UNIVERSITÀ DEGLI STUDI DI PADOVA
Department of Land, Environment Agriculture and Forestry

Second Cycle Degree (MSc)
in Food and Health

Tittle: Molecular characterization of plasmids carrying AmpC β-lactamases (pAmpCs) and extended-spectrum β-lactamase (ESBLs) genes using hybrid genome assembly analysis.

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ACADEMIC YEAR 2023/2024

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ABSTRACT

To improve the ambiguity that short-read data often gives for plasmids genetic elements characterization, the hybrid assembly of whole genome sequences (WGS) belonging to *Escherichia coli* strains obtained by using two different platforms, namely Illumina for short reads sequencing (Miseq system) and Oxford Nanopore for long reads (MinON device) was performed. These bacteria were isolated from poultry and showed phenotypic resistance to Extended-spectrum cephalosporins (ESCs), antimicrobials used as last-resort drugs to treat humans and animals infected by multidrug-resistant (MDR) bacteria. The main objective of this study was to comprehend their genetic makeup by characterizing the plasmids carrying extended-spectrum β-lactamases (ESBLs) genes and plasmid-mediated AmpC β-lactamases (pAmpCs) resistance. To this aim, the resistance and virulence genes repertoire of each plasmid was determined. Furthermore, to perform a comparative analysis, plasmid assemblies were screened against NCBI (National Center for Biotechnology Information) BLAST service.

Keywords: Whole genome sequence, Hybrid assembly, Antimicrobial resistance, Extended-spectrum β-lactamases, AmpC β-lactamases

INTRODUCTION

ANTIBIOTIC USE PROBLEMATIC

Antibiotics are chemical compounds produced by microorganisms (MOs), which either kill by aiming the essential cellular processes (bactericidal) or inhibit the growth of other MOs (bacteriostatic) (Paul et al. 2022) (Manyi-Loh et al. 2018). Antibiotics classification is extensive, one basic categorization is by nature of source (Manyi-Loh et al. 2018) (Pancu et al. 2021), divided in 1) naturals, compounds extracted from MOs, 2) semi-synthetic, natural products that have been structurally modified and 3) synthetic, made completely in the laboratory with the aim of augment therapeutical effect (Pancu et al. 2021).

Antibiotics were first discovered by Alexander Fleming in 1928, who by mistake noticed penicillin, a broad-spectrum bactericidal (Manyi-Loh et al. 2018). Penicillin's first introduction into the clinical setting started in 1940, and no longer after (around 10 years later) penicillin resistance became a substantial clinical problem (Ventola 2015) giving the popular idea that antibiotics have two general opposite effects: 1) microbial growth inhibition and 2) the emergence of antimicrobial resistance (AMR) bacteria (Paul et al. 2022).

AMR refers to the ability of MOs to withstand antimicrobial treatments. Some driving factors that generate AMR bacteria are poor hygiene environments and practices in healthcare settings or the food chain that help the diffusion of resistant MOs (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) 2024). Furthermore, the absence of public awareness about the consequences of inappropriate antibiotic use, which triggers misuse and abuse, such as the imprudent use of antibiotics by physicians (Salam et al. 2023) (Manyi-Loh et al. 2018) (Malik and Mundra 2022) for an inadequate diagnostic process. For instance, in 2019 when the COVID-19 global pandemic stopped by, antibiotic usage, increased more than 72% even though the bacterial co-infection rate was around 16%. (Malik and Mundra 2022).

Antibiotics, however, are not only used for human health care but also employed in livestock farming for treatment of diseased animals or prevention (Manyi-Loh et al. 2018).

Therefore, AMR problem is contemplated as one health approach (Aslam et al. 2021) (Manyi-Loh et al. 2018) (Laskey et al. 2020) because it can be transmitted from a myriad of pathways between humans-animal-environment (Graham et al. 2019) (Medugu et al. 2022), such as, direct contact, via food chain, or across animal/human waste dissemination in the environment (Manyi-Loh et al. 2018). In addition, another concern rise since antibiotics are not strictly selective against only pathogens, hence every time is taken, resistance commensal microbiota is emerging that may later act as reservoirs of antimicrobial resistance genes (ARGs) and transfer this genes to pathogenic bacteria (Juricova et al. 2021).

ESCHERICHIA COLI (E. COLI) STRAINS RESISTANT TO A CRITICAL IMPORTANCE ANTIBIOTIC

The emergence of multidrug-resistant (MDR) bacteria (MOs resistant to 3 or more groups of antimicrobials) (Kayastha et al. 2020) has given rise to limited therapeutic options for common infectious diseases (Manyi-Loh et al. 2018) (Kayastha et al. 2020) (European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) 2024) (Weber et al. 2019). One example is *E. coli*, a commensal bacteria of the gastrointestinal tract of human and animal that by virtue of acquiring ARGs (Kayastha et al. 2020) have developed resistance to extended-spectrum cephalosporins (ESCs), antibiotic used as last-resort drugs to treat MDR bacteria (Zamudio et al. 2024) (Apostolakos et al. 2019) (Apostolakos et al. 2020) (Weber et al. 2019)

ESCs are antimicrobials of critical importance in humans (Laconi et al. 2023) (Paul et al. 2022) (Apostolakos et al. 2019) (Furusawa et al. 2024) (Apostolakos et al. 2020) and veterinary medicine (Furusawa et al. 2024). In addition, according to the European Medicines Agency (EMA) who established a classification of antibiotics for animal use to support veterinarians decision-making, ESCs are category A (Kayastha et al. 2020), hence usage is forbidden for food-producing animals and only utilized in exceptional cases with companion animals (European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) 2024) (Apostolakos et al. 2019) (Zamudio et al. 2024).

E. coli resistance to ESCs is considered a human health threat (Laconi et al. 2023) (Furusawa et al. 2024) because it can serve as a reservoir of antibiotic-resistant genes (ARGs) and pass these genes to commensal bacteria (Furusawa et al. 2024) (Laconi et al. 2023). Besides, it has been detected in food-producing animals, especially in broiler (Apostolakos et al. 2020) (Shirakawa et al. 2020) and chicken meat (Furusawa et al. 2024). Its resistance is predominantly mediated by two main groups of inactivating enzymes: extended-spectrum beta-lactamases (ESBLs) (Paul et al. 2022) (Zamudio et al. 2024), (Evans et al. 2022). (Weber et al. 2019) and plasmid-mediated AmpC β-lactamases (pAmpCs) (Paul et al. 2022) (Zamudio et al. 2024), (Evans et al. 2022) (Zamudio et al. 2022).

ANTIBIOTIC RESISTANCE DISSEMINATION

Bacteria can transfer ARGs in two different ways: vertical gene transfer (VGT), where genetic material is inherited from parent to offspring (Hsu et al. 2023), or horizontal gene transfer (HGT) (Manyi-Loh et al. 2018) (Tokuda and Shintani 2024), when DNA is moved between MOs in the same generation (Hsu et al. 2023). HGT plays a crucial role in evolution and adaptation of bacteria because it provides recipients with advantageous traits. For instance, ARGs, virulence factors (VF), metal resistance genes, and/or catabolic genes that allows this bacteria to expand and diversify species (Khedkar et al. 2022), besides surviving in different environmental niches (Tokuda and Shintani 2024) (Hsu et al. 2023) (Khedkar et al. 2022).

ARGs dissemination are possible mainly due to its location inside DNA molecules capable of moving within the genome (intracellular mobility) or between bacterial cells (intercellular mobility). These sequences are named mobile genetic elements (MGE) (Tokuda and Shintani 2024) (Manyi-Loh et al. 2018) (Paul et al. 2022). Five major classes of MGEs are often found in the genome of MDR bacteria (Paul et al. 2022): 1) plasmids, circular or linear extrachromosomal replicons with the ability of intercellular mobility by means of conjugation mechanism. 2) bacteriophages, viral protein capsids that infect prokaryotic cells via transduction and can exchange their nucleic acid with host genome 3) conjugative elements (ICEs), self-transmissible conjugative elements that are normally incorporated into the host chromosome and capable of intercellular transfer 4) transposons, genetic elements that carry a transposase enzyme gene that catalyzes intracellular transposition, but in case of being inserted into plasmids or ICEs it can be

transferred to another cell. 5) integrons, genetic elements that cannot either be transposed or transferred without the help of other MGE (Tokuda and Shintani 2024) (Paul et al. 2022). (Khedkar et al. 2022).

HYBRID GENOME ASSEMBLY

Next generation sequencing (NGS) is an innovative technology used to read base pairs of DNA and RNA segments or read the whole genome of MOs (Dahui 2019) (Jagadeesan et al. 2019) in an easier, faster, and cheaper manner (Adewale 2020). In the beginning, NGS was only employed as a research tool, however, nowadays it is widely applied in many fields, including food authenticity, agriculture, outbreak investigation, AMR, etcetera (Jagadeesan et al. 2019). On top of that, in 2021, the European Union authorized whole genome sequencing (WGS) as an alternative method to monitor isolates displaying phenotypic resistance to ESBL/pAmpC producing *E. coli,* aiming to comprehend the spread of ARGs to develop appropriate strategies and actions for its management (European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) 2024).

WGS can be divided into 2 different methods, short-read sequencing when DNA is fragmented in 100-300 bp (Dahui 2019) and then assembled to a draft genome by computer programs (Adewale 2020), or long-read sequencing producing 10 to 50Kb reads of length to form a full sequence (Jagadeesan et al. 2019). Each presents different advantages and disadvantages because errors in assembly can occur. To illustrate, short-read sequencing is the most common technology used in comparative genomics and phylogenetic analysis due to its high accuracy (Hernandez et al. 2024) (Jagadeesan et al. 2019) and template coverage (Hernandez et al. 2024), but for determining complex genomic regions it undergoes some difficulties. For instance, repetitive regions can be seen by the program as mistakes and consequently being eliminated or else joined in the wrong place or orientation (Jagadeesan et al. 2019). Also, short-read sequencing struggles to assemble high GC regions (Hernandez et al. 2024). For this reason, reliable complete plasmid and other MGE sequences are often unreachable (Zamudio et al. 2024). On the other hand, long-read sequencing resolves better complex sequences, nevertheless it has low sensitivity which means it is error-prone (Sharon et al. 2021) with a higher incidence of insertions and deletions

(Hernandez et al. 2024) that can mislead variant analysis results (Sharon et al. 2021). Besides, it can struggle with smaller templates (Jagadeesan et al. 2019) (Hernandez et al. 2024).

Recently, a new tool namely hybrid assembly which combines both short and long reads has emerged (Sharon et al. 2021) (Hernandez et al. 2024) (Zamudio et al. 2024). (Jagadeesan et al. 2019). This technology combines the accurate yet fragmented genome of short reads with long reads scaffolds to join contingents and obtain a reliable and highly accurate genome sequence (Sharon et al. 2021). As plasmids are especially relevant for the HGT of AMR bacteria (Stein et al. 2024), its genetic characterization using hybrid assembly provides important information about its composition and maintenance systems (Darphorn et al. 2021), as well as to better define the extent of their diversity (Negeri et al. 2023) to monitor ARGs that can be transferred to pathogenic bacteria (Zamudio et al. 2024).

OBJETIVES

The objectives of this study were i) to perform a hybrid assembly of WGS belonging to eight *E. coli* strains showing phenotypic resistance to ESC antibiotic because regardless of the increased amount of genomic studies according this topic, most are based on short-reads (Zamudio et al. 2024). Additionally, ii) characterize plasmids harboring ESBL and pAmpC genes to comprehend better its genetic makeup, iii) along with a comparative plasmid analysis using NCBI BLAST service.

MATERIALS AND METHODS

STUDY DESIGN

Eight *E. coli* strains showing phenotypic and genotypic resistance to ESCs isolated from cloacal swabs on live birds were selected. This assortment was done considering ESBL/pAmpC resistance genes and plasmid replicon typing (Inc Type), choosing the most diverse possible between them. The isolation and characterization of ESBL/pAmpC strains was done in a previous study conducted by Apostolakos *et al* (Apostolakos et al. 2019); here 820 samples were collected from three production chains (parent stock chicken, offspring broiler flocks and carcasses) of an integrated broiler company in Italy with the objective of quantify ESBL/pAmpC producing *E.coli* occurrence and transfer.

DE NOVO GENOME ASSEMBLY ANALYSIS

Two different WGS technologies were utilized for this study: 1) Illumina for short reads sequencing (Miseq system), and 2) Oxford Nanopore for long reads (MinON device). Each sample was assembled following the hybrid genome assembly Nanopore and Illumina protocol by the University of Melbourne (more details in supplementary 1). In this protocol, a web-based platform called Galaxy (https://usegalaxy.eu/) is used to produce a high-quality bacterial genome sequence. The first function used was Flye v2.9.3 to create a draft genome assembly from Nanopore reads. Secondly, its quality was assessed by Quast v5.2.0 function using as reference genome *E. coli* (accession number NC_008563.1). Thirdly, the BWA-MEM v0.7.17.2 tool mapped Illumina reads to a draft assembly. Subsequently, the Illumina assembly genome was polished by pilon v1.2.01 function, followed by Quast v5.2.0 analysis. Lastly, hybrid assembly of short and long reads was done by Unicycler tool v0.5.0 in normal mode. All these steps were repeated for each of the samples labeled as: EC07, EC33, EC40, EC56, EC78, EC91, EC94, EC115.

Furthermore, Galaxy platform was also used to find plasmid sequences by MOB-Recon v3.0.3 function.

GENOMIC AND PLASMID TYPING IDENTIFICATION

To understand the genetic elements associated with phenotypic resistance to ECSs, an open access website called Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) was employed. It provides bioinformatics resources to analyze sequencing data. MGE, AMR and VF genes identification were done through MGE v1.0.3 and ResFinder v4.6.0 tools by default parameters. Information such as: plasmid Inc type, position, length, coverage, plasmid form (circular or linear), insertion sequence types, transposon and AMR genes inside plasmids carrying ESBL or pAmpC genes were collected, along with other Inc type plasmids included in each isolate strain (**Table 1**).

The same web tool was applied to obtained bacterial sequence type (ST) utilizing Multilocus sequence typing (MLST 2.0.9) service, selecting *E. coli* #1 configuration and a minimum of 5x depth for an allele. MLST is a type of classification based on housekeeping genes, encoding essentials proteins for bacterial survival and therefore conserved in a given bacterial species to record variation in the bacterial gene level (Wang et al. 2021) (Tung, Pan, and Lin 2024). Also, plasmid sequence type (pST) assessment was done thru plasmid Multilocus sequence typing (pMLST 2.0) tool, where only 2 isolates (EC07 and EC40), were able to analyze using IncA/C as plasmid group for EC07, and IncI1 for EC40.

Lastly, PlasmidFinder v2.0.1 service was utilized to obtain plasmid sequences of isolates not found by the previews MOB-Recon analysis. This were EC56, EC94 and EC115. Enterobacteriales, 95% minimum of identity and at least 60% of coverage were the parameters selected for the analysis. Table representation showing genomic and plasmid typing identification (**Table 1**), as well as MGE localization (**Table 2**) were made in a web-based software named canvas for digital learning (https://www.canva.com).

COMPARATIVE ANALYSIS: BLAST SERVICE

Plasmid assemblies of EC07, EC33, EC40, EC56, EC78, EC91, EC94, EC115 isolates were screened against NCBI BLAST (https://blast.ncbi.nlm.nih.gov) using nucleotide BLAST (BLASTn, default parameters) with 100 as maximum target sequences to find the closest matching plasmid defined by uppermost query coverage, followed by identity. Location, host, bacteria

species, plasmid name and length (bp), matches, query cover, identity, author and doi data of the assembly sequences were filed. Besides, a specific attention on location, host and bacteria species was made. Graphical representation (stack bars) was made thru SRplot website for data analysis and visualization (https://www.bioinformatics.com.cn/srplot).

RESULTS

GENOME AND PLASMID TYPE IDENTIFICATION

MLST analysis of whole genome sequences identified the following STs types: ST88, ST695, ST155, ST38, ST4980, ST69, ST4937 and ST3107 (**Table 1**).

After analyzing MGE finder results of the eight *E. coli* isolates (EC07, EC33, EC40, EC56, EC78, EC91, EC94 and EC115), can be concluded that 100% of the samples contained at least one plasmid, which 29 were typeable **(Figure 1).** A total of 15 different Inc Type plasmids were identified: InA/C2 (n=1), IncX1 (n=1), IncQ1 (n=2), IncFIB(AP001918) (n=6), col8282 (n=1), IncI1(n=3), Col440l (n=3), Col(MG828) (n=4), Incl2 (n=1), ColpVC (n=1), p0111(n=2), IncFII (n=1), IncHI2/ IncHI2A (n=1), IncFIC(FII) (n=1), IncY (n=1). The most abundant replicon type was IncF, specifically IncFIB(AP001918). Also, 48% (n=14/29) of the plasmids contained at least one ARG. None of the strains contained two plasmids belonging to the same incompatibility group.

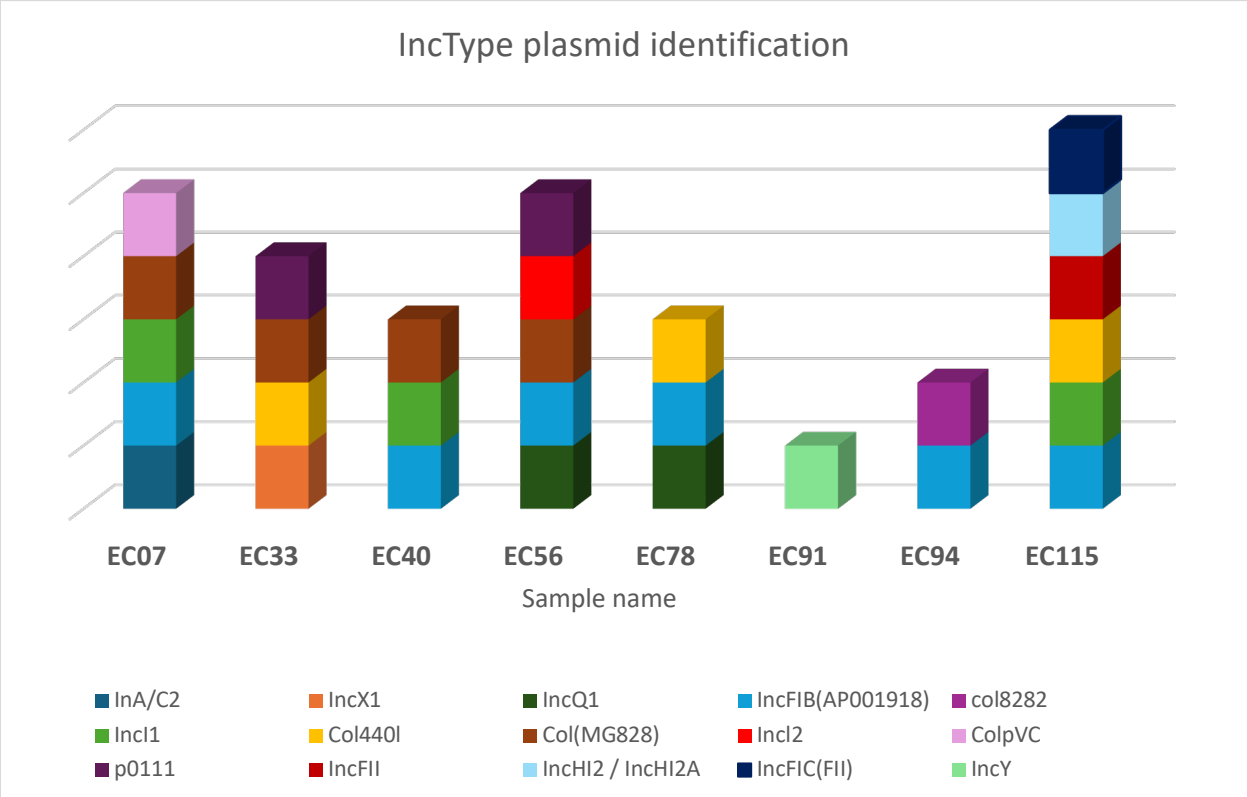

Figure 1. Typeable plasmids that were identified from hybrid assembly sequencing on eight *E. coli* samples (EC07, EC33, EC40, EC56, EC78, EC91, EC94, EC115) isolated from poultry in Italy.

Overall, five ESBL genes were identified: *blaCTX-M-1* (n=3) *blaCTX-M-2* (n=1), *blaCTX-M-15* (n=1), *bla-TEM-52B* (n=1) and *blaSHV-12* (n=1). On the other hand, only one pAmpC gene *blaCMY-2* (n=1) was detected. Almost all ESBL/ pAmpC genes were inserted as one copy, except for *blaCTX-M-15* which was localized 2 times in the same plasmid IncY. Also, three ESBL genes (*blaTEM-52B, blaCTX-M-15,* and *blaCTX-M-1*) were localized inside a transposon sequence (**Table 1**).

Sample name	EC07	EC33	EC40	EC56	EC78	EC91	EC94	EC115
ESBL/AmpC genes	*blaCMY-2*	*blaTEM-52B**	*blaSHV-12*	*blaCTX-M-1*	*blaCTX-M-2*	*blaCTX-M-15**	*blaCTX-M-1**	*blaCTX-M-1*
MLST	ST88	ST695	ST155	ST38	ST4980	ST69	ST4937	ST3107
IncType	IncA/C2	IncX1	IncI1	NF	IncHI2A	IncY	InFIB(AP001918)	NF
Plasmid length (bp)	163,204	38,611	114,680	24,683	226,048	81,890	152,994	29,681
Circular (Yes/No)	Yes	Yes	Yes	No	Yes	Yes	No	No
pMLST	pST3	NF	pST26	NF	NF	NF	NF	NF

Table 1. Genomic and plasmid typing identification of eight *E. coli* samples (EC07 EC33, EC40, EC56, EC78, EC91, EC94, EC115) conferring phenotypic resistance to ESCs and isolated from poultry in Italy. Table shows the ESBL/pAmpC genes identified, MLST classification number, as well as Inc Type plasmid and pMLST discovered using hybrid sequencing analysis. Abbreviations: NF= Not found. ***** ESBL genes located inside transposons.

CHARACTERIZATION OF PLASMID-ASSOCIATED TO ESC RESISTANCE GENES

A total of eight different plasmids harboring at least one ESBL/pAmpC gene were found, of which 62.5% (n=5/8) were circular. Inc Type identification was accomplished in 75% (n=6/8) of plasmids harboring ESBL/pAmpC genes. ESBL/pAmpC carrying plasmids harbored by strains EC56 and EC115 were not typeable, even though 2 different web tools were used (PlasmidFinder and MOB-suite). Five Inc plasmid types were found with 100% of coverage: IncA/C2 (n=1), IncI1 (n=1), IncX1 (n=1), IncHI2A (n=1), and IncY (n=1). Only one Inc type, IncFIB(AP001918), resulted in 99.71% coverage. Furthermore, PlasmidFinder analysis of isolates EC78 and EC94 showed 2 possible plasmids Inc Type. For instance, EC78 showed IncQ1 with 66.46% of coverage/

100% identity, and IncHI2A with 100% coverage/ 99.52% identity. On the other hand, the EC94 isolate showed IncFIB(AP001918) with 99.71% coverage/ 96.77% identity, and IncFII(pSE11) with 97.73% coverage/ 95.47% identity. Plasmids with a higher coverage percentage were used for further analysis. As it was mentioned, pSTs identification was only accomplished for isolate EC07 and EC40 with 100% coverage, obtaining pST3 for IncA/C2, and pST26 for Incl1 **(Table 2)**.

Moreover, a high variability of plasmid length was observed. The largest plasmid length was 226,048 bp (EC78 IncHI2A), followed by 163,204 bp (EC07 IncA/C2), 152,994 bp [EC94 IncFIB(AP001918)], 114,680 bp (EC40 IncI1), 81,890 bp (EC91 IncY), 38,611 bp (EC33 IncX1), 29,681 bp (EC115 not found), and the shortest 24,682 bp (EC56 not found).

In addition to ESBL/pAmpC resistance genes, 62.5% of these plasmids (n=5/8) carry at least one other ARG (**Figure 2**). Overall, 17 ARGs that do not confer resistance to ESCs were identified conferring resistance to tetracyclines [*tet(A)*], florfenicol and chloramphenicol (*floR and cmlA1*), streptomycin [*aph(3")lb, aph(6)Id, aadA1, aadA2b*], sulfamethoxazole (*sul1, sul2, sul3*), ciproflaxin (*qnrS1*), erythromycin [*mph(A)*], chlorhexidine (*qacE, qacL*) , gentamicin [*aac(3)-Vla*], ampicillin (*blaTEM-1B*), and andrimethoprim (*dfrA1*). Individually, IncA/C2 and IncHI2A were the plasmids with a highest number of different ARGs in its sequence (n=9) not counting *blaCMY-2* [i.e, *tet(A), sul2, aph(6)Id, qacE, aph(3")lb, floR, sul1, aac(3)-Vla,* and *aadA1*] for IncA/C2 and *blaCTX-M-2* [i.e, *dfrA1, aadA1, aph(6)-ld, qacE, tet(A), aph(3")-lb, sul, sul2, blaTEM-1B*] for IncHI2A. Secondly, IncI1 and IncY with other seven ARGs [i.e, *cmlA1, sul3, tet(A), qacL, aadA2b, aadA1]* and [i.e, *qnrS, aph(6)-ld, aph(3")-lb, sul2* and *tet(A), blaTEM-1B*] respectively, and lastly for IncFIB(AP001918) only one [*mph(A)*]. It is important to mention that all ARGs in IncY were localized inside transpon sequences (**Table 2**).

Virulence profile of the samples was limited; indeed, only six different genes in total were identified: *cib, tetC, astA, traT anr,*and *traJ,* localized in 37% of the isolates (n=3/8), all with 100% coverage. EC40 sample contained *cib,* a structural gene for a polypeptide toxin called colicin. EC78 contained *tecC* gene that codes to tellurium ion resistance protein (Afset et al. 2006). Finally, EC94 was the sample with the most VF genes: *traT anr traJ,* and *astA*, where last one was inserted inside a transposon **(Table 2).**

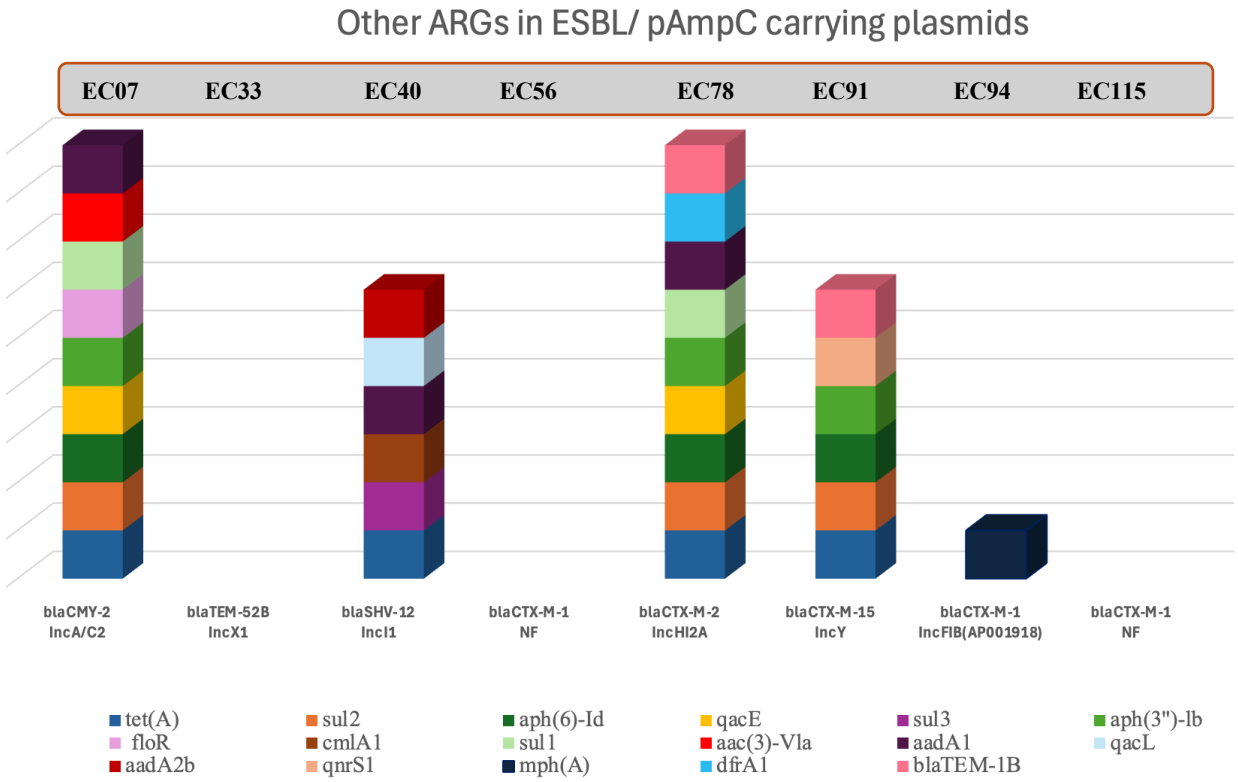

Figure 2. Bar graph of other ARGs that do not confer resistance to ESCs identified in ESBL/pAmpC producing *E. coli* plasmids. Horizontal axis shows eight Inc Type plasmids with its respective ESBL/pAmpC gene name and sample name (EC07 EC33, EC40, EC56, EC78, EC91, EC94, EC115). ARGs are separated by color.

In terms of IS and transposons, EC56 was the only sample with no IS or transposon found, therefore 87.5% (n=7/8) of plasmids contained at least one IS or transposon. Overall, nine different insertion sequences were identified: IS*Vsa3*, IS*Ec9*, IS*26*, IS*Kpn19*, IS*5075*, IS*629*, IS*Sbo1*, IS*Sen6* and IS*Ec37*. IS*26* was the most repetitive, placed in six of eight plasmids as 4 copies [EC94 IncFIB(AP001918)], 3 copies (EC91 IncY), 2 copies (EC07 IncA/C2), and one copy (EC40 IncI1, EC78 IncHI2A, EC115 not found) respectively. Secondly, IS*Sbo1* was identified 3 times on EC78 IncHI2A plasmid, meanwhile, IS*629* was found 2 times in EC94 IncFIB(AP001918) plasmid. All resting IS were found as one copy as it is shown in **Table 2**. In addition, composite transposons and unit transposons were identified on 62.5% (n=5/8) of plasmids. In general two unit transposons were identified: Tn*2* (n=1) Tn*6196* (n=1) and 13 composite transposons: cn_3556_IS*26* (n=1), cn_2162_IS*Sbo1*(n=1), cn_3422_IS*Sbo1* (n=1), cn_2064_IS*26* (n=1) cn_31036_IS*26* (n=1), cn_29961_IS*26* (n=1), cn_31544_IS*5075* cn_5331_IS*26* (n=1) cn_45457_IS*629* (n=1),

cn_10298_IS*629* (n=1), cn_24265_IS*26* (n=1), cn_3781_IS*26* (n=1), cn_17266_IS*26* (n=1). Details on location and relation to AMR and VF can be seen in **Table 2.**

Sample name	EC07	EC33	EC40	EC56	EC78	EC91	EC94	EC115
Plasmid IncType	IncA/C2	IncX1	IncI1	NF	IncHI2A	IncY	InFIB(AP001918)	NF
IS type	IS*Vsa3* IS*Ec9* IS*26* *(2)	None	IS*26*	None	IS*26* IS*Sbo1* *(3)	IS*26* *(3) IS*Kpn19* IS*Ec9* IS*5075*	IS*26* *(4) IS*629* *(2) IS*Sen6* IS*Ec37*	IS*26*
Transposon name and type	Unit tranposase, Tn*6196* Compose transposase, cn_2064_IS*26*	Unit transposase, Tn*2*	None	None	Compose transposase, cn_3556_IS*26* Composase transpose, cn_21962_IS*Sbo1* Composase transpose, cn_3422_IS*Sbo1*	Compose transposase, cn_29961_IS*26* *(2) Compose transposase, cn_31544_IS*5075* Compose transposase, cn_31036_IS*26* *(2)	Compose trasnposase, cn_45457_IS*629* Compose trasnposase, cn_10298_IS*629* Compose trasnposase, cn_24265_IS*26* Compose trasnposase, cn_3781_IS*26* Compose trasnposase, cn_17266_IS*26* Compose trasnposase, cn_5331_IS*26*	None
AMR or VF inserted into transpon sequence	None	*blaTEM-52B* inside Tn*2*	None	None	*blaTEM-1B* inside cn_3556_IS*26*	*blaTEM-1B, qnrS1, aph(6)-Id, aph(3'')-Ib, sul2, blaCTX-M-15* inside cn_29961_IS*26* *(2) *tet(A)* inside cn_31544_IS*5075* *tet(A)* inside cn_31036_IS*26* *(2)	*astA* inside cn_45457_IS*629* *mph(A)* and *blaCTX-M-1* inside cn_5331_IS*26*	None

Table 2. MGE identified in ESBL/pAmpC producing *E. coli* plasmids, besides its relation to ARGs and VF. Table shows MGE identified in eight E. *coli* samples (EC07 EC33, EC40, EC56, EC78, EC91, EC94, EC115) conferring phenotypic resistant to ESCs isolated from poultry in Italy, as well as ARG and VF location. Abbreviation: *(Number of copies).

COMPARATIVE ANALYSIS: BLAST SERVICE

NCBI BLASTn database results showed 100 hits for five out of eight *E. coli* samples. Therefore, 62.5% (n=5/8) of the plasmids [IncA/C2, IncI1, IncX1, IncY and IncF(AP001918)] were able to screen, resulting in 500 similar assemblies sequences in total. It was not possible to analyze the EC78 plasmid sequence, mainly due to a size limit in the database. Besides, EC56 and EC115 plasmid sequences were not found. Also, EC94 was the only sample using Inc Type sequence obtained from PlasmidFinder to run BLASTn search against the NCBI database.

Sequences derived from diverse sources, which were classified into nine main categories: 1) human, 2) cattle (domestic bovine, cow, bull and yak) 3) poultry (turkey, chicken, goose), 4) food (beef, chicken, turkey meat, pre-washed mixed salad, basil), 5) swine (pig and wild board), 6) environment (fresh/ waste/ river water, pine shaving in broiler litter, floor on pig farm, market, alcohol foam dispenser in hospital), 7) dog, 8) other [bacteria strain, mouse, shrimp, snake, horse, sheep, goat, cockroach, panda, avian (parrot and bird) and cat], and lastly 9) data not specified

(NS). Generally, most of the isolates were from humans (32%), NS (20%), cattle (10%) poultry (11%), swine (8%), and small percentage from the environment (6%) food (6%), other (4%) and dog (2%) (**Figure 3**). The observed data presented a high degree of dispersion between samples in all strains meaning a high variability among the strains. Only 2 categories (human and NS) were always present as the 3 most repetitive sources. For instance, the three main sources for EC07 IncA/C2 assemblies were human (n=34/100), cattle (n=17/100) and NS (n=14/100); in the case of EC33 IncX1 was cattle (n=22/100), human (n=19/100), and NS and food (n=16/100), regarding EC40 IncI1 firstly was poultry (n=27/100), then human (n=21/100) next swine, NS and food (n=11/100), in the case of EC91 IncY, primary source was human (n=52/100), next NS (n=26/100), and environmental (n=7/100), and lastly, for EC94 IncF(AP001918) firstly was NS (n=34/100), then human (n=33/100), and swine (n=12/100).

In addition, assemblies were located from diverse countries, distributed in a minimum of 18 different countries [EC07 IncA/C2] and a maximum of 28 [EC94 IncF(AP001918)]; therefore, countries were classified into continents: North America (USA, Mexico, Canada, Guadeloupe, Haiti), South America (Trinidad and Tobago, Chile, Ecuador and Argentina) Europe (Italy, Sweden, Switzerland, Germany, Greece, United Kingdom, France, Denmark, Norway, Belgium, Belarus, Czech republic, Romania, Poland, Russia, Spain and Heidelberg), Asia (China, Kuwait, Thailand, Afghanistan, India, Pakistan, Japan, South Korea, Taiwan, Singapore, Lebanon, Hong Kong, Vietnam, Thailand, Bangladesh, Myanmar, India, Saudi Arabia, Cambodia, Israel, Egypt), Africa (Burundi, Koulikoro, Kenya, Mozambique and Gaza strip). Antarctica was not included since no assemblies were found from here. Most of the sequences were isolated from North America (32%) Asia (23%), NS (21%) and Europe (20%), independently of the sample name. A better distribution can be seen in **Figure 4.**

In general, 25 different bacteria species were found in the 500 assemblies: *Escherichia coli* (n=259), *Salmonella enterica* (n=133), *Klebsiella pneumoniae* (n=50), *Escherichia fergusonii* (n=10), *Enterobacter hormaechei* (n=12), and *Escherichia albertii* (n=5) were the most abundant **(Figure 5)**, but some other bacteria species were identified in a small percentage, such as *Providencia stuatii* (n=5), *Enterobacter bugandesis* (n=3), *Shigella sonnei* (n=3), *Aeromonas hydrophila* (n=2), *Citrobacter freundii* (n=2), *Enterobacter cloacae* (n=2), *Klebsiella variicola*

(n=1), *Aeromonas salmonicisa* (n=1), *Enterobacter asburiae* (n=1), *Enterobacter chuandaensis* (n=1), *Klebsiella oxytoca* (n=1), *Morganella morganii* (n=1), *Proteus mirabilis* (n=1), *Proteus vulgaris* (n=1), *Providencia rettgeri* (n=1), *Vibrio alginolyticus* (n=1), *Vibrio Cholerae* (n=1), *Vibrio parahaemolyticus* (n=1), and NS (n=1). All bacteria species belonged to 3 different families: Enterobateriacea (98.8%), and Aeromonadaceae (0.6%) Vibrionaceae (0.6%). Important to mention that assemblies belonging to Vibrionaceae and Aeromonadaceae families came from sample EC07.

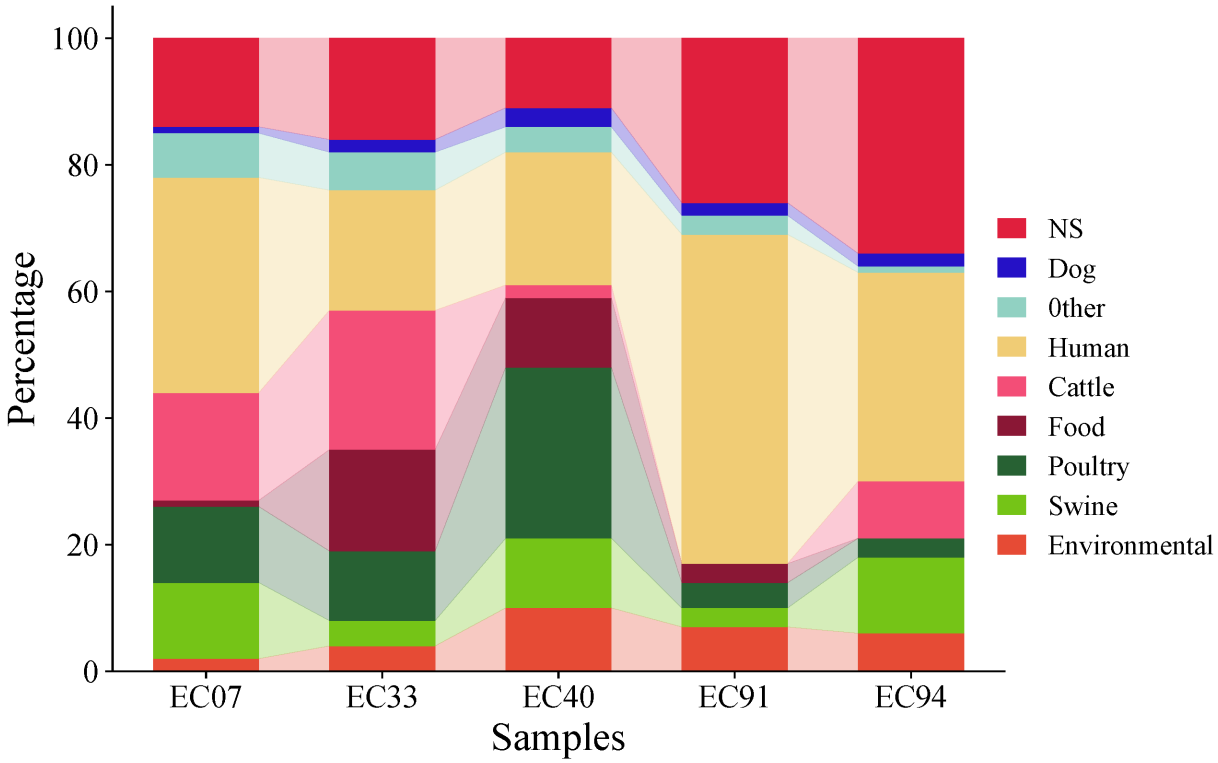

Figure 3. NCBI BLASTn database analysis results of ESBL/pAmpC producing *E. coli* samples (EC07 EC33, EC40, EC91, EC94) focused on host source. Stack bar shows homologous plasmids sequence classified by sources into nine main categories [human, cattle, food, poultry, swine, environment, dog, other and NS)] separated by color. Abbreviations: NS= Not specified.

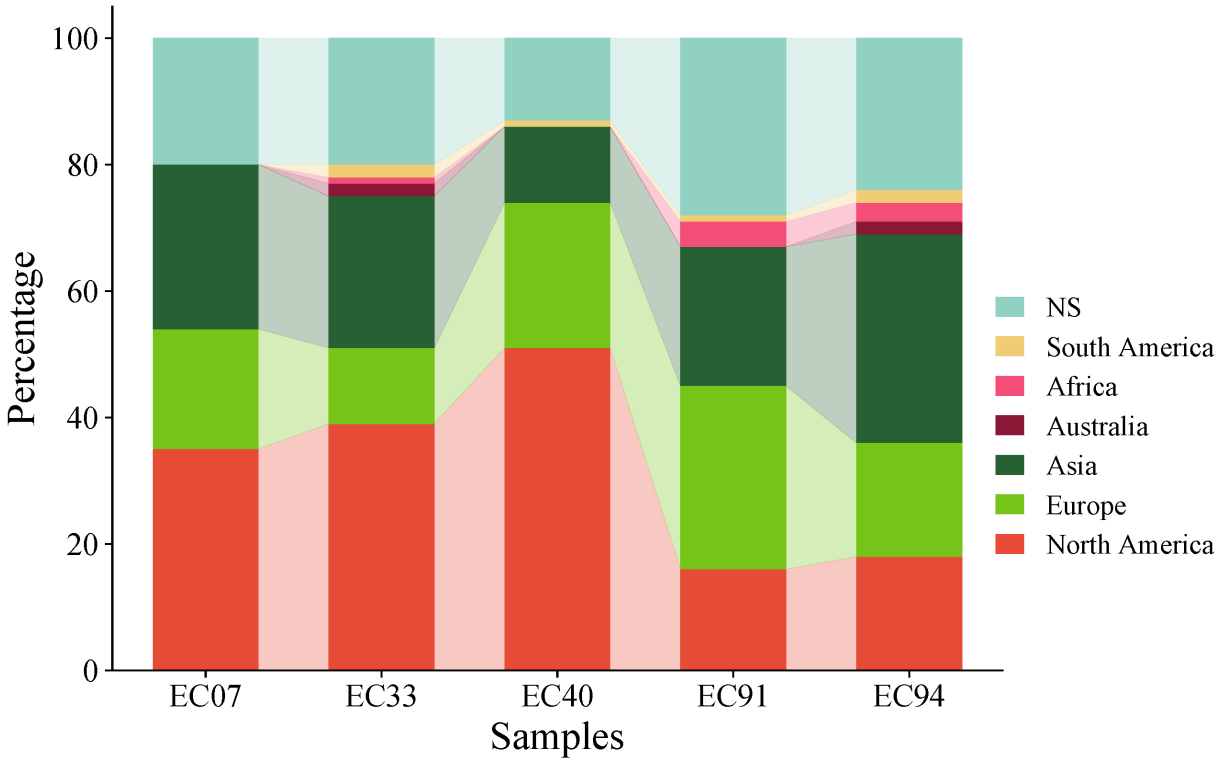

Figure 4. NCBI BLASTn database analysis results of ESBL/pAmpC producing *E. coli* samples (EC07, EC33, EC40, EC91, EC94) focused on location. Stack bar shows homologous plasmids sequences classified into continents. NS= not specified.

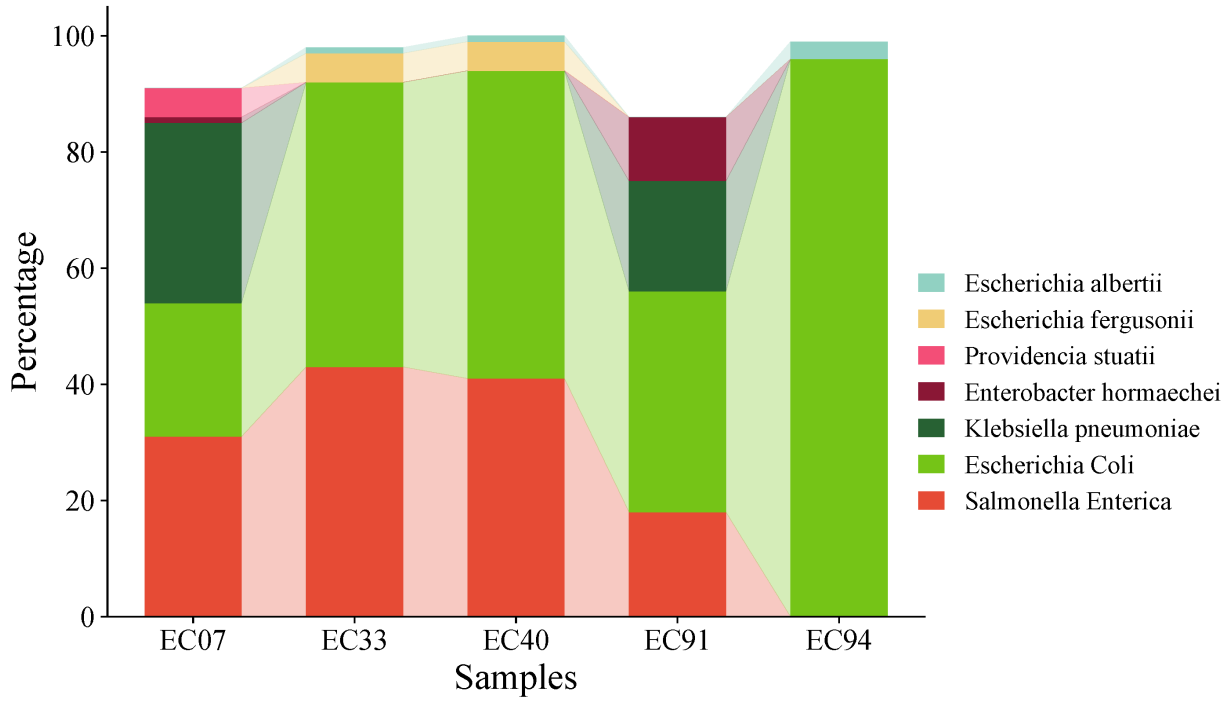

Figure 5. NCBI BLASTn database analysis results of ESBL/pAmpC producing *E. coli* samples (EC07 EC33, EC40, EC91, EC94) focused on bacteria species. Stack bar shows the most significant bacterial species found in the BLAST analysis.

Notably, NCBI BLASTn research identified highly similar plasmids for each of the eight *E. coli* samples. For instance, sample EC07 IncA/C2 was aligned with 99% coverage and 99.98% identity to a plasmid isolated from bovine in Tennessee, USA (GenBank accession: CP051316.1). This plasmid was obtained from *Salmonella Enterica*. It was identified with the same Inc Type (IncA/C2), but its length was bigger (170,832 bp). Interestingly, it presents one pAmpC gene *blaCMY*-2 and three *β* lactamases genes: *blaTEM-1B, blaTEM-206, blaTEM-214* located inside two composite transposons (cn_35873_IS*5075* and cn_5765_IS*26*).

Regarding samples EC33, EC91 and EC94 the highest homologous plasmids had a coverage and identity of 100%, besides isolated from *E. coli*. EC33 IncX1 similar plasmid was isolated from beef in Denmark (GenBank accession: JQ269336.1). In the case of EC91 sample, the highest homologous plasmid was isolated from a human stool sample in Mali, Koulikoro, Africa (GenBank accession: CP117003.1). Inc Type was the same (IncY) with 95,344 bp of length. Its ARGs and IS elements repertoire were identical, with the addition of *dfrA1A* and IS*4*. Besides, *blaTEM-1B* and *blaCTX-M15* where inserted in cn_31869_IS*26* composite transposon. The highest homologous plasmids of sample EC94 were 23, all isolated came from Germany (n=3),

USA (n=7) and NS (n=13). Finally, the highest similar plasmid of sample EC40 was isolated from gallus in Germany (GenBank accession: LT669764.1) with 100% coverage and 99.95% of identity. This plasmid was obtained from *E. coli*. identified with the same Inc Type (IncA/C2), and bigger length (117,387 bp).

DISCUSSION

Hybrid assembly technology, combining short reads sequencing with long reads, provided a highly accurate genome sequence of eight *E. coli* strains conferring resistance to ESCs isolated from poultry in Italy. This approach allowed the genomic location of ESBL and pAmpC genes, besides the characterization of eight plasmids carrying these genes which is rarely achieved by the only use of short reads technology due to a high number of repetitive regions in MGE (Jagadeesan et al. 2019) (Hernandez et al. 2024) (Zamudio et al. 2024).

Inc plasmid Typing identification discovered incompatibility groups associated with ESBL/ pAmpC genes (Darphorn et al. 2021). For instance, IncF, which is one of the most common Inc Type associated with ESBL genes (Darphorn et al. 2021) with a high incidence of *blaCTX-M-15* gene (Zamudio et al. 2024). It plays an important role in disseminating ARGs in Enterobacteriaceae from multiple sources such as humans, environment, food and animals (Stein et al. 2024). Besides, it normally presents high variability in its genetic structure with multiple copies of insertion sequences (Zamudio et al. 2024). In the present analysis, this was the case, since plasmid IncFIB(AP001918) was the plasmid with most IS elements (IS*26*, IS*629*, IS*Sen6* and IS*Ec37*) and composite transposons diversity (cn_31544_IS*5075*, cn_45457_IS*629*, cn_10298_IS*629*, cn_24265_IS*26*, cn_3781_IS*26*, cn_17266_IS*26*). Alternatively, IncF and IncA/C2 usually co-harbor large collections of genetic AMR determinants (Weber et al. 2019), but in this study, only two ARGs [*blaCTX-M-1* and *mph(A)*] were found in IncFIB(AP001918). On the other hand, plasmid IncA/C2 was indeed the plasmid with most ARGs diversity [*blaCMY-2, tet(A), sul2, aph(6)Id, qacE, aph(3")lb, floR, sul1, aac(3)-Vla, aadA1*]. In addition, IncA/C2 plasmids have previously been associated with *blaCMY-2* in multiple sources and countries (Zamudio et al. 2024).

IncI and IncY also represent widespread plasmids associated with ESBL genes in Enterobacteriaceae (Zamudio et al. 2024) (Duggett et al. 2020). Both have been associated with *blaCTX-M-15* gene in bacteria isolated from food-producing animals, mainly in poultry (Darphorn et al. 2021) and often in association with other ARG. (Negeri et al. 2023) (Zamudio et al. 2024). In particular, IncI is linked to *blaSHV-12* from *E. coli* of poultry origin, as well as with the spread of AMR (Kurittu et al. 2021). In addition, one study conducted by Zamudio *et al* in 2024 using

hybrid assembly revealed that IncI plasmids isolated from *E. coli* in Canada and France frequently mobilized *blaCMY-2* and *blaCTX-M-1* genes (Zamudio et al. 2024).

IncHI2A, on the contrary, is not commonly associated with ESBL/pAmpC genes, nevertheless, in our study this plasmid was found to carry ESBL genes inside a composite transposon sequence. This is concerning since transposons have the capacity for extracellular mobility when are located inside plasmids (Tokuda and Shintani 2024).

The most common ESBL genes reported in the European Union from poultry and derivate meat are *blaCTX-M-1*, followed by *blaCTX-M-15* and *blaSHV-12* (European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) 2024). In the main findings of our study, the *blaCTX-M-1* gene was the most common in the eight *E. coli* strains. Additionally, together with *blaCTX-M-15*, they were localized within composite transpons. Apart from ESBL genes, other ARGs such as *aph(3")lb, aph(6)Id, sul2, qnrS1*, *mph(A) tet(A) and blaTEM-1B* were identified inside composite transposons (**Table 2**). *Tet(A)* was identified in five *bla*-carrying plasmids, and it is considered a common tetracycline-resistance gene associated with MGE that can be easily spread among bacteria (Gargano et al. 2021). Conversely, only one VF gene, *astA*, remained in a composite transposon. The *AstA* gene encodes for a heat-stable toxin widespread in pathogens linked with diarrhea in animals, with the capacity to enhance the pathogenicity of other VF. (J. Wang et al. 2023)

MGEs are responsible for gene dissemination and bacterial adaptation (Tansirichaiya, Mullany, and Roberts 2016). It is well known that multiple copies of the same IS element can promote genomic rearrangements via homologous recombination (Tokuda and Shintani 2024) and mobilizing the neighboring genes (Paul et al. 2022). In particular, the insertion sequence IS*26* plays a key role in disseminating AMR (Harmer, Moran, and Hall 2014) (Zamudio et al. 2024) (Paul et al. 2022), and MDR enterobacteria commonly carry large regions of IS*26* (Harmer, Moran, and Hall 2014) (Zamudio et al. 2024). The reason behind this high prevalence is still unclear, but there are 2 hypotheses. 1) “the founded effect” which says that the first IS*26* element was inserted close to an ARG helping it to spread. 2) An unknown factor in the bacteria makes IS*26* element move easily (He et al. 2015). IS*26* was indeed the most common and repetitive IS between the plasmids identified in our study. Likewise, there is a clear relationship between IS*26* element and

ARG content since the strains containing at least one copy of IS*26* were the ones with higher diversity of ARG.

In this study, BLASTn search against the NCBI database showed ESBL genes distribution only in the Enterobacteriaceae family, meanwhile, pAmpC gene (*blaCMY-2*) was also identified, in a low percentage, in Aeromonadaceae and Vibrionaceae families. Therefore, it appears that ESBL genes are mainly shared between the Enterobacteriaceae family. Moreover, BLAST NCBI analysis, focused on location, revealed a larger number of similar plasmids mainly in North America, Europe and Asia. These results come with an enormous bias since 58% of these data came from developed countries (USA, United Kingdom, China, Germany and Switzerland) which intensified the necessity of improving surveillance globally. Furthermore, around 20% of total plasmid sequence assemblies did not specify source and location, making it hard to monitor. One promising solution is the installation of open and reproducible bioinformatic pipelines, which are systems that methodically process massive sequence data in a systematic manner to be able to interpret and visualize reproducible results (Roy et al. 2018) (Kuznetsova et al. 2024). This approach can consolidate information to ensure data comparability between countries.

In addition, BLASTn search against NCBI database showed many different sources for each of the strains, meaning that these genes are not specific to one host and can adapt to different hosts (Duggett et al. 2020) (Zamudio et al. 2024). Importantly, a high percentage of similar plasmids were isolated from humans, and 176 highly similar plasmids came from *Salmonella Enterica,* the second most common zoonotic pathogen that can cause infections in humans and animals. (Li et al. 2021). (European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) 2024).

It is evident that the presence of ESBL/ pAmpC producing *E. coli* is undesired due to the potential risk of transmission of AMR bacteria from livestock animals and food to humans. Fortunately, from 2014 to 2021 the prevalence of ESBL producing *E. coli* in broilers, broiler meat and pig meat at the EU level had a statistically decreased trend (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) 2024). Besides, mathematical transmission models showed that human consumption of chicken meat carrying

ESBL/ pAmpC producing *E. coli* is not a major risk factor, since only 0.12% to 0.15% of these genes identified in humans were linked to poultry (Furusawa et al. 2024). This does not imply that special surveillance across the world should not be carried out. On the contrary, it ought not to be underestimated and better investigation using methods such as WGS for comparison are indispensable to tackle this issue from a One Health. (European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA), and European Medicines Agency (EMA) 2024). Likewise, biosecurity and hygiene management in farms are the most effective control to reduce the number of bacteria in the environment, and therefore decrease the occurrence of ESBL/ pAmpC producing *E. coli* bacteria (Furusawa et al. 2024) (Kurittu et al. 2021).

Finally, although it was not one of the main objectives of this analysis, since none of the strains contained two plasmids belonging to the same incompatibility group, this study helped to rectify the theory of incompatibility. This concept says that two plasmids belonging to the same Inc Type cannot coexist in the same strains since there is competition for the same replication and partitioning machinery (Darphorn et al. 2021). In addition, can be inferred that the Inc Types coexisting in each strain contained a less similar conserved region.

CONCLUSION

The use of Hybrid assembly, an analysis of WGS data that combines short and long reads, produced highly accurate genome sequences of eight *E. coli* strains isolated from poultry in Italy. Eight plasmids carrying ESBL and pAmpC genes were identified and characterized, discovering incompatibility groups already associated with ESBL and pAmpC genes in Enterobacteriaceae, such as IncA/C2, Incl1, IncX1, IncF and IncY, and one not so common IncHI2A. In the main finding of this study, six ESBL/ pAmpC genes were identified, of which three (*blaTEM*-52B *blaCTX-M-1*, *blaCTX-M-15*) were localized inside composite tranposon. This is alarming since transposons have the capacity for extracellular mobility when are located within plasmids. Furthermore, this study identified a positive potential relationship between the IS*26* element and ARGs content. In addition, BLAST NCBI analysis, showed ESBL genes distribution only in Enterobacteriaceae family, meanwhile, pAmpC gene (*blaCMY-2*) was also identified, in a very low percentage, in Aeromonadaceae and Vibrionaceae families. Importantly, ESBL/ pAmpC producing *E. Coli* displayed no specificity to a type of location and host source, concluding that these plasmids can adapt to different scenarios; and unfavorable, the second most abundant bacteria species in highly homologous plasmids assembly were isolated from *Salmonella Enterica,* the second most common zoonotic pathogen. Considering all these outcomes, the improvement of global surveillance in a One Health perspective should be performed to enhance monitoring and reduction of ESBL/ pAmpC producing *E. Coli.*

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