrisome and could represent a serious threat to public health, as witnessed in different important human outbreaks (Bielaszewska *et al.*, 2011; Mariani-Kurkdjian *et al.*, 2014; Toval *et al.*, 2014; Lindstedt *et al.*, 2018). Recombination of ExPEC and IPEC VAGs could lead to the transition from IPEC to ExPEC pathotype and vice versa (Toval *et al.*, 2014), or the emergence of strains able to cause

both intestinal and extraintestinal infections. Further studies are needed to better understand this phenomenon and the potential animal sources involved in hybrid pathotype evolution and diffusion.

Poultry source strains (11/25; 44%) belonged to some of the most prominent ExPEC sequence types (ST131, 1; ST69, 1; ST10, 1; ST23, 3; ST117; 5), all of which were isolated from poultry meat. ExPEC lineages carried the highest number of VAGs (mean=22) among poultry source and a high number of ARGs (mean=5). *mcr1*, ESBL genes and quinolone resistance mutations were not identified among these lineages. Notably, ST117 was the most common sequence type among poultry source. This lineage is considered an avian pathogenic extraintestinal *E. coli* (APEC) lineage and a human pathogen (Leverstein-van Hall *et al.*, 2011; Mora *et al.*, 2012). All the above mentioned ExPEC STs have been previously isolated from poultry animals and meat (Cortés *et al.*, 2010; Vincent *et al.*, 2010; Leverstein-van Hall *et al.*, 2011; Giufrè *et al.*, 2012; Manges and Johnson, 2012; Mora *et al.*, 2012; Maluta *et al.*, 2014). In particular, poultry meat was suspected to represent a source of ExPEC *E. coli*, which can be transmitted to consumers through food consumption (Mitchell *et al.*, 2015). It has been hypothesised that the poultry gastrointestinal tract could represent a reservoir of APEC, coexisting with commensal bacteria (Dziva and Stevens, 2008). Faecal contamination of poultry carcasses during slaughter could allow potential ExPEC diffusion through the food chain (Mor-Mur and Yuste, 2010; Lyhs *et al.*, 2012). Our findings underline the role of poultry as a source of potential ExPEC lineages and the importance of related meat as an ExPEC carrier to humans.

The literature mainly focuses on the role of wildlife in AMR maintenance and diffusion, overshadowing its possible involvement in virulence dissemination. The spread of VAG may exhibit similar dynamics involved in AMR diffusion, with the interplay between humans and human-associated animals/wildlife being the most important factor. Wild animals can represent an infection source, where potential pathogens can emerge in wild ecosystems or spread in turn to urban and agricultural environments. Indeed, wildlife has been recognized as a reservoir of emerging infectious diseases in humans (Jones *et al.*, 2008).

A relatively high number of VAGs (mostly associated to ExPEC pathotype) were observed in wild animal (mean=11) and wild boar (mean=8), with 10/47 (21,3%) isolates carrying ≥15. Notably, four

strains belonged to some of the predominant ExPEC lineages (2, ST23; 1, ST117; 1, ST648). Concerningly, ST648 strain carried the highest number of VAGs (37) in the entire collection. ST648 has previously been isolated from wild birds (Guenther *et al.*, 2010a), humans, surface water, fish, vegetables and companion animals (Ewers *et al.*, 2014; Müller *et al.*, 2016). It has been related to disease in both human and animals (pets, horses and wildlife) (https://enterobase.warwick.ac.uk/, last accessed 19/10/2019) and frequently reported as an ESBL gene carrier (Cortés-Cortés *et al.*, 2016; Müller *et al.*, 2016).

Interestingly, wild animal strains carrying the highest number of VAGs and ARGs were isolated from birds. These findings could be explained considering bird's synanthropic behaviour, due to many advantages such as availability of food resources/refuges and absence of natural predator. Human related environments are exposed to higher antimicrobial selective pressure compared to wild habitats. For this reason, synanthropic birds could come in contact and be colonised with resistant bacteria and different genetic determinants (ARGs and VAGs), typical of human and agricultural settings. Birds are often chosen as an AMR dispersion indicator for their important epidemiological role in AMR diffusion (Guenther *et al.*, 2011). Migratory behavior could allow the dissemination of AMR bacteria/genes in other human related environments or in wildlife (Bonnedahl and Järhult, 2014). However, the high number of VAGs identified in birds could suggest their epidemiological importance also in virulence diffusion.

Briefly, our study reaffirmed the role of food-producing animals as a reservoir of potential zoonotic pathogens, with variable antimicrobial and virulence traits among the sources investigated.

In particular, rabbit and poultry represented the most worrisome sources, carrying the highest number of ARGs and VAGs. Poultry was associated with potential ExPEC strains, meanwhile rabbit was suggested as a source of potential hybrid pathogens and an *mcr1* carrier. Moreover, the role of wildlife in maintenance and diffusion of virulence determinants was hypothesised.

It should be noted that our study has two important limitations. Firstly, the small sample size of each source (in particular for companion animals) prevented the comparison between antimicrobial and

virulence profiles and the evaluation of their possible transmission routes between environments. Secondly, our data originated from short read sequencing analysis. Therefore, we could not determine the location of all ARGs identified and their association with MGEs. Moreover, we were not able to establish similarities/dissimilarities between F plasmids, potentially responsible for VAGs and ARGs carriage in our collection.

Despite these limitations, our study provided basic information about AMR and virulence determinants circulating in various environments in Italy. Further investigations can add a deeper understanding of AMR and virulence epidemiological traits in Gram-negative bacterial population in different environments.

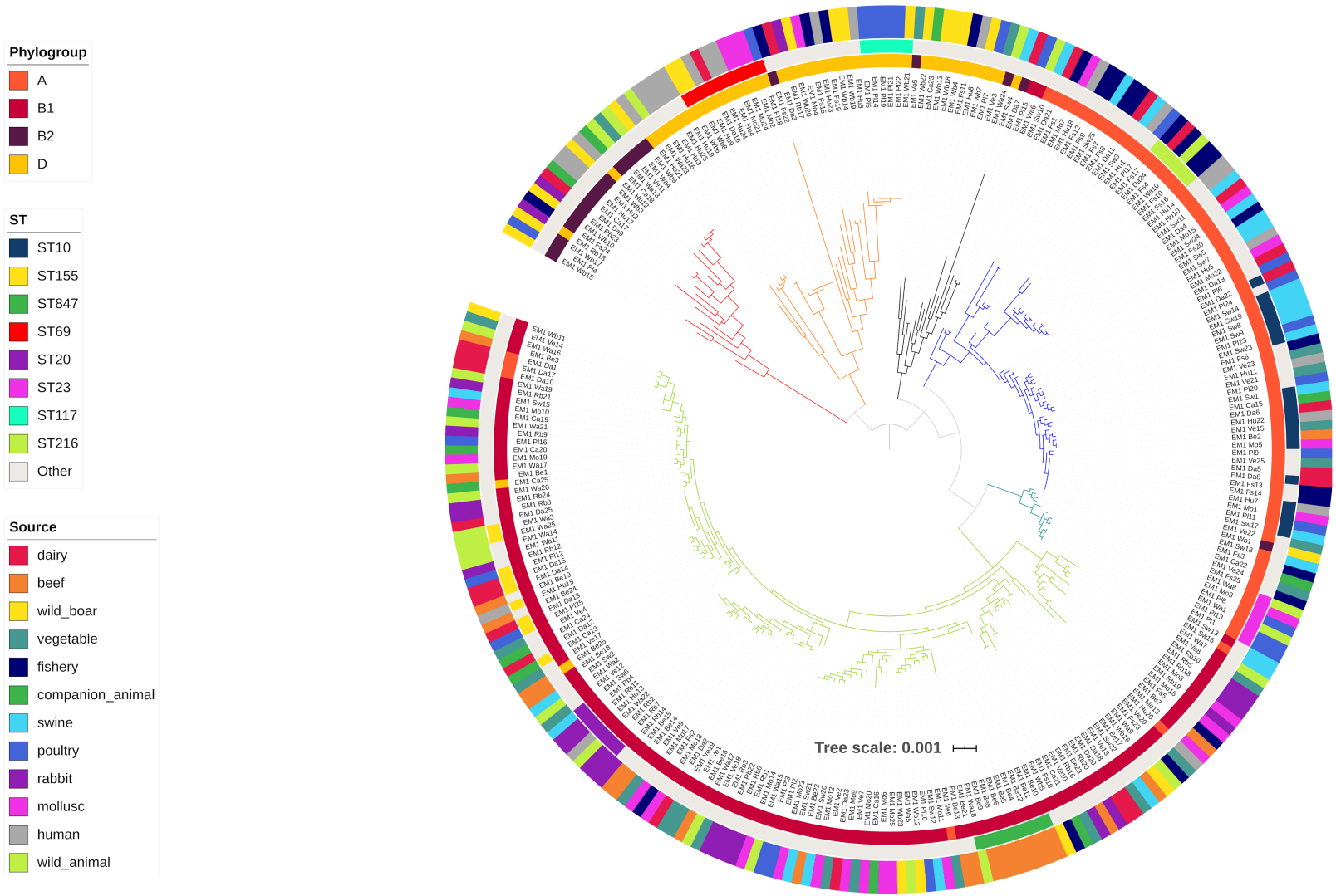


Figure 4.1 A mid-point rooted, maximum-likelihood phylogenetic tree of 279 commensal *E. coli* included in the study. Branches are coloured by clade and subclade (red: clade 1, subclade 1; orange: clade 1, subclade 2; black: clade2, subclade 1; blue: clade 2, subclade 2; sea green: clade 2, subclade 3; light green: clade 2, subclade 4). Phylogroups (inner ring), sequence types (middle ring) and sources (outer ring) are annotated according to the legend. Presumptive STs are shown with the same colour as their corresponding definitives.

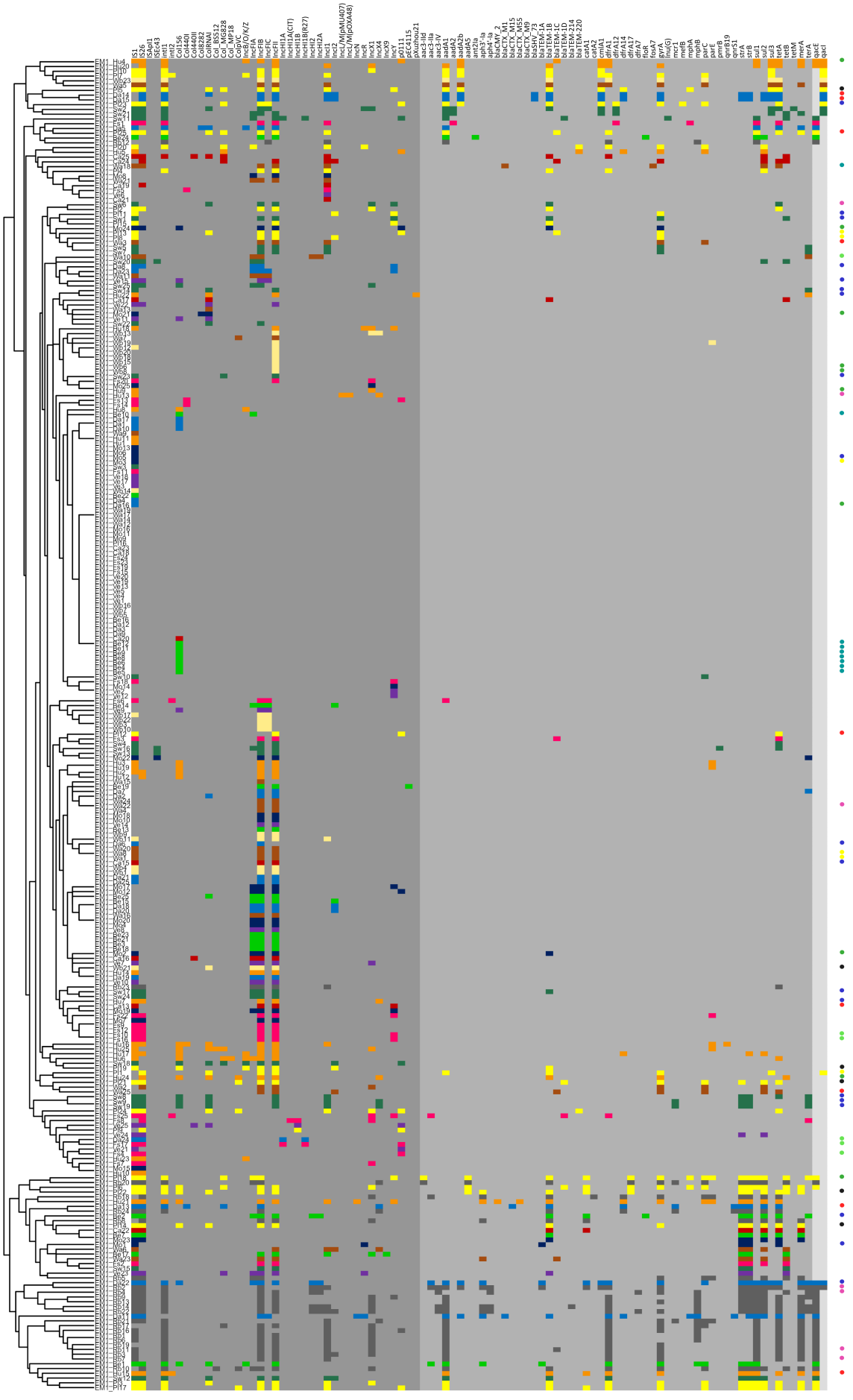


Figure 4.2 Heat map depicting carriage of insertion sequences, plasmid incompatibility groups, ARGs and plasmid mediated QAC resistance genes in the strain collection. In the heatmap, sources are identified by different colours (light green = beef; companion animal = red; dairy = light blue; fishery = fuchsia; human = orange; mollusc = blue; poultry = yellow; rabbit = grey; swine = dark green; vegetable = violet; wild animal = brown; wild boar = cream). The most common STs observed in the collection are displayed with coloured circles (ST10 = blue; ST155 = red; ST847 = light sea green; ST69 = green; ST20 = fuchsia; ST23 = yellow; ST117 = dark green; ST216 = light green). Presumptive STs are shown with the same colour as their corresponding definitives.

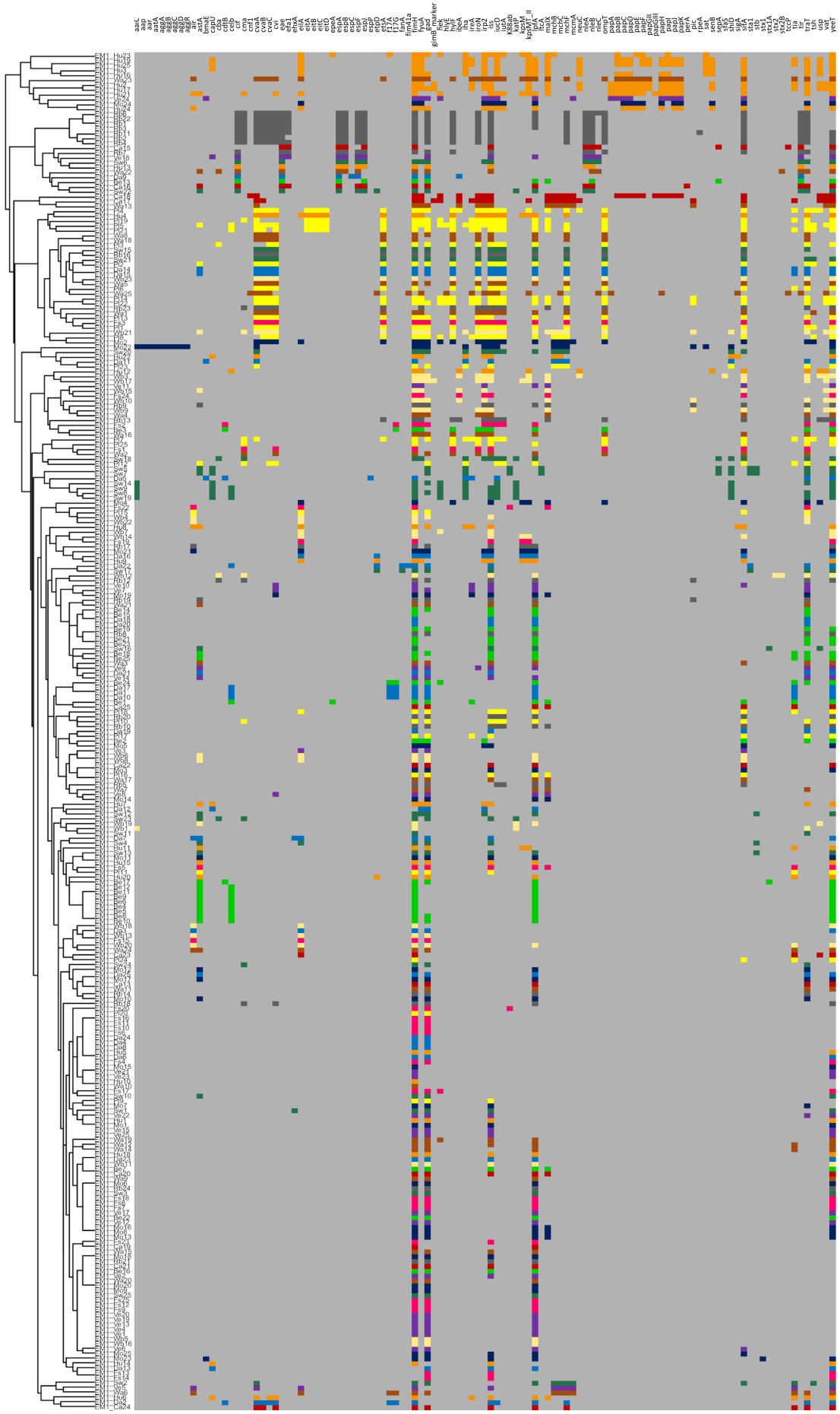
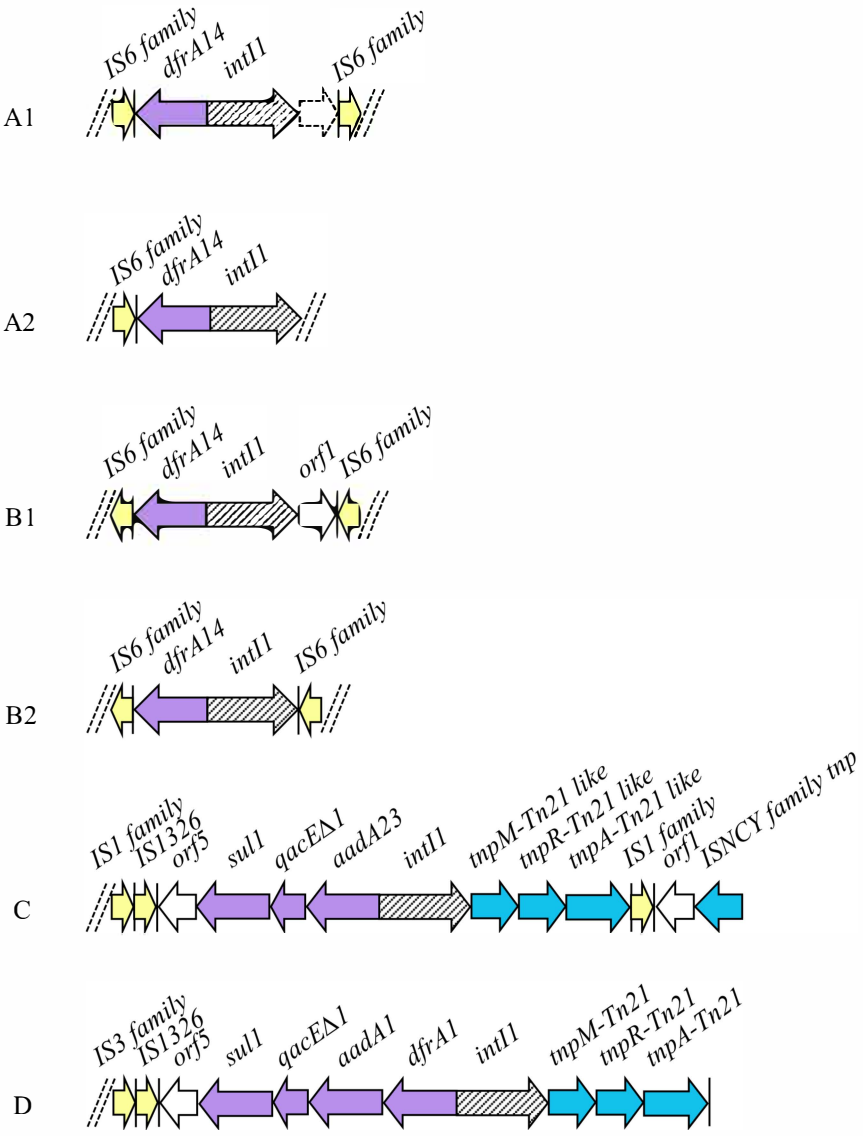


Figure 4.3 Heat map depicting carriage of VAGs in the strain collection. In the heat map, sources are identified by different colours (light green = beef; companion animal = red; dairy = light blue; fishery = fuchsia; human = orange; mollusc = blue; poultry = yellow; rabbit = grey; swine = dark green; vegetable = violet; wild animal = brown; wild boar = cream). The most common STs observed in the collection are displayed with a coloured circles (ST10 = blue; ST155 = red; ST847 = light sea green; ST69 = green; ST20 = fuchsia; ST23 = yellow; ST117 = dark green; ST216 = light green). Presumptive STs are shown with the same colour as their corresponding definitives.



Rb21, ST1431

Da13, ST155

Rb24, ST1727

Hu21, ST457

Sw11, ST871

Rb11, ST20

Figure 4.4 Schematic diagram (not to scale) of integron structures identified in 34 commensal E. coli. Arrows represent ORFs. Arrows with broken lines indicate hypothetical proteins. Vertical bars represent inverted repeats. Dashed double diagonal lines represent sequence scaffold breaks. Elements displayed are colour coded (ARGs = purple; IS elements = yellow; transposon elements = light blue).

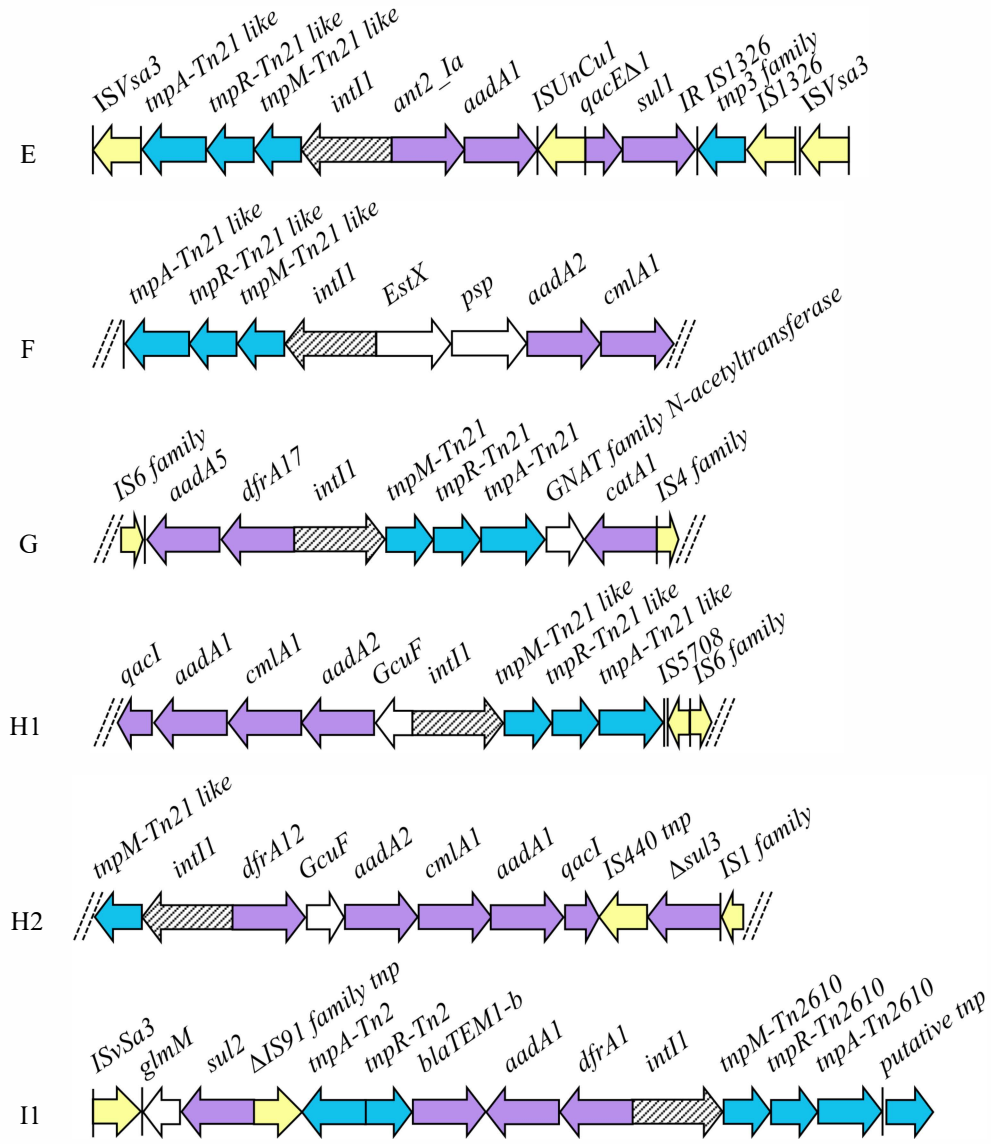
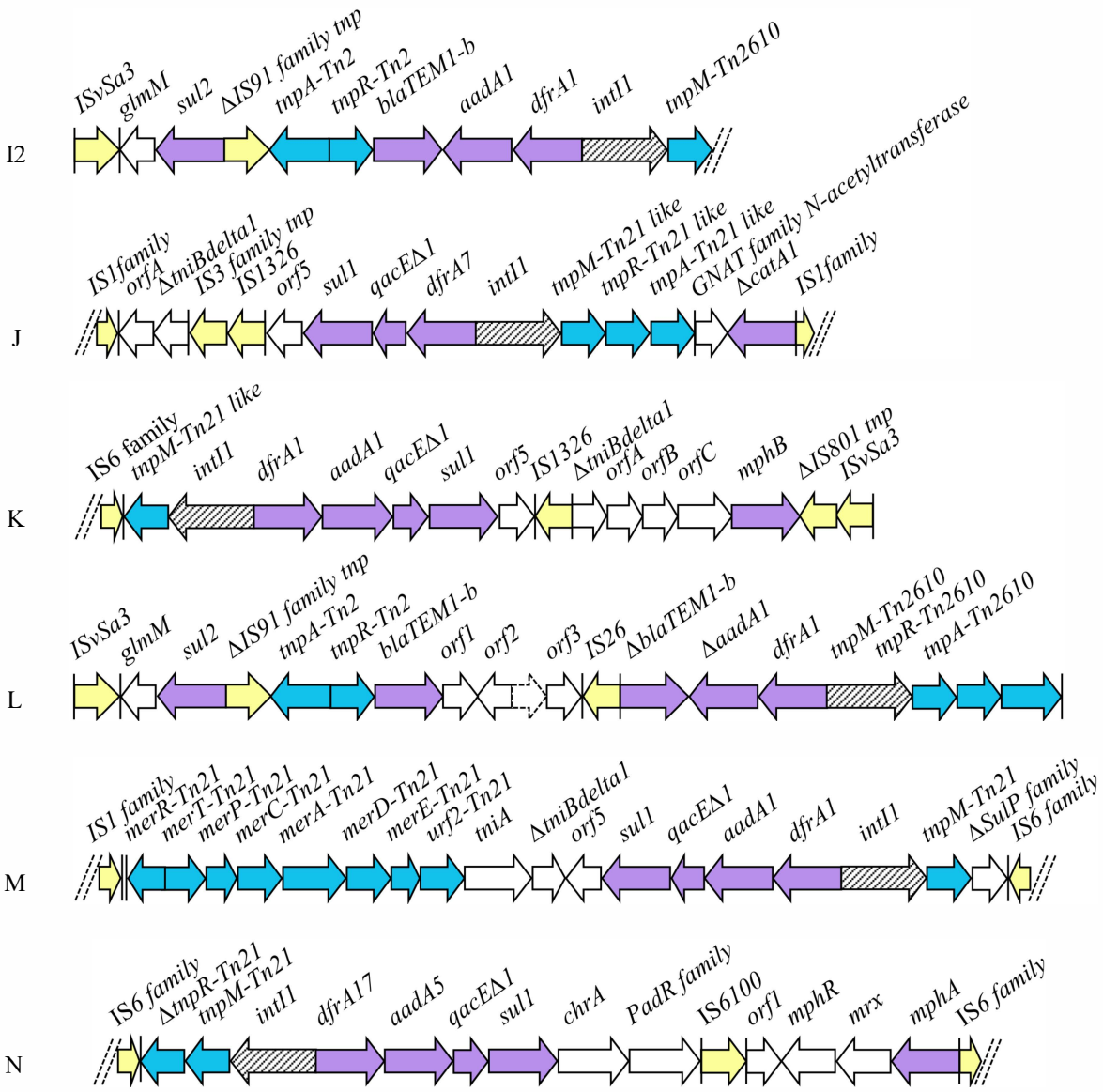

ISVsa3
tnpA-Tn21 like
tnpR-Tn21 like
tnpM-Tn21 like
intI1
ant2_Ia
aadA1
ISUnCu1
qacEΔ1
sul1
IR IS1326
tnp3 family
IS1326
ISVsa3
E
Be24, ST58
tnpA-Tn21 like
tnpR-Tn21 like
tnpM-Tn21 like
intI1
EstX
psp
aadA2
cmlA1
F
Pl7, ST57
IS6 family
aadA5
dfrA17
intI1
tnpM-Tn21
tnpR-Tn21
tnpA-Tn21
GNAT family N-acetyltransferase
catA1
IS4 family
G
Pl22, ST117
qacI
aadA1
cmlA1
aadA2
GcuF
intI1
tnpM-Tn21 like
tnpR-Tn21 like
tnpA-Tn21 like
IS5708
IS6 family
H1
Sw2 , ST641
tnpM-Tn21 like
intI1
dfrA12
GcuF
aadA2
cmlA1
aadA1
qacI
IS440 tnp
Δsul3
IS1 family
H2
Pl23, ST10
ISvSa3
glmM
sul2
ΔIS91 family tnp
tnpA-Tn2
tnpR-Tn2
blaTEM1-b
aadA1
dfrA1
intI1
tnpM-Tn2610
tnpR-Tn2610
tnpA-Tn2610
putative tnp
I1
Pl4, ST131

Figure 4.4 ..\..



Pl20, ST1286

Rb4, ST20

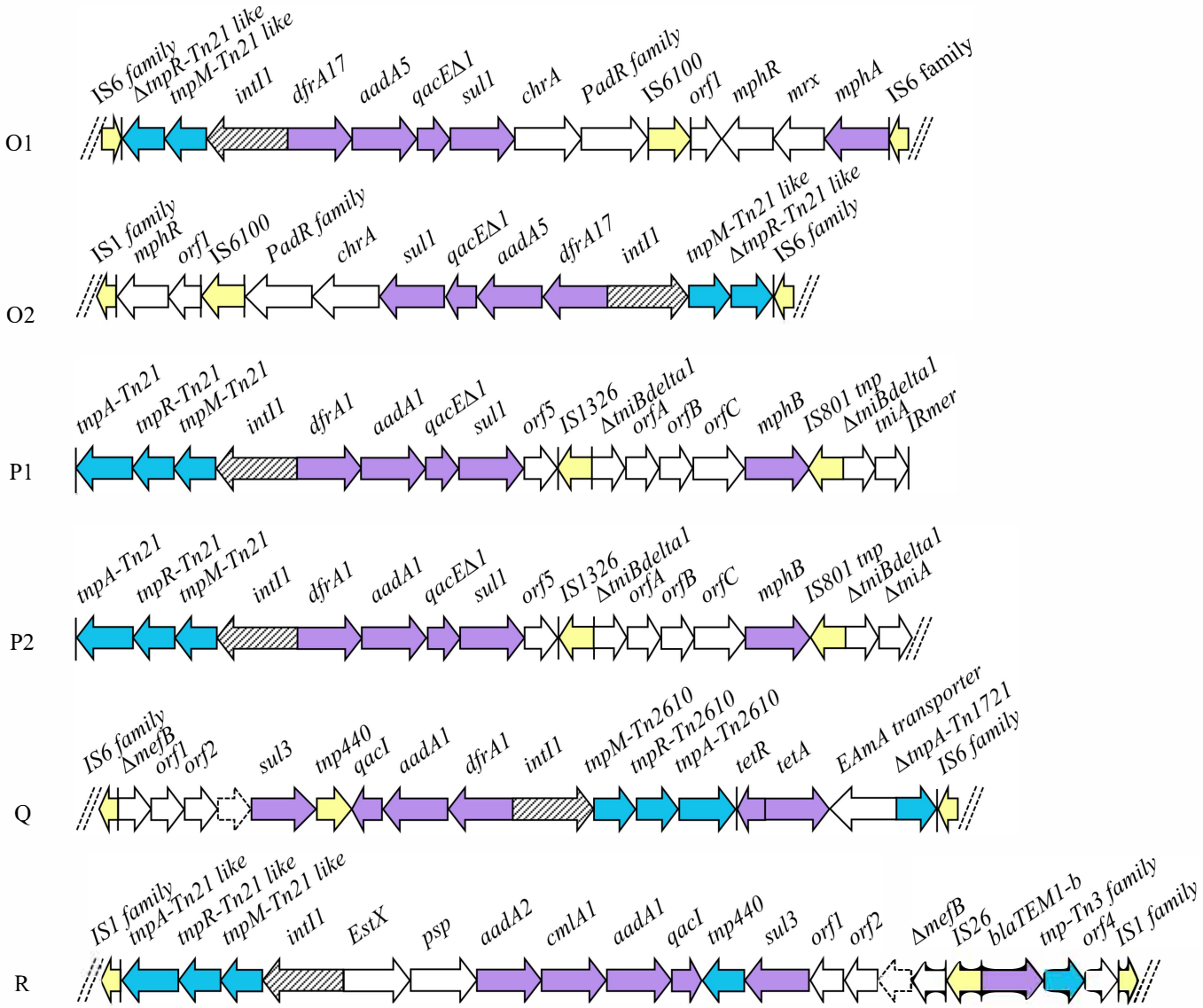
Be1, ST297

Ca25, ST297

Rb10, ST533

Pl6, ST744

Figure 4.4 ..\..



O1 — Pl18, ST69

O2 — Rb20, ST539

P1 — Rb12, ST129

P2 — Rb1 , ST40; Rb6, ST40; Rb16, ST351; Rb17, ST501

Q — Wb23, ST162

R — Pl10, ST162

Figure 4.4 ..\..

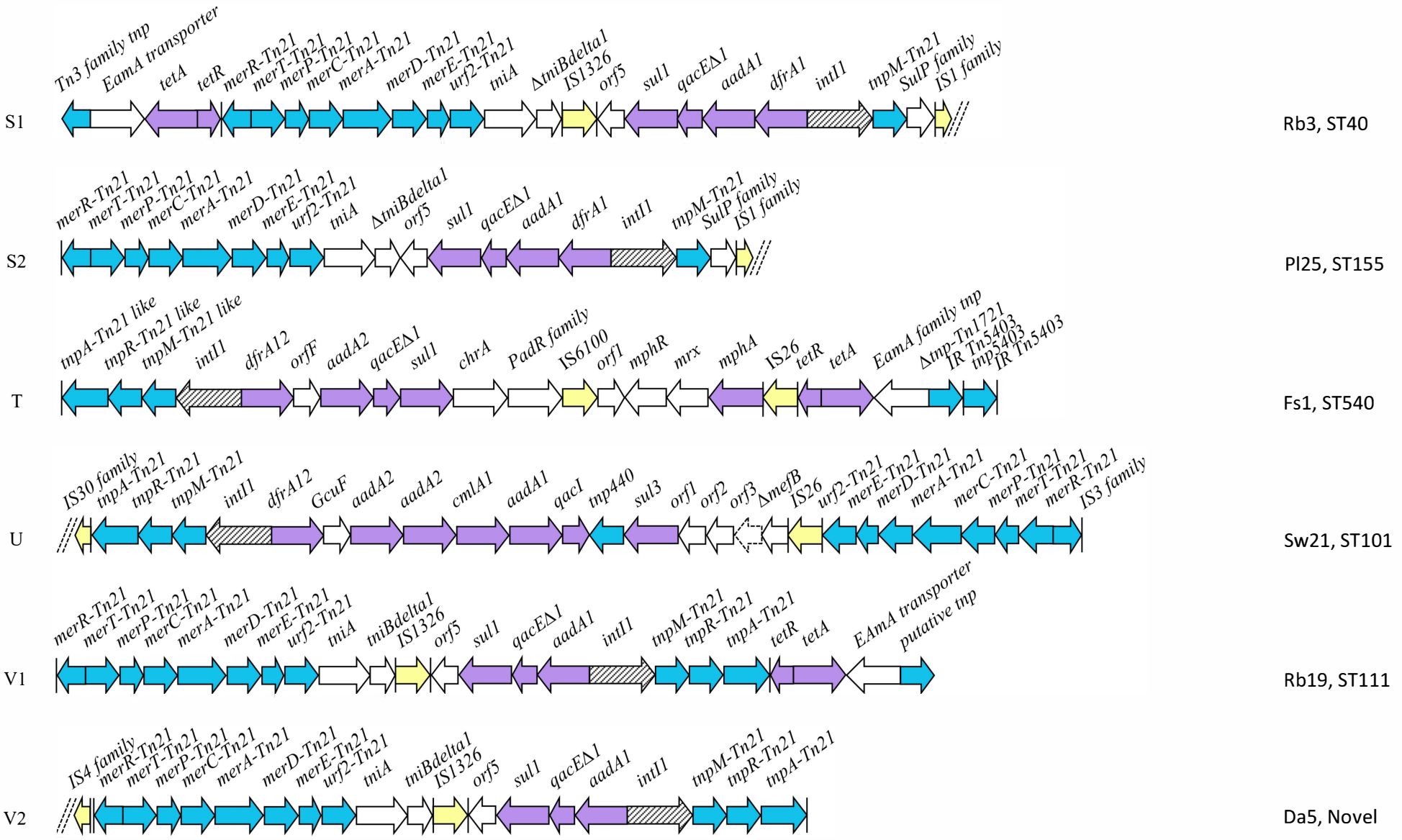
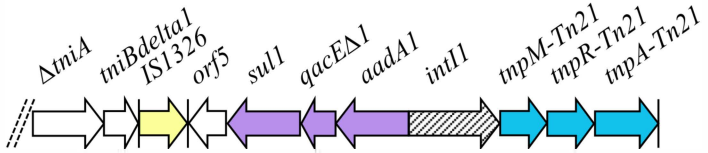

S1
Tn3 family tnp
EamA transporter
tetA
tetR
merR-Tn21
merT-Tn21
merP-Tn21
merC-Tn21
merA-Tn21
merD-Tn21
merE-Tn21
urf2-Tn21
tniA
ΔtniBdelta1
IS1326
orf5
sul1
qacEΔ1
aadA1
dfrA1
intI1
tnpM-Tn21
SulP family
IS1 family
Rb3, ST40
S2
merR-Tn21
merT-Tn21
merP-Tn21
merC-Tn21
merA-Tn21
merD-Tn21
merE-Tn21
urf2-Tn21
tniA
ΔtniBdelta1
orf5
sul1
qacEΔ1
aadA1
dfrA1
intI1
tnpM-Tn21
SulP family
IS1 family
Pl25, ST155
T
tnpA-Tn21 like
tnpR-Tn21 like
tnpM-Tn21 like
intI1
dfrA12
orfF
aadA2
qacEΔ1
sul1
chrA
PadR family
IS6100
orf1
mphR
mrx
mphA
IS26
tetR
tetA
EamA family tnp
Δtnp-Tn1721
IR Tn5403
tnp5403
IR Tn5403
Fs1, ST540
U
IS30 family
tnpA-Tn21
tnpR-Tn21
tnpM-Tn21
intI1
dfrA12
GcuF
aadA2
aadA2
cmlA1
aadA1
qacI
tnp440
sul3
orf1
orf2
orf3
ΔmefB
IS26
urf2-Tn21
merE-Tn21
merD-Tn21
merA-Tn21
merC-Tn21
merP-Tn21
merT-Tn21
merR-Tn21
IS3 family
Sw21, ST101
V1
merR-Tn21
merT-Tn21
merP-Tn21
merC-Tn21
merA-Tn21
merD-Tn21
merE-Tn21
urf2-Tn21
tniA
tniBdelta1
IS1326
orf5
sul1
qacEΔ1
aadA1
intI1
tnpM-Tn21
tnpR-Tn21
tnpA-Tn21
tetR
tetA
EAmA transporter
putative tnp
Rb19, ST111
V2
IS4 family
merR-Tn21
merT-Tn21
merP-Tn21
merC-Tn21
merA-Tn21
merD-Tn21
merE-Tn21
urf2-Tn21
tniA
tniBdelta1
IS1326
orf5
sul1
qacEΔ1
aadA1
intI1
tnpM-Tn21
tnpR-Tn21
tnpA-Tn21
Da5, Novel

Figure 4.4 ..\..



Rb7, ST20

Figure 4.4 ..\..

4.2. Phylo-typing methods in comparison

Among the collection, phylogroup evaluation was performed using: (Clermont *et al.*, 2015)

- *In silico* genome screening with ARIBA in order to detect 2 genes (*chuA* and *yjaA*) and 1 DNA fragment (TspE4.C2), targets of a triplex PCR (Clermont *et al.*, 2000) able to identify 4 different phylogroups (A, B1, B2, D);
- Laboratory PCR using a derivative of the triplex PCR method (Clermont *et al.*, 2000) characterised by a quadruplex PCR (targeting *arpA*, *chuA*, *yjaA* genes and TspE4.C2 DNA fragment), followed by possible additional testing (Clermont *et al.*, 2011; Clermont *et al.*, 2013). This method is able to detect 7 different phylogroups (A, B1, B2, C, D, E, F) and 5 cryptic lineages (clade I-II-III-IV-V).

Comparison of the data was implemented considering that some phylogroups/clades (E, F, clade I-II-II-IV-V) identified in Clermont *et al.* (2011, 2013) would be included in the 4 main phylogroups (A, B1, B2, D) in the original triplex phylogroup assignment method (Clermont *et al.*, 2000). Clermont *et al.*, (2015) provided a table of phylogroup correspondences between the two methods, hereafter summarised:

Triplex PCR (Clermont *et al.,* 2000)	Quadruplex PCR and other allele-specific PCR genotypes (Clermont et *al.,* 2011; Clermont *et al.,* 2013)
A	A
	C
	clade I-II-III-IV-V
B1	B1
B2	B2
	clade I
D	D
	E
	F

Table 4.1 Correspondences between phylogroups identified in the triplex and quadruplex PCR phylo-typing methods.

The comparison of the data obtained with both phylo-typing methods showed a complete concordance of the results. Only exeptions are represented by 4 isolates designated as unknown with the quadruplex PCR method and properly assigned to a phylotype with ARIBA.

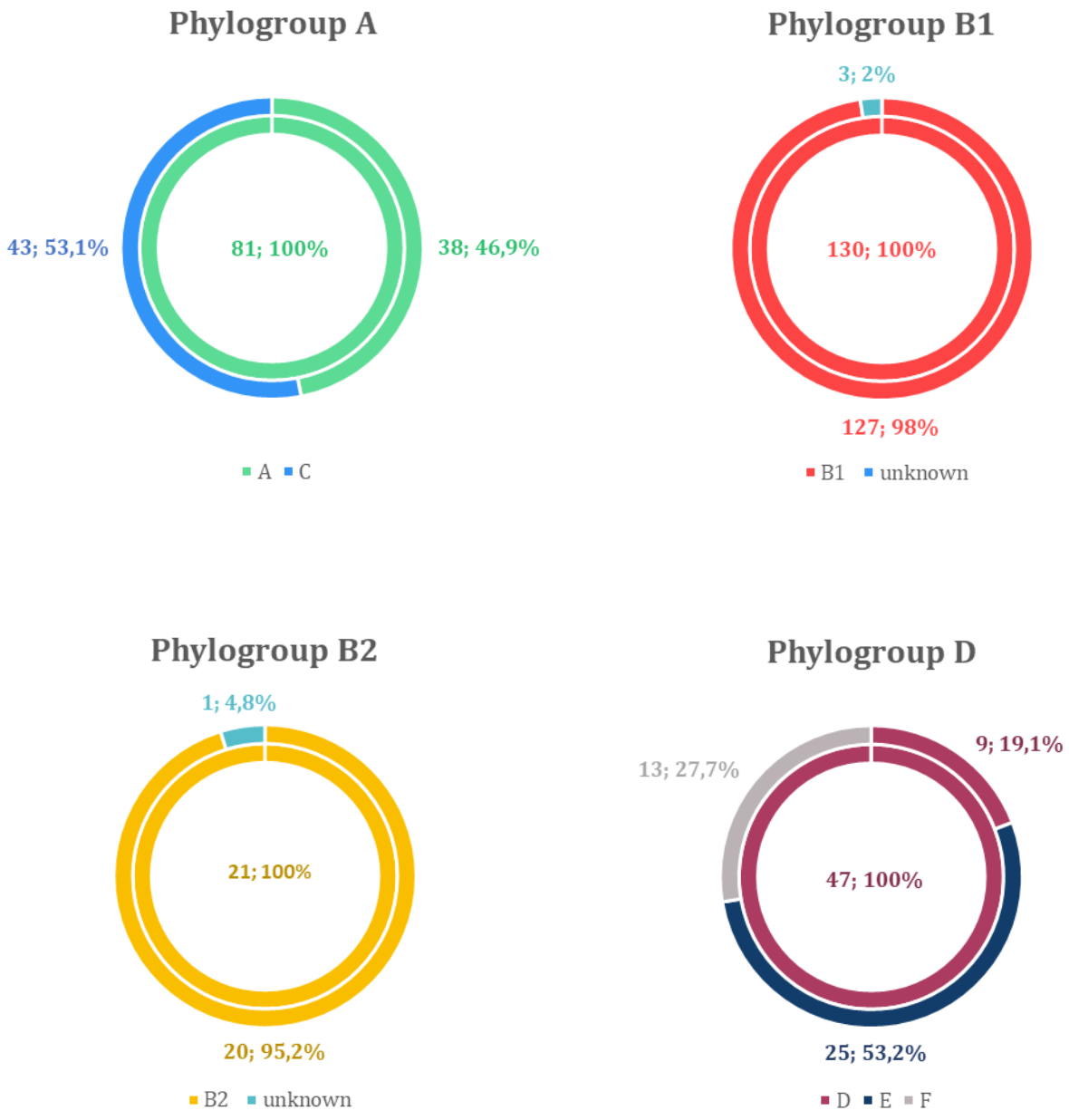


Figure 4.5 Schematic representation of phylogroups identified with triplex PCR (the inner circle) and quadruplex PCR (the outer circle) phylo-typing methods in 279 strains.

4.3. Agreement between phenotypic resistance and ARGs identification

Discrepancies were observed for all the antimicrobials tested when comparing phenotypic and genetic resistance profiles of the strain collection. There were numerous cases in which phenotypic resistances were not explained by the identification of related ARGs and/or gene presence was not accompanied by the expected resistance phenotype (Figure 4.6.).

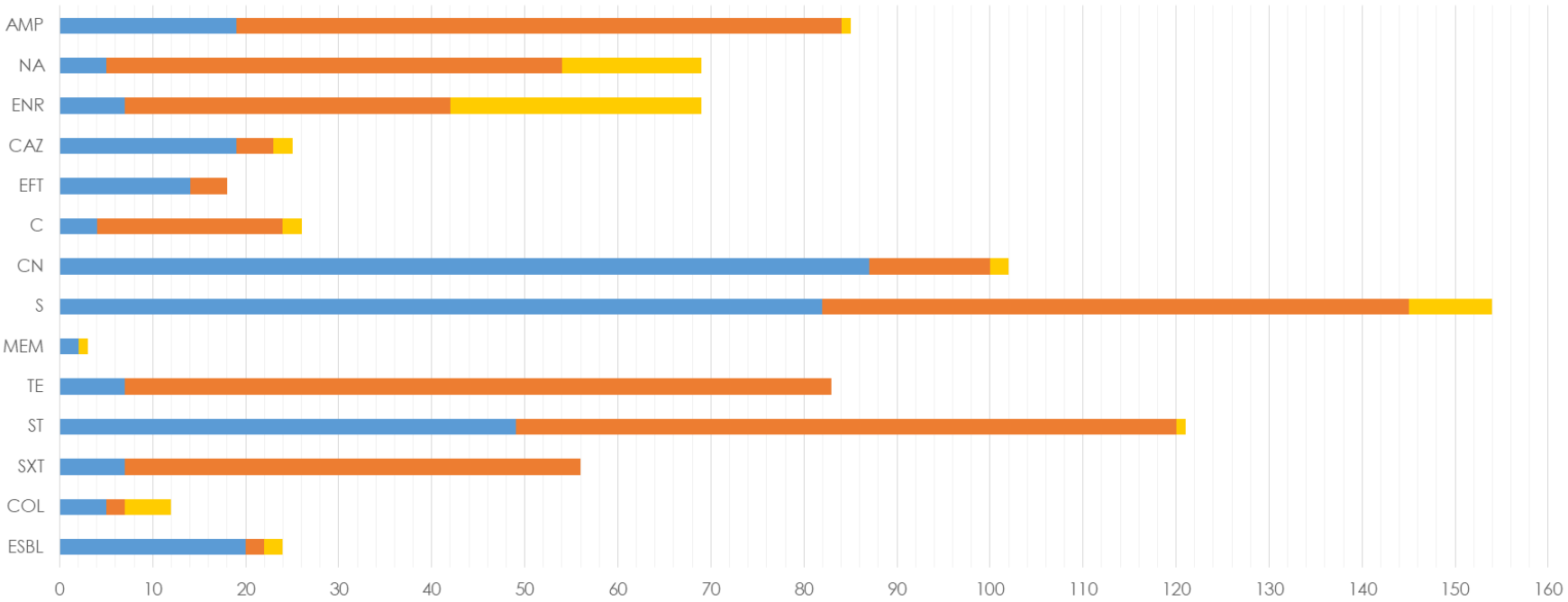
Generally, known antimicrobial genetic determinants complemented phenotypic resistance for tetracycline (91.5%), sulfamethoxazole/trimethoprim (87.5%), chloramphenicol (76.9%), ampicillin (75%), nalidixic acid (71%). In most cases, phenotypic resistance to sulfisoxazole (58.7%) and enrofloxacin (50%) was accompanied by a relevant ARGs detection.

In contrast, resistance to streptomycin (40.9%), colistin (25%), ceftazidime (22.2%), ceftiofur (16%), gentamicin (12.7%) and ESBL production (8.3%) was often not confirmed by related ARGs presence. Considering meropenem, phenotypic resistance was not supported by ARG identification.

Two possible reasons for these discrepancies could be:

- Absence of the relevant resistance genes in the reference database;
- Involvement of other genetic mechanisms associated with phenotypic antimicrobial resistance such as efflux pump up-regulation.

AMR is a variable phenomenon, characterised by continuing genetic changes and rearrangements (deletion, insertions, inversions, duplications). It has been estimated that mutations could occur at a rate of 10^{-3} - 10^{-5} per cell per generation, accelerating antimicrobial resistance acquisition (Andersson *et al.*, 1998; Roth, 2011; Durão *et al.*, 2018). Genetic mutations and their accumulation in the genome



	AMP	NA	ENR	CAZ	EFT	C	CN	S	MEM	TE	ST	SXT	COL	ESBL
phenotypic R	20 (23.8%)	5 (7,2%)	7 (10%)	19 (76%)	14 (77.8%)	4 (15.4%)	87 (85.3%)	82 (53.2%)	2 (66.7%)	7 (8.5%)	49 (40.5%)	7 (12.5%)	2 (25%)	20 (83.3%)
phenotypic R/ARGs	63 (75%)	49 (71%)	35 (50%)	4 (16%)	4 (22.2%)	20 (76.9%)	13 (12.7%)	63 (40.9%)	0 (0%)	75 (91.5%)	71 (58.7%)	49 (87.5%)	2 (25%)	2 (8.3%)
ARGs	1 (1.2%)	15 (21.7%)	28 (40%)	2 (8%)	0 (0%)	2 (7.7%)	2 (2%)	9 (5.8%)	1 (33.3%)	0 (0%)	1 (0.8%)	0 (0%)	4 (50%)	2 (8.3%)

Figure 4.6 Graphic representation of the number of isolates showing phenotypic resistance (R) not supported by carriage of related ARGs (blue), phenotypic R and carriage of related ARGs (orange), carriage of ARGs without phenotypic expression, for all the antimicrobials tested (included ESBL profile). Only 279 strains are shown in the figure.

AMP: ampicillin; NA: nalidixic acid; ENR: enrofloxacin; CAZ: ceftazidime; EFT: ceftiofur; C: chloramphenicol; CN: gentamicin; S: streptomycin; MEM: meropenem; TE: tetracycline; ST: sulfisoxazole; SXT: trimethoprim/sulfamethoxazole; COL: colistin; ESBL: extended spectrum beta-lactamase profile.

are the force driving the constant AMR evolution (Weinreich *et al.*, 2006), also leading to the development of novel ARGs variants. Their occurrence and assembly have been frequently described in antimicrobial genetic determinants (Gniadkowski and infection, 2008).

ResFinder, used to identify horizontally-acquired ARGs in our collection, is able to detect 1862 different resistance genes from 12 different antimicrobial classes (Zankari *et al.*, 2012). Despite its continuous update (to include additional and novel variants of genetic determinants) it is possible that novel genes were not included in the databases at the time of the analysis.

The high number of strains showing genetically unexplained antimicrobial resistance could suggest the occurrence of important evolution events, leading to the emergence of new antimicrobial resistance genes. This is of particular concern, because of the involvement of different HP-CIAs (in particular ceftazidime, ceftiofur, colistin and meropenem) and ESBL producers. Further studies are required in order to understand the genetic mechanisms underlying these antimicrobial resistance phenotypes and to allow their detection.

Another program we have utilised is Pointfinder. It used to detect chromosomal point mutations associated with AMR but also has some limitations (Zankari *et al.*, 2017). For example, it doesn't allow the detection of chromosomal mutations in genes coding bacterial cell lipopolysaccharide-modifying enzymes (*pmrC*, *pmrE*, *phoP*, *phoQ*) or in associated negative regulators (*mgrB*, *crrB*), which could explain the phenotypic colistin resistance (not associated to related ARGs) identified in two isolates (Poirel *et al.*, 2017).

The antimicrobial genetic determinants identified in our collection are mostly genes encoding well-understood antimicrobial resistance mechanisms (Partridge *et al.*, 2009). However, in the environment AMR may arise through other pathways, involving other aspecific genetic "machinery" such as efflux pumps.

Efflux pumps are extremely widespread transport proteins involved in the extrusion of a wide range of molecules, including different antimicrobial classes (Webber and Piddock, 2003). Five groups of efflux pump have been reported (the ATP binding cassette superfamily - ABC, the major facilitator superfamily - MFS, the multidrug and toxic-compound extrusion family - MATE, the small multidrug

resistance (SMR) family - SMR and the resistance nodulation division family – RND) (Piddock, 2006). Among these categories, RND family and MFS superfamily represent some of the most implicated in AMR in *E. coli* (Slipski *et al.*, 2018). In particular, RND family are the most common in Gram-negative bacteria (Kumar and Schweizer, 2005) and have been associated to clinically significant MDR (Piddock, 2006).

The biocide gene screening (see pages 101-2) identified different genetic determinants involved in the expression of RND and MFS efflux pumps and related regulators (Figure 4.2.).
In particular, the presence of genes coding for the TolC-associated AcrAB/AcrEF and the MdfA/TolC-associated AcrD efflux pumps could explain our results, because of their potential involvement in resistance to beta-lactams (Ma *et al.*, 1994; Piddock, 2006) and aminoglycosides (Rosenberg *et al.*, 2000; Poole, 2005) respectively. However, phenotypic resistance is strictly related to efflux pump overexpression (influenced by different regulatory genes) (Piddock, 2006), which was not evaluated in this study.

These efflux pumps could also be involved in resistance to numerous other antimicrobials (Table 3.2.), explaining the inconsistencies of strains with ampicillin, nalidixic acid, enrofloxacin, chloramphenicol, tetracycline, sulfisoxazole and sulfamethoxazole/trimethoprim phenotypic expression, without carriage of related antimicrobial genetic determinants.

Efflux pump	**Phenotypic resistance**	**References**
AcrAB (RND)	chloramphenicol, fluoroquinolones, fusidic acid, lipophilic beta-lactam antibiotics, nalidixic acid, novobiocin, rifampin and tetracycline	Piddock *et al.,* 2006
AcrD (RND)	amikacin, gentamicin, neomycin, kanamycin, and tobramycin	Rosenberg *et al*., 2000
AcrEF (RND)	beta-lactams, novobiocin, erythromycin, chloramphenicol, tetracycline, fluoroquinolones	Ma *et al*., 1994
MdfA (MFS)	chloramphenicol, erytrhomycines, tetracyclines, fluoroquinolones	Poole *et al*., 2005

Table 4.2 Efflux pumps of MFS superfamily and RND family and associated phenotypic antimicrobial resistance pattern.

Other specific resistance mechanisms (already reported in *Enterobacteriaceae*) not evaluated and potentially involved in colistin phenotypic resistance are:

- hyperproduction of capsule polysaccharide (CPS), which limited bacterial cell and polymyxin interaction, developing resistance (Campos *et al.*, 2004);
- porin activity, allowing diffusion of molecules across the bacterial cell. For example, the interaction between OmpD porin and the periplasmatic protein YdeI (regulated by the PhoPQ and PmrAB two-component systems) leads to an increase in polymyxin resistance (Pilonieta *et al.*, 2009).

Finally, for almost all antimicrobials (with the exception of ceftiofur, tetracycline and sulfamethozaxole/trimethoprim) a variable number of isolates carried ARGs without showing susceptibility to the related antimicrobials. This was most pronounced for colistin (4/8; 50%), enrofloxacin (28/70; 40%), nalidixic acid (15/69; 21.7%) and meropenem (1/3; 33,3%).

These findings could have different possible explanations. Expression of integron-associated ARGs (including those conferring resistance to aminoglycosides, chloramphenicol, trimethoprim, quinolones)(Partridge *et al.*, 2009) depends on their location in the integron structure. In particular, the proximity to the integron promoter (Pc) is essential for gene expression (Kaushik *et al.*, 2018). Therefore, ARGs located distal to the integron Pc could be weakly or not expressed. At the same time, ARGs typically not included in the integron structure (colistin, meropenem, quinolone resistance genes) could be silent because of the lack or the weakness of their promoter (Carattoli, 2001).

Lastly, other promoter-unrelated silencing mechanisms have been described in bacteria. Currently, little information is available aside from the involvement of silencer elements (AT-rich regions or plasmid centromeres) (Yarmolinsky, 2000) or chromosomal alteration (Enne *et al.*, 2006).

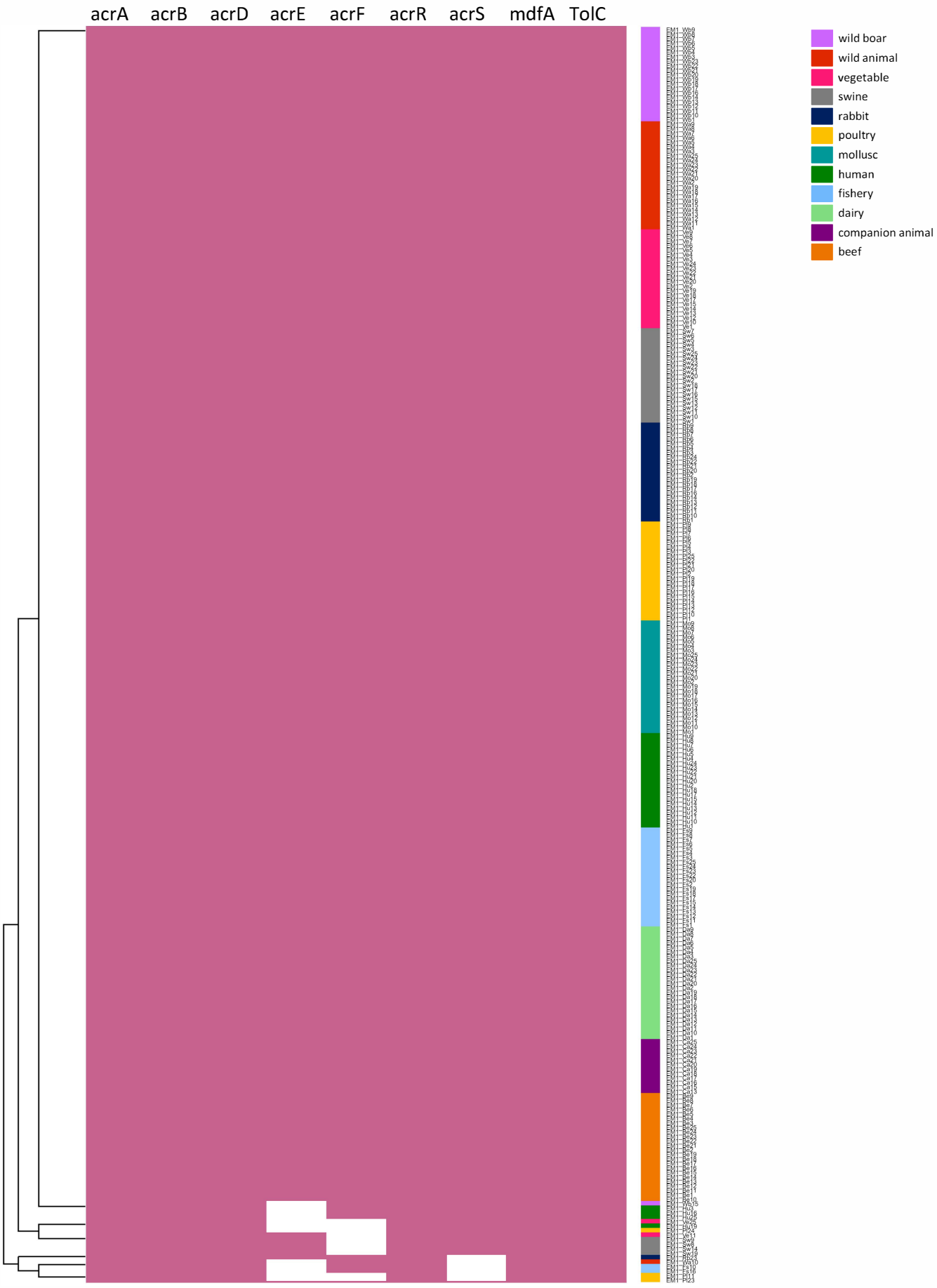


Figure 4.7 Heatmap depicting carriage (violet) of efflux pump associated genes identified in the strain collection. White colour indicates the absence of a specific gene. Colour coded indication of the strain origin (source) is shown before each isolate name.

4.4 References

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5. Conclusion

This PhD thesis provides an important overview of AMR in *E. coli* in Italy, delving deeper into the genetic mechanisms behind phenotypic resistance. The potential epidemiological role played by different environments (animal, food, human) in AMR maintenance and diffusion was explored. In particular, our study supported the role of food-producing animals as a reservoir of potential zoonotic pathogens, with variable antimicrobial resistance and virulence traits.

Our findings demonstrated a general wide diffusion of AMR in the different sources investigated. Among livestock, companion animals, wildlife and human, the most common phenotypic AMR profile was characterised by resistance to tetracycline, ampicillin, sulfisoxazole and aminoglycosides (streptomycin and gentamicin), confirming the general European AMR trend for these niches.

This resistance pattern was congruent with high selective pressure induced by common therapeutic antimicrobial treatments in the sources investigated (tetracycline, ampicillin, sulfisoxazole in food-producing animals; penicillin in humans and companion animals).

An exception was streptomycin/gentamicin resistance, which was widely observed among the collection, despite rare use in both animals and humans. The reasons behind the wide identification of phenotypic resistance to these antimicrobials require further investigation. However, the involvement and diffusion of plasmid-mediated complex antimicrobial genetic structures (like integrons) among the collection could have led to a co-selection and co-transmission of multiple ARGs (including those conferring resistance to aminoglycosides).

Notably, phenotypic resistance to different HP-CIAs (3-GCs, meropenem, colistin, quinolones) was detected among the collection, in particular in food-producing animals. High quinolone resistance was observed in rabbit and poultry strains. Swine carried the highest number of phenotypic colistin resistance isolates. However, colistin resistance gene *mcr1* was mainly identified in rabbit strains, although with no phenotypic expression. Beef, poultry, RTE vegetable, and human were the sources responsible for most 3-GC resistance and ESBL producers. Notably, meropenem resistance was observed in one swine and one human strain.

Phenotypic tetracycline, sulfonamides, quinolones, chloramphenicol and ampicillin resistance were generally explained by identification of related ARGs. However, discrepancies between phenotypic AMR pattern and antimicrobial genetic profile have been observed. In particular, most cases of phenotypic aminoglycosides, meropenem, 3-GCs resistance and ESBL profile did not have a genetic explanation.

This data could suggest the involvement of other genetic mechanisms leading to resistance (i.e. efflux pumps) or AMR evolutional events, responsible for the emergence and diffusion of new genetic variants of ARGs associated to these antimicrobial classes.

Finally, we identified different antimicrobial genetic determinants (in particular those conferring resistance to colistin), without corresponding phenotypic expression, suggesting the potential involvement of different gene silencing mechanisms.

Although the collection was represented by commensal *Escherichia coli*, worrisome genetic virulence traits have been observed. VAGs, typical of ExPEC pathotype, were widespread among all the sources investigated. Moreover, different pandemic (ST69, ST95, ST131) and emerging (ST10, ST23 ST58, ST117, ST405, ST648) ExPEC lineages were identified.

Rabbit and poultry represented the most worrisome sources, presenting the highest phenotypic antimicrobial resistance rate. Moreover, they carried the highest number of ARGs and VAGs among the collection.

Rabbit was suggested as a possible reservoir of colistin resistance *mcr1* gene. The identification of *mcr1* in strains isolated from rabbit meat highlighted the potential for its transmission through the food chain. Notably, rabbit harboured different potential hybrid pathotypes belonging to ST20 and ST40.

Poultry source was characterised by genetic virulence features, typical of ExPEC pathotype, and by different ExPEC lineages (ST10; ST23; ST69, ST117; ST131). Finally, it was suggested as a potential reservoir of ESBL producers, together with beef, RTE vegetable and human niches.

Overall antimicrobial resistance in companion animals was low. However, 4 MDR strains showed an important AMR pattern, with resistance up to 9 different antimicrobial classes. These findings are concerning, considering that potential transmission and exchange of AMR bacteria between pets and humans could be promoted by sharing the same household and by their frequent interaction.

Wildlife AMR profiles reflected those identified in the remaining collection. Moreover, an important number of ExPEC VAGs were identified. Our findings suggest the role of wildlife (in particular birds) in maintenance and diffusion of ARGs and VAGs between human-related and remote environments (and vice versa).

The 2 main limitations of our study are represented by the low sample size and the intrinsic features of the short read sequencing method. In the future, further analysis, grounded on our findings, could be performed to increase the population size and the representativeness of our data. Moreover, the potential assessment of long read sequencing analysis could allow an extensive evaluation of whole bacterial genomes, supplying important epidemiological information related to whole antimicrobial and virulence genetic pool and association with MGEs.

6. Supplementary Material

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Da1	milk	A														
EM1_Da2	in-line milk filter	B1														
EM1_Da3	in-line milk filter	E														
EM1_Da4	in-line milk filter	A														
EM1_Da5	faeces	C														
EM1_Da6	milk	C														
EM1_Da7	in-line milk filter	B2														
EM1_Da8	milk	C														
EM1_Da9	milk	B2														
EM1_Da10	milk	A														
EM1_Da11	intestine	A														
EM1_Da12	intestine	B1														
EM1_Da13	faeces	B1														
EM1_Da14	faeces	B1														
EM1_Da15	faeces	B1														
EM1_Da16	faeces	D														
EM1_Da17	faeces	A														
EM1_Da18	faeces	B1														
EM1_Da19	faeces	C														
EM1_Da20	faeces	B1														
EM1_Da21	milk	A														
EM1_Da22	intestine	A														
EM1_Da23	milk	B1														
EM1_Da24	caciotta cheese	A														
EM1_Da25	milk	B1														

Table S1 Antimicrobic resistance pattern of *E. coli* isolated from dairy source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Be1	faeces	B1														
EM1_Be2	minced meat	C														
EM1_Be3	muscle	B1														
EM1_Be4	skin sponge	B1														
EM1_Be5	skin sponge	B1														
EM1_Be6	skin sponge	B1														
EM1_Be7	skin sponge	B1														
EM1_Be8	skin sponge	B1														
EM1_Be9	skin sponge	B1														
EM1_Be10	skin sponge	B1														
EM1_Be11	skin sponge	B1														
EM1_Be12	skin sponge	B1														
EM1_Be13	faeces	B1														
EM1_Be14	skin sponge	B1														
EM1_Be15	skin sponge	B1														
EM1_Be16	skin sponge	B1														
EM1_Be17	skin sponge	B1														
EM1_Be18	skin sponge	B1														
EM1_Be19	skin sponge	B1														
EM1_Be20	skin sponge	B1														
EM1_Be21	skin sponge	B1														
EM1_Be22	skin sponge	B1														
EM1_Be23	skin sponge	B1														
EM1_Be24	skin sponge	B1														
EM1_Be25	skin sponge	D														

Table S2 Antimicrobic resistance pattern of *E. coli* isolated from beef source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Wb1	diaphragm	B2														
EM1_Wb2	diaphragm	unknown														
EM1_Wb3	diaphragm	B2							■							
EM1_Wb4	diaphragm	E														
EM1_Wb5	diaphragm	B1						■								
EM1_Wb6	diaphragm	E														
EM1_Wb7	diaphragm	E							■	■						
EM1_Wb8	diaphragm	E														
EM1_Wb9	diaphragm	B2														
EM1_Wb10	diaphragm	B2														
EM1_Wb11	diaphragm	B1							■							
EM1_Wb12	diaphragm	unknown														
EM1_Wb13	diaphragm	E														
EM1_Wb14	diaphragm	E														
EM1_Wb15	diaphragm	B2							■							
EM1_Wb16	diaphragm	B1							■	■						
EM1_Wb17	diaphragm	B2				■			■			■				
EM1_Wb18	diaphragm	E							■	■						
EM1_Wb19	diaphragm	E	■						■							
EM1_Wb20	diaphragm	E														
EM1_Wb21	diaphragm	E		■					■	■				■		
EM1_Wb22	diaphragm	E							■							
EM1_Wb23	diaphragm	unknown	■	■				■	■	■		■	■	■		
EM1_Wb24	diaphragm	clade I-II							■							
EM1_Wb25	diaphragm	clade I-II							■			■				

Table S3 Antimicrobic resistance pattern of *E. coli* isolated from wild boar source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Ve1	spinach (RTE)	B1														
EM1_Ve2	celery	B1														
EM1_Ve3	rosemary (RTE)	E														
EM1_Ve4	arthicoke	B1														
EM1_Ve5	arugula (RTE)	unknown														
EM1_Ve6	artichoke	A														
EM1_Ve7	spinach	B1														
EM1_Ve8	parsley	B1														
EM1_Ve9	celery	B1														
EM1_Ve10	gentile salad	B1														
EM1_Ve11	roman salad	B2														
EM1_Ve12	spinach	B1														
EM1_Ve13	arugula (RTE)	B1														
EM1_Ve14	parsley	B1														
EM1_Ve15	celery	C														
EM1_Ve16	spinach	unknown														
EM1_Ve17	rosemary (RTE)	B1														
EM1_Ve18	rosemary (RTE)	B1														
EM1_Ve19	salad "roman"	B1														
EM1_Ve20	arugula (RTE)	B1														
EM1_Ve21	rosemary (RTE)	C														
EM1_Ve22	spinach	C														
EM1_Ve23	spinach (RTE)	C														
EM1_Ve24	parsley	C														
EM1_Ve25	rosemary (RTE)	C														

Table S4 Antimicrobic resistance pattern of *E. coli* isolated from vegetable source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Fs1	*Salmo trutta*	A							■	■		■	■	■		
EM1_Fs2	*Salmo trutta*	B1	■						■	■		■		■		
EM1_Fs3	*Dicentrarchus labrax*	C	■									■		■		
EM1_Fs4	*Salmo salar*	A														
EM1_Fs5	*Engraulis encrasicolus*	B1							■			■				
EM1_Fs6	*Sardina pilchardus*	C							■	■		■				
EM1_Fs7	*Dicentrarchus labrax*	A							■	■						
EM1_Fs8	*Salmo salar*	A							■	■						
EM1_Fs9	*Sardina pilchardus*	A														
EM1_Fs10	*Engraulis encrasicolus*	A														
EM1_Fs11	*Trachurus Trachurus*	E										■				
EM1_Fs12	*Salmo salar*	A														
EM1_Fs13	*Mullus surmuletus*	C														
EM1_Fs14	*Sardina pilchardus*	C														
EM1_Fs15	*Sparus aurata*	E														
EM1_Fs16	*Sarpa salpa*	A														
EM1_Fs17	*Platichthys flesus*	A				■			■	■						■
EM1_Fs18	*Sparus aurata*	B1	■		■	■	■		■	■		■				■
EM1_Fs19	*Trisopterus capelanus*	E										■				
EM1_Fs20	*Engraulis encrasicolus*	A								■						
EM1_Fs21	*Sparus aurata*	B1								■				■		
EM1_Fs22	*Salmo trutta*	B2							■							
EM1_Fs23	*Engraulis encrasicolus*	B1					■							■		
EM1_Fs24	*Sardina pilchardus*	B2								■		■				
EM1_Fs25	*Gobius paganellus*	C	■						■	■		■	■			

Table S5 Antimicrobic resistance pattern of *E. coli* isolated from fishery source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Ca13	faeces	B1														
EM1_Ca14	faeces	B2														
EM1_Ca15	faeces	C														
EM1_Ca16	faeces	B1														
EM1_Ca17	faeces	B2	■											■		
EM1_Ca18	faeces	B2														
EM1_Ca19	faeces	B1														
EM1_Ca20	faeces	B1														
EM1_Ca21	faeces	B1	■				■					■				
EM1_Ca22	faeces	C	■							■		■	■	■		
EM1_Ca23	faeces	E														
EM1_Ca24	faeces	B1	■	■		■		■	■	■		■	■	■		■
EM1_Ca25	faeces	E	■		■				■	■		■	■	■		

Table S6 Antimicrobic resistance pattern of *E. coli* isolated from companion animal source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Sw1	faeces	C														
EM1_Sw2	faeces	B1														
EM1_Sw3	faeces	A														
EM1_Sw4	faeces	E														
EM1_Sw5	faeces	A														
EM1_Sw6	faeces	B1														
EM1_Sw7	faeces	A														
EM1_Sw8	faeces	A														
EM1_Sw9	faeces	A														
EM1_Sw10	faeces	C														
EM1_Sw11	faeces	A														
EM1_Sw12	faeces	B1														
EM1_Sw13	faeces	B1														
EM1_Sw14	faeces	C														
EM1_Sw15	faeces	B1														
EM1_Sw16	faeces	C														
EM1_Sw17	faeces	C														
EM1_Sw18	faeces	C														
EM1_Sw19	faeces	C														
EM1_Sw20	muscle	B1														
EM1_Sw21	sausage	B1														
EM1_Sw22	meat	B1														
EM1_Sw23	meat	C														
EM1_Sw24	minced meat	A														
EM1_Sw25	meat	A														

Table S7 Antimicrobic resistance pattern of *E. coli* isolated from swine source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Pl1	breast	C	■	■		■		■	■	■		■				■
EM1_Pl2	thigh	B1	■			■			■					■		■
EM1_Pl3	thigh	B1	■	■		■		■	■	■		■	■	■		■
EM1_Pl4	thigh	B2	■						■	■		■	■			
EM1_Pl5	wing	F	■	■			■	■		■		■	■	■		
EM1_Pl6	wing	C	■	■			■	■		■		■	■	■		
EM1_Pl7	breast	E	■	■			■	■		■		■	■			
EM1_Pl8	hamburger	C		■				■	■			■				
EM1_Pl9	thigh	C	■	■						■		■	■			
EM1_Pl10	faeces	B1	■				■		■	■		■				
EM1_Pl11	meat	A		■												
EM1_Pl12	faeces	unknown							■	■				■		
EM1_Pl13	breast	C	■	■				■								
EM1_Pl14	thigh	F	■	■				■	■	■		■		■		
EM1_Pl15	hamburger	B1	■		■				■							
EM1_Pl16	wing	B1							■	■						
EM1_Pl17	wing	A	■	■			■	■		■		■	■	■		
EM1_Pl18	breast	E	■	■			■	■	■	■		■	■	■		
EM1_Pl19	thigh	F	■						■							
EM1_Pl20	breast	C	■	■				■	■	■		■	■			
EM1_Pl21	thigh	F	■	■				■	■	■		■	■	■		
EM1_Pl22	hamburger	F	■				■		■	■		■	■	■		
EM1_Pl23	breast	C	■	■		■	■	■		■		■		■	■	■
EM1_Pl24	thigh	C	■											■		
EM1_Pl25	wing	B1	■									■	■	■		

Table S8 Antimicrobic resistance pattern of *E. coli* isolated from poultry source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Rb1	intestine	B1														
EM1_Rb2	intestine	B1														
EM1_Rb3	intestine	B1														
EM1_Rb4	intestine	B1														
EM1_Rb5	intestine	B1														
EM1_Rb6	intestine	B1														
EM1_Rb7	intestine	B1														
EM1_Rb8	intestine	B1														
EM1_Rb9	intestine	B1														
EM1_Rb10	meat	B1														
EM1_Rb11	meat	B1														
EM1_Rb12	meat	B1														
EM1_Rb13	meat	D														
EM1_Rb14	meat	B1														
EM1_Rb15	meat	B1														
EM1_Rb16	liver	B1														
EM1_Rb17	meat	D														
EM1_Rb18	meat	B1														
EM1_Rb19	meat	B1														
EM1_Rb20	meat	B1														
EM1_Rb21	meat	B1														
EM1_Rb22	meat	B1														
EM1_Rb23	intestine	B2														
EM1_Rb24	intestine	B1														
EM1_Rb25	intestine	B1														

Table S9 Antimicrobic resistance pattern of *E. coli* isolated from rabbit source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Mo1	mussel	C														
EM1_Mo2	mussel	D														
EM1_Mo3	mussel	C														
EM1_Mo4	mussel	D														
EM1_Mo5	clam	C														
EM1_Mo6	clam	B1														
EM1_Mo7	clam	A														
EM1_Mo8	clam	B1														
EM1_Mo9	clam	B1														
EM1_Mo10	clam	B1														
EM1_Mo11	clam	B1														
EM1_Mo12	mussel	B1														
EM1_Mo13	mussel	B1														
EM1_Mo14	clam	B1														
EM1_Mo15	clam	A														
EM1_Mo16	clam	B1														
EM1_Mo17	mussel	B1														
EM1_Mo18	mussel	B1														
EM1_Mo19	mussel	B1														
EM1_Mo20	clam	B1														
EM1_Mo21	clam	E														
EM1_Mo22	clam	C														
EM1_Mo23	clam	B1														
EM1_Mo24	clam	D														
EM1_Mo25	clam	B1														

Table S10 Antimicrobic resistance pattern of *E. coli* isolated from mollusc source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Matrices	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Hu1	faeces	A														
EM1_Hu2	faeces	B2														
EM1_Hu3	faeces	F														
EM1_Hu4	faeces	E														
EM1_Hu5	faeces	A														
EM1_Hu6	faeces	F														
EM1_Hu7	faeces	C														
EM1_Hu8	faeces	E														
EM1_Hu9	faeces	D														
EM1_Hu10	faeces	A														
EM1_Hu11	faeces	C														
EM1_Hu12	faeces	F														
EM1_Hu13	faeces	B1														
EM1_Hu14	faeces	A														
EM1_Hu15	faeces	B1														
EM1_Hu16	faeces	F														
EM1_Hu17	faeces	B2														
EM1_Hu18	faeces	A														
EM1_Hu19	faeces	F														
EM1_Hu20	faeces	A														
EM1_Hu21	faeces	F														
EM1_Hu22	faeces	C														
EM1_Hu23	faeces	E														
EM1_Hu24	faeces	D														
EM1_Hu25	faeces	F														

Table S11 Antimicrobic resistance pattern of *E. coli* isolated from human source.
AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Animal	Phylogroup	AMP	NA	EFT	CAZ	C	ENR	CN	S	MEM	ST	SXT	TE	COL	ESBL
EM1_Wa1	seagull	C														
EM1_Wa2	seagull	B1														
EM1_Wa3	heron	B1														
EM1_Wa4	roe deer	B2														
EM1_Wa5	Podiceps spp.	B1														
EM1_Wa6	roe deer	B1														
EM1_Wa7	fox	B1														
EM1_Wa8	roe deer	C														
EM1_Wa9	fox	B1														
EM1_Wa10	dolphin	A														
EM1_Wa11	fox	B1														
EM1_Wa12	badger	B1														
EM1_Wa13	fox	B2														
EM1_Wa14	fox	B1														
EM1_Wa15	fallow deer	B1														
EM1_Wa16	fallow deer	B1														
EM1_Wa17	carrion crow	B1														
EM1_Wa18	magpie	B1														
EM1_Wa19	magpie	B1														
EM1_Wa20	fallow deer	B1														
EM1_Wa21	turtledove	B1														
EM1_Wa22	turtledove	B1														
EM1_Wa23	stork	F														
EM1_Wa24	magpie	B2														
EM1_Wa25	pheasant	B1														

Table S12 Antimicrobic resistance pattern of *E. coli* isolated from wild animal source. Matrices: miscellaneous.

AMP: ampicillin; NA: nalidixic acid; EFT: ceftiofur; CAZ: ceftazidime; C: chloramphenicol; ENR: enrofloxacin; CN: gentamicin; S: streptomycin; MEM: meropenem; ST: sulfisoxazole; TE: tetracycline; SXT: trimethoprim/sulfamethoxazole.

Strain	Source	Matrice	Sequence type	Serotype	NCBI ID
EM1_Da1	dairy	milk	1510	O93:H19	SAMN11246364
EM1_Da2	dairy	in-line milk filter	Novel	O88:H8	SAMN11246365
EM1_Da3	dairy	in-line milk filter	Novel	O88:H31	SAMN11246366
EM1_Da4	dairy	in-line milk filter	398	O8:H20	SAMN11246367
EM1_Da5	dairy	faeces	Novel	O123:H16	SAMN11246368
EM1_Da6	dairy	milk	10	ONT:H12	SAMN11246369
EM1_Da7	dairy	in-line milk filter	Novel	O51:H37	SAMN11246370
EM1_Da8	dairy	milk	10	O107:HNT	SAMN11246371
EM1_Da9	dairy	milk	583	O71:H6	SAMN11246372
EM1_Da10	dairy	milk	1510	O93:H19	SAMN11246373
EM1_Da11	dairy	intestine	1303	ONT:H18	SAMN11246374
EM1_Da12	dairy	intestine	58	ONT:H27	SAMN11246375
EM1_Da13	dairy	faeces	155	O8:H21	SAMN11246376
EM1_Da14	dairy	faeces	155	ONT:H45	SAMN11246377
EM1_Da15	dairy	faeces	155	ONT:H45	SAMN11246378
EM1_Da16	dairy	faeces	69	O15:H18	SAMN11246379
EM1_Da17	dairy	faeces	1510	O93:H19	SAMN11246380
EM1_Da18	dairy	faeces	278	O105:H7	SAMN11246381
EM1_Da19	dairy	faeces	1091	O53:H10	SAMN11246382
EM1_Da20	dairy	faeces	278	O105:H7	SAMN11246383
EM1_Da21	dairy	milk	731	O15:H40	SAMN11246384
EM1_Da22	dairy	intestine	10	O89*/O162:H9	SAMN11246385
EM1_Da23	dairy	milk	2328	O54:H2	SAMN11246386
EM1_Da24	dairy	caciotta cheese	216	O3:H4	SAMN11246387
EM1_Da25	dairy	milk	1125	O139:H19	SAMN11246388
EM1_Be1	beef	faeces	297	ONT:H37	SAMN11246389
EM1_Be2	beef	minced meat	10	O13-O53*/O13_O135-O53*:H10-H11	SAMN11246390
EM1_Be3	beef	muscle	392	O109:H2	SAMN11246391
EM1_Be4	beef	carcass sponge	847	ONT:H2	SAMN11246392
EM1_Be5	beef	carcass sponge	847	ONT:H2	SAMN11246393
EM1_Be6	beef	carcass sponge	847	ONT:H2	SAMN11246394
EM1_Be7	beef	carcass sponge	6345	O83:H7	SAMN11246395
EM1_Be8	beef	carcass sponge	847	ONT:H2	SAMN11246396
EM1_Be9	beef	carcass sponge	847	ONT:H2	SAMN11246397
EM1_Be10	beef	carcass sponge	847	ONT:H2	SAMN11246398
EM1_Be11	beef	carcass sponge	847	ONT:H2	SAMN11246399
EM1_Be12	beef	carcass sponge	847	ONT:H2	SAMN11246400
EM1_Be13	beef	faeces	2178	O170:H49	SAMN11246401
EM1_Be14	beef	carcass sponge	906	O74:H8	SAMN11246402
EM1_Be15	beef	carcass sponge	906	O74:H8	SAMN11246403
EM1_Be16	beef	carcass sponge	2280	O185:H16	SAMN11246404
EM1_Be17	beef	carcass sponge	187	O110:H2	SAMN11246405
EM1_Be18	beef	carcass sponge	1665	ONT:H2	SAMN11246406
EM1_Be19	beef	carcass sponge	58	O58:H40	SAMN11246407
EM1_Be21	beef	carcass sponge	154	O154:H38	SAMN11246409

Table S13 Details of the strain collection.

Strain	Source	Matrice	Sequence type	Serotype	NCBI ID
EM1_Be22	beef	carcass sponge	101	O174:H40	SAMN11246410
EM1_Be23	beef	carcass sponge	1463	O18ac*/O18*:H7	SAMN11246411
EM1_Be24	beef	carcass sponge	58	ONT:H12	SAMN11246412
EM1_Be25	beef	carcass sponge	1665	ONT:H2	SAMN11246413
EM1_Wb1	wild boar	diaphragm	3553	O105:H32	SAMN11246414
EM1_Wb3	wild boar	diaphragm	141	O2:H6	SAMN11246416
EM1_Wb4	wild boar	diaphragm	Novel	O151:H20	SAMN11246417
EM1_Wb5	wild boar	diaphragm	4623	ONT:H16	SAMN11246418
EM1_Wb6	wild boar	diaphragm	69*	O17/O44/O77:H18	SAMN11246419
EM1_Wb7	wild boar	diaphragm	5913	O87:H19	SAMN11246420
EM1_Wb8	wild boar	diaphragm	69*	O17/O44/O77:H18	SAMN11246421
EM1_Wb9	wild boar	diaphragm	681	O8:H10	SAMN11246422
EM1_Wb10	wild boar	diaphragm	680	O169:H1	SAMN11246423
EM1_Wb11	wild boar	diaphragm	13	O49:H10	SAMN11246424
EM1_Wb12	wild boar	diaphragm	201	O8:H19	SAMN11246425
EM1_Wb13	wild boar	diaphragm	5597	O75:H31	SAMN11246426
EM1_Wb14	wild boar	diaphragm	973	O11:H15	SAMN11246427
EM1_Wb15	wild boar	diaphragm	134*	ONT:H10	SAMN11246428
EM1_Wb16	wild boar	diaphragm	2522	ONT:H8	SAMN11246429
EM1_Wb17	wild boar	diaphragm	1170	ONT:H4	SAMN11246430
EM1_Wb18	wild boar	diaphragm	5597	O75:H31	SAMN11246431
EM1_Wb19	wild boar	diaphragm	4541	O146:H28	SAMN11246432
EM1_Wb20	wild boar	diaphragm	Novel	O9:H31	SAMN11246433
EM1_Wb21	wild boar	diaphragm	117	O24:H4	SAMN11246434
EM1_Wb22	wild boar	diaphragm	Novel	ONT:H9	SAMN11246435
EM1_Wb23	wild boar	diaphragm	162	O29:H9	SAMN11246436
EM1_Ve1	vegetable	spinach (RTE)	2280	O185:H16	SAMN11246439
EM1_Ve2	vegetable	celery	708	O8:H19	SAMN11246440
EM1_Ve3	vegetable	rosemary (RTE)	1508	O172:H45	SAMN11246441
EM1_Ve4	vegetable	arthicoke	2313	ONT:H7	SAMN11246442
EM1_Ve5	vegetable	arugula (RTE)	1819	O166:H28	SAMN11246443
EM1_Ve6	vegetable	artichoke	191	O48:H20	SAMN11246444
EM1_Ve7	vegetable	spinach	2005	ONT:H7	SAMN11246445
EM1_Ve8	vegetable	parsley	2161	O180:H14	SAMN11246446
EM1_Ve9	vegetable	celery	602	ONT:H21	SAMN11246447
EM1_Ve10	vegetable	gentile salad	348	O181:H28	SAMN11246448
EM1_Ve11	vegetable	roman salad	3601	O8:H6	SAMN11246449
EM1_Ve12	vegetable	spinach	5296	O112ac:H20	SAMN11246450
EM1_Ve13	vegetable	arugula (RTE)	278*	O153_O178:H7	SAMN11246451
EM1_Ve14	vegetable	parsley	13	ONT:H10	SAMN11246452
EM1_Ve15	vegetable	celery	10	O113:H4	SAMN11246453
EM1_Ve17	vegetable	spinach	56	O38:H21	SAMN11246455
EM1_Ve18	vegetable	rosemary (RTE)	40	O109:H21	SAMN11246456
EM1_Ve19	vegetable	rosemary (RTE)	164	ONT:H8	SAMN11246457
EM1_Ve20	vegetable	roman salad	3574	O105:H23	SAMN11246458
EM1_Ve21	vegetable	arugula (RTE)	48	O81:H11	SAMN11246459

Table S13 ..\..

Strain	Source	Matrice	Sequence type	Serotype	NCBI ID
EM1_Ve22	vegetable	rosemary (RTE)	6223	O21:H12	SAMN11246460
EM1_Ve23	vegetable	spinach	34	O62:H30	SAMN11246461
EM1_Ve24	vegetable	spinach (RTE)	88	O9:H19	SAMN11246462
EM1_Ve25	vegetable	parsley	48*	O184:H11	SAMN11246463
EM1_Fs1	vegetable	rosemary (RTE)	540	O9:H30	SAMN11246464
EM1_Fs2	fishery	*Salmo trutta*	446	013353_O103*/ O103* :H21	SAMN11246465
EM1_Fs3	fishery	*Dicentrarchus labrax*	88	O8:H17	SAMN11246466
EM1_Fs4	fishery	*Salmo salar*	216	O10:H26	SAMN11246467
EM1_Fs5	fishery	*Engraulis encrasicolus*	2073	O180:H14	SAMN11246468
EM1_Fs6	fishery	*Sardina pilchardus*	34	O62:H30	SAMN11246469
EM1_Fs7	fishery	*Dicentrarchus labrax*	399	O15:H12	SAMN11246470
EM1_Fs8	fishery	*Salmo salar*	399	O15:H12	SAMN11246471
EM1_Fs9	fishery	*Sardina pilchardus*	635	O8*/O40:H25	SAMN11246472
EM1_Fs10	fishery	*Engraulis encrasicolus*	216	O7:H39	SAMN11246473
EM1_Fs11	fishery	*Trachurus Trachurus*	1140	O38:H39	SAMN11246474
EM1_Fs12	fishery	*Salmo salar*	635	O8*/O40:H25	SAMN11246475
EM1_Fs13	fishery	*Mullus surmuletus*	1137	O21:H25	SAMN11246476
EM1_Fs14	fishery	*Sardina pilchardus*	1137	O21:H25	SAMN11246477
EM1_Fs15	fishery	*Sparus aurata*	132	ONT:H1	SAMN11246478
EM1_Fs16	fishery	*Sarpa salpa*	216	O7:H39	SAMN11246479
EM1_Fs17	fishery	*Platichthys flesus*	216	O19:H4	SAMN11246480
EM1_Fs18	fishery	*Sparus aurata*	212	O18ac*/O18*:H49	SAMN11246481
EM1_Fs19	fishery	*Trisopterus capelanus*	973	O11:H15	SAMN11246482
EM1_Fs20	fishery	*Engraulis encrasicolus*	1408	O13:H30	SAMN11246483
EM1_Fs22	fishery	*Salmo trutta*	3910	O8:H33	SAMN11246485
EM1_Fs23	fishery	*Engraulis encrasicolus*	Novel	O112ac:H16	SAMN11246486
EM1_Fs24	fishery	*Sardina pilchardus*	Novel	O8/O30:H10	SAMN11246487
EM1_Fs25	fishery	*Gobius paganellus*	783	O83:H9	SAMN11246488
EM1_Ca13	dog	faeces	155	O86:H51	SAMN11246489
EM1_Ca15	dog	faeces	10	ONT:H16	SAMN11246501
EM1_Ca16	dog	faeces	327	O23:H8	SAMN11246503
EM1_Ca17	dog	faeces	80	O75:H7	SAMN11246504
EM1_Ca18	dog	faeces	372	O2:H31	SAMN11246505
EM1_Ca19	dog	faeces	2521	ONT:H7	SAMN11246506
EM1_Ca20	dog	faeces	297	O149:H45	SAMN11246507
EM1_Ca21	dog	faeces	212	O18ac*/O18*:H49	SAMN11246508
EM1_Ca22	dog	faeces	410	O8:H9	SAMN11246509
EM1_Ca23	dog	faeces	1629	ONT:H31	SAMN11246510
EM1_Ca24	cat	faeces	58	O9:H9	SAMN11246511
EM1_Ca25	cat	faeces	297	O49:H49	SAMN11246512
EM1_Sw1	swine	faeces	10	O16:H48	SAMN11246513
EM1_Sw2	swine	faeces	641	O9/O30:H25	SAMN11246514
EM1_Sw3	swine	faeces	3744	O157:H42	SAMN11246515
EM1_Sw4	swine	faeces	5759	ONT:H20	SAMN11246516
EM1_Sw5	swine	faeces	100	O149:H10	SAMN11246517

Table S13 ..\..

Strain	Source	Matrice	Sequence type	Serotype	NCBI ID
EM1_Sw6	swine	faeces	20	O177:H49	SAMN11246519
EM1_Sw7	swine	faeces	100	O149:H10	SAMN11246520
EM1_Sw8	swine	faeces	10	O98:H12	SAMN11246521
EM1_Sw9	swine	faeces	10	O98:H12	SAMN11246522
EM1_Sw10	swine	faeces	206	ONT:H5	SAMN11246523
EM1_Sw11	swine	faeces	871	O16:H5	SAMN11246524
EM1_Sw12	swine	faeces	641	O45:H10	SAMN11246525
EM1_Sw13	swine	faeces	410	O15:H12	SAMN11246526
EM1_Sw14	swine	faeces	10	O98:H12	SAMN11246527
EM1_Sw15	swine	faeces	7093	O8:H14	SAMN11246528
EM1_Sw16	swine	faeces	410*	O15:H12	SAMN11246529
EM1_Sw17	swine	faeces	10	O89*/O162:H9	SAMN11246530
EM1_Sw18	swine	faeces	88	O8/O160:H4	SAMN11246531
EM1_Sw19	swine	faeces	10*	O98:H12	SAMN11246532
EM1_Sw20	swine	muscle	101	O109:H40	SAMN11246533
EM1_Sw21	swine	sausage	101	ONT:H21	SAMN11246534
EM1_Sw22	swine	meat	795	O76:H7	SAMN11246535
EM1_Sw23	swine	meat	10	O69:H32	SAMN11246536
EM1_Sw24	swine	minced meat	1434*	O8*/O40:H27	SAMN11246537
EM1_Sw25	swine	meat	399	O25:H12	SAMN11246538
EM1_Pl1	poultry	breast	23	O78:H4	SAMN11246539
EM1_Pl2	poultry	thigh	101	O88:H8	SAMN11246540
EM1_Pl3	poultry	thigh	359	O115:H21	SAMN11246541
EM1_Pl4	poultry	thigh	131	O25:H4	SAMN11246542
EM1_Pl5	poultry	wing	117	O143:H4	SAMN11246543
EM1_Pl6	poultry	wing	744	O89*/O162:H9	SAMN11246544
EM1_Pl7	poultry	breast	57	O11:H25	SAMN11246545
EM1_Pl8	poultry	hamburger	23	O78:H4	SAMN11246546
EM1_Pl9	poultry	thigh	48	O8/O152:H11	SAMN11246547
EM1_Pl10	poultry	faeces	162	O8/O134*/O46*:H19	SAMN11246548
EM1_Pl11	poultry	meat	10*	O16:H48	SAMN11246549
EM1_Pl12	poultry	faeces	155	O8:H20	SAMN11246550
EM1_Pl13	poultry	breast	23	O78:H4	SAMN11246551
EM1_Pl14	poultry	thigh	117	O161:H4	SAMN11246552
EM1_Pl15	poultry	hamburger	2614	O138:H28	SAMN11246553
EM1_Pl16	poultry	wing	297	O149:H8	SAMN11246554
EM1_Pl17	poultry	wing	93	O7:H4	SAMN11246555
EM1_Pl18	poultry	breast	69	O17/O44/O77:H18	SAMN11246556
EM1_Pl19	poultry	thigh	117	O102:H4	SAMN11246557
EM1_Pl20	poultry	breast	1286	O16:H32	SAMN11246558
EM1_Pl21	poultry	thigh	117	O35:H4	SAMN11246559
EM1_Pl22	poultry	hamburger	117	O161:H4	SAMN11246560
EM1_Pl23	poultry	breast	10	ONT:H48	SAMN11246561
EM1_Pl24	poultry	thigh	48*	O25*:H11	SAMN11246562
EM1_Pl25	poultry	wing	155	O29:H10	SAMN11246563
EM1_Rb1	rabbit	intestine	40	O109:H21	SAMN11246564

Table S13 ..\..

Strain	Source	Matrice	Sequence type	Serotype	NCBI ID
EM1_Rb2	rabbit	intestine	20	54Cigleris_O128ab*/O128ac*:H2	SAMN11246565
EM1_Rb3	rabbit	intestine	40	O109:H21	SAMN11246566
EM1_Rb4	rabbit	intestine	20	54Cigleris_O128ab*/O128ac*:H2	SAMN11246567
EM1_Rb5	rabbit	intestine	1642	O8:H7	SAMN11246568
EM1_Rb6	rabbit	intestine	40	O109:H21	SAMN11246569
EM1_Rb7	rabbit	intestine	20	O145:H2	SAMN11246570
EM1_Rb8	rabbit	intestine	1611	O125ab:H19	SAMN11246571
EM1_Rb9	rabbit	intestine	297	O85:H8	SAMN11246572
EM1_Rb10	rabbit	meat	533	O76:H14	SAMN11246573
EM1_Rb11	rabbit	meat	20	O49:H2	SAMN11246574
EM1_Rb12	rabbit	meat	129	O104:H7	SAMN11246575
EM1_Rb13	rabbit	meat	706*	O85:H1	SAMN11246576
EM1_Rb14	rabbit	meat	906	O150:H8	SAMN11246577
EM1_Rb16	rabbit	liver	351	O18ac*/O18*:H7	SAMN11246579
EM1_Rb17	rabbit	meat	501	O17/O44/O77:H1	SAMN11246580
EM1_Rb18	rabbit	meat	224	O102:H23	SAMN11246581
EM1_Rb19	rabbit	meat	111	O8:H49	SAMN11246582
EM1_Rb20	rabbit	meat	539	ONT:H28	SAMN11246583
EM1_Rb21	rabbit	meat	1431	O8:H19	SAMN11246584
EM1_Rb22	rabbit	meat	40	O109:H21	SAMN11246585
EM1_Rb23	rabbit	intestine	491	O54:H45	SAMN11246586
EM1_Rb24	rabbit	intestine	1727	O83:H7	SAMN11246587
EM1_Mo1	mollusc	mussel	10	O9:H9	SAMN11246589
EM1_Mo2	mollusc	mussel	69	O15:H6	SAMN11246590
EM1_Mo3	mollusc	mussel	23	O8:H30	SAMN11246591
EM1_Mo4	mollusc	mussel	1406	O51:H14	SAMN11246592
EM1_Mo5	mollusc	clam	10	O13:H11	SAMN11246593
EM1_Mo6	mollusc	clam	661	O174:H2	SAMN11246594
EM1_Mo7	mollusc	clam	540	O9:H30	SAMN11246595
EM1_Mo8	mollusc	clam	3576*	O8/O113:H7	SAMN11246596
EM1_Mo9	mollusc	clam	345	O8/O134*/O46*:H21	SAMN11246597
EM1_Mo10	mollusc	clam	2521	O147:H7	SAMN11246598
EM1_Mo11	mollusc	clam	442	O91:H21	SAMN11246599
EM1_Mo12	mollusc	mussel	6188	ONT:H8	SAMN11246600
EM1_Mo13	mollusc	mussel	6096	O39:H28	SAMN11246601
EM1_Mo14	mollusc	clam	4481	ONT:H7	SAMN11246602
EM1_Mo15	mollusc	clam	398	O159:H21	SAMN11246603
EM1_Mo16	mollusc	clam	4088	O113:H7	SAMN11246604
EM1_Mo17	mollusc	mussel	602	ONT:H21	SAMN11246605
EM1_Mo18	mollusc	mussel	446	ONT:H8	SAMN11246606
EM1_Mo19	mollusc	mussel	Novel	O148:H8	SAMN11246607
EM1_Mo20	mollusc	clam	2768	O65:H19	SAMN11246608
EM1_Mo21	mollusc	clam	69	O15:H18	SAMN11246609
EM1_Mo22	mollusc	clam	34	O92:H33	SAMN11246610

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Strain	Source	Matrice	Sequence type	Serotype	NCBI ID
EM1_Mo23	mollusc	clam	101	O55:H12	SAMN11246611
EM1_Mo24	mollusc	clam	69	O25:H4	SAMN11246612
EM1_Mo25	mollusc	clam	942	O39:H28	SAMN11246613
EM1_Hu1	human	faeces	Novel	O7:H4	SAMN11246614
EM1_Hu2	human	faeces	95	O1:H7	SAMN11246615
EM1_Hu3	human	faeces	59	O1:H7	SAMN11246616
EM1_Hu4	human	faeces	69	O15:H18	SAMN11246617
EM1_Hu5	human	faeces	165	O80:H19	SAMN11246618
EM1_Hu6	human	faeces	657	O183:H18	SAMN11246619
EM1_Hu7	human	faeces	10*	O78:H12	SAMN11246620
EM1_Hu8	human	faeces	543	O169:H9	SAMN11246621
EM1_Hu9	human	faeces	69	O15:H18	SAMN11246622
EM1_Hu10	human	faeces	Novel	O159:H5	SAMN11246623
EM1_Hu11	human	faeces	48	O88:H5	SAMN11246624
EM1_Hu12	human	faeces	420*	O134*/O46*:H31	SAMN11246625
EM1_Hu13	human	faeces	20	54Cigleris_O128ab*/ O128ac*:H2	SAMN11246626
EM1_Hu14	human	faeces	398	O8:H20	SAMN11246627
EM1_Hu15	human	faeces	155	FJ539194_O28ac_O42*/ O28ac_O42*:H21	SAMN11246628
EM1_Hu16	human	faeces	59	O1:H7	SAMN11246629
EM1_Hu17	human	faeces	95	O1:H7	SAMN11246630
EM1_Hu18	human	faeces	1072	O4:H4	SAMN11246631
EM1_Hu19	human	faeces	59	O1:H7	SAMN11246632
EM1_Hu20	human	faeces	Novel	O187:H11	SAMN11246633
EM1_Hu21	human	faeces	457	O11:H25	SAMN11246634
EM1_Hu22	human	faeces	10	O168:H4	SAMN11246635
EM1_Hu23	human	faeces	405	O102:H6	SAMN11246636
EM1_Hu24	human	faeces	69	O8*/O25:H4	SAMN11246637
EM1_Hu25	human	faeces	59	O1:H7	SAMN11246638
EM1_Wa1	seagull	miscellaneous	23	O8/O160:H12	SAMN11246639
EM1_Wa2	seagull	miscellaneous	4388	O180:H14	SAMN11246640
EM1_Wa3	heron	miscellaneous	155	O109:H51	SAMN11246641
EM1_Wa4	roe deer	miscellaneous	7900	O8/O147:H5	SAMN11246642
EM1_Wa5	Podiceps spp.	miscellaneous	162*	O45:H2	SAMN11246643
EM1_Wa6	roe deer	miscellaneous	2614	O138:H28	SAMN11246644
EM1_Wa7	fox	miscellaneous	2602	O8:H16	SAMN11246645
EM1_Wa8	roe deer	miscellaneous	23	O8/O8:H9	SAMN11246646
EM1_Wa9	fox	miscellaneous	2522	ONT:H8	SAMN11246647
EM1_Wa10	dolphin	miscellaneous	216	O7:H39	SAMN11246648
EM1_Wa11	fox	miscellaneous	4054	O88:H28	SAMN11246649
EM1_Wa12	badger	miscellaneous	2354	O103:H21	SAMN11246650
EM1_Wa13	fox	miscellaneous	136	O8:H10	SAMN11246651
EM1_Wa14	fox	miscellaneous	5177*	O88:H25	SAMN11246652
EM1_Wa15	fallow deer	miscellaneous	Novel	ONT:H19	SAMN11246653
EM1_Wa16	fallow deer	miscellaneous	392	O8:H2	SAMN11246654
EM1_Wa17	carrion crow	miscellaneous	4392	O149:H8	SAMN11246655

Table S13 ..\..

Strain	Source	Matrice	Sequence type	Serotype	NCBI ID
EM1_Wa18	magpie	miscellaneous	847	ONT:H2	SAMN11246656
EM1_Wa19	magpie	miscellaneous	4198	O75:H38	SAMN11246657
EM1_Wa20	fallow deer	miscellaneous	196	O8:H7	SAMN11246658
EM1_Wa21	turtledove	miscellaneous	2521	ONT:H7	SAMN11246659
EM1_Wa22	turtledove	miscellaneous	20	54Cigleris_O128ab* /O128ac*:H2	SAMN11246660
EM1_Wa23	stork	miscellaneous	648	O25:H4	SAMN11246661
EM1_Wa24	magpie	miscellaneous	5825	O148:H39	SAMN11246662
EM1_Wa25	pheasant	miscellaneous	155	O37:H10	SAMN11246663

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