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Transmission of β-lactamases in the pork food chain: A public health concern

Martina Rega [a,*], Laura Andriani [a], Antonio Poeta [b], Chiara Casadio [c], Giuseppe Diegoli [d], Silvia Bonardi [a], Mauro Conter [a], Cristina Bacci [a]

[a] Food Hygiene and Inspection Unit, Veterinary Science Department, University of Parma, Strada del Taglio, 10, 43126 Parma, Italy
[b] Azienda Unità Sanitaria Locale (AUSL) sede Reggio Emilia, via Amendola 2, 42122 Reggio Emilia, Italy
[c] Azienda Unità Sanitaria Locale (AUSL) sede Modena, Via S. Giovanni del cantone, 23 41121 Modena, Italy
[d] Emilia-Romagna Region, Collective Prevention and Public Health Service, viale Aldo Moro 21, 40127 Bologna, Italy

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ABSTRACT

Antimicrobial resistance (AMR) is a risk for public health that requires management in a One Health perspective, including humans, animals, and the environment. The food production chain has been identified as a possible route of transmission of AMR bacteria to humans. The most critical issue regards resistance to the Critically Important Antimicrobials (CIAs), such as β-lactams antibiotics. Here, pigs were analysed along the entire food producing chain, including feces, carcasses and pork products (fresh meat, fermented and seasoned products) ensuring treaciability of all samples.

Escherichia coli were isolated and their ability to produce ESBL and AmpC β-lactamases was evaluated both phenotypically and genotypically. Strains with the same AMR profile from feces, carcasses, and meat products were selected for phylogenetic and comparative genomic analyses to evaluate the possible "farm-to-fork" transmission of β-lactams resistant bacteria. Results showed that the percentage of ESBL strains in fecal *E. coli* was approximately 7% and increased slightly in the pork food chain: the 10% of ESBL *E. coli* isolated from carcasses and the 12.5% of isolates from fresh meat products. AmpC *E. coli* were found only in feces, carcasses, and fresh meat with a low prevalence.

Results showed that of the 243 pigs followed along the entire food chain genetic similarities in *E. coli* isolated from farm-to-fork were found in only one pig (feces, carcasses and fresh meat). Frequent similarities were shown in resistant *E. coli* isolates from carcasses and fresh meat or fermented product (three pork food chain). Moreover, in one case, bacteria isolated from fresh meat and fermented product were genotypically similar. Concluding, direct transmission of β-lactams resistance from farm-to-fork is possible but not frequent. Further studies are needed to improve risk communication to consumers and access to clear and reliable information and health concerns on food.

1. Introduction

Antimicrobial resistance (AMR) is one of the leading public health risks of the 21st century. Prevalence of hard-to-treat infections (bacteria, viruses, parasites, and fungi) is increasing due to resistance to many commonly used drugs [1]. The selection of antimicrobial resistant microorganisms in particular is caused by excessive antibiotic consumption, inappropriate prescription, poor adherence to prescribed therapy, use of counterfeit and low-quality antibiotics and poor hygiene practices in hospitals [2]. At the same time, the extensive use of antimicrobials in

Abbreviations: AMR, Antimicrobial Resistance; CBP, Clinical Breakpoints; CIA, Critically Important Antimicrobials; PBP, Penicillin Binding Protein; ARG, Antimicrobial Resistance Genes; BPW, Buffered Peptone Water; TBX, Triptone Bile X-gluc; CDT, Combination Disk test; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CTX, Cefotaxime; CAZ, Ceftazidime; TSA, Triptic Soy Agar; ERIC-PCR, Enterobacterial Repetitive Intergenic Consensus PCR; HCL, Hierarchical Clustering Analysis.

* Corresponding author.
E-mail addresses: martina.rega@unipr.it (M. Rega), laura.andriani@unipr.it (L. Andriani), antonio.poeta@ausl.re.it (A. Poeta), c.casadio@ausl.mo.it (C. Casadio), giuseppe.diegoli@regione.emilia-romagna.it (G. Diegoli), silvia.bonardi@unipr.it (S. Bonardi), mauro.conter@unipr.it (M. Conter), cristina.bacci@unipr.it (C. Bacci).

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livestock production can further promote the growth of resistant microorganisms [3].

Current European legislation [4] requires mandatory monitoring of AMR for *Salmonella, Campylobacter jejuni* and the commensal indicator *Escherichia coli (E. coli)* in food producing animals and derived meat products [5]. Particular attention is given to resistance to Critically Important Antimicrobials (CIAs), associated with extended-spectrum-β-lactamases, AmpC and carbapenemase enzymes [5].

The antibacterial activity of β-lactams is based on their ability to bind Penicillin Binding Proteins (PBPs) that catalyze the D-alanyl D-alanine cross linkages of the peptidoglycan wall, thus interfering with bacterial cell wall synthesis [6]. Intrinsic resistance due to insensitivity of PBPs (e.g. *Enterococcus* spp.) and non-specific resistance mechanisms such as impermeability or efflux pump synthesis or target modifications can occur [7]. At the same time, selective pressure has allowed the development of resistance through spontaneous mutations or DNA transfer, leading to the production of β-lactamases, enzymes that hydrolase the β-lactam ring rendering the antibiotic unable to target PBPs [8]. β-lactamases are frequently encoded on plasmids and can be readily disseminated through horizontal gene transfer [9]. CTX-M is the most predominant plasmid-mediated enzyme followed by TEM-1, TEM-2, and SHV [10]. Transmissible plasmid-mediated AmpC β-lactamases are enzymes that are capable of hydrolyzing a wide range of cephalosporin antibiotics, including narrow-, broad-, and extended-spectrum cephalosporins. The most common plasmid-mediated AmpC β-lactamases are found in families known as CMY, FOX, and DHA. These enzymes have been identified in various microorganisms, including but not limited to *Klebsiella* spp., *Salmonella* spp., *Citrobacter freundii, Enterobacter aerogenes, Proteus mirabilis*, and *Escherichia coli (E. coli)* [11].

The environment can be a source of AMR bacteria that can spread from animals to humans. Moreover, horizontal gene transfer can occur through the consumption of raw- or poorly cooked food or by cross contamination. Monitoring of AMR is thus necessary in animals and derived products, in order to better understand the implications of the food production chain in the spread of AMR [12].

A recent study compared microbiomes from workers in pig farms and in slaughterhouses with those of a control population [13]. Workers showed a significant increase in tetracycline, β-lactam and macrolide resistance genes [13]. However, comparison between pig farm workers' vs. pig resistomes showed no similarity, suggesting that direct contact with pigs' feces is not the main route of antimicrobial resistance gene (ARGs) transmission [13]. Possible environmental ARGs transmission may be due to materials and workers harbouring AMR microorganisms and a possible co-resistance to sanitation products used for cleaning and disinfection [14]. Finally, humans can be exposed to pig-derived ARGs during food production and consumption [15]. Contamination of raw and ready to eat products can occur in production and market environments, and several AMR bacteria have been found in pork ham, pork sausage, salami, and pork luncheon meat sliced at shops [16].

The present study focuses on the pork food chain through sampling of pig feces, carcasses and pork food products (fresh meat, fermented and seasoned product) for *E. coli* isolation. AMR profiles were evaluated both phenotypically and genotypically for the bacteria's ability to produce ESBL and AmpC enzymes. Phylogenetical analyses were performed to understand the possible relation of isolates in the different stages of the food chain. Strains that showed phylogenetic similarities were confirmed by sequencing analysis using Average Nucleotide Identity technique.

2. Material and methods

Samples were collected from eight different farms (A, B, C, D, E, F, G, H) located in Emilia Romagna region (North Italy). Pigs were marked with an ear tag and followed along the food production chain. Samples were performed twice, firstly in 2019/2020 and then in 2020/2022.

Feces, carcasses and pork products, including fresh meat, seasoned and fermented meat product, were analysed.

Fecal samples were firstly collected using sterile fecal swabs. Fifteen pigs per farm were selected except for Farm B (17 pigs-2019/2020), Farm E (16 pigs-2019/2020 and 16 pigs-2020/2022) and Farm H (16 pigs-2019/2020). Fecal samples were collected at least 30/40 days before slaughtering, together with information on pharmacological treatments administered to pigs in the last six months before slaughter. Given the aim of the study, only treatments with β-lactams antibiotics were considered.

Carcasses were sampled using prewetted- sponges, according to UNI EN ISO 17604:2015 [17] and Reg. CE 2073/05 [18]. Seventeen pigs from Farm B-2019/2020 could not be reached and one pig from Farm E-2019/2020 died before slaughter and was not sampled; because of two *E. coli*-free fecal samples in 2019/2020, the related carcasses and meat products were not considered due to the inability to evaluate the pork food chain.

Fresh meat samples were collected at the slaughterhouse only in pigs that showed AMR strains in fecal swabs. Seasoned (coppa, pancetta) and fermented (salami) products were sampled and analysed after proper transformation (performed from 30 to 70 days). However, traceability is always guaranteed. Pork fresh meat and seasoned meat products derived from a single animal and have been selected according to resistance found in fecal isolates. Due to production necessity, fermented products gather together parts of different animals belonging to the same batch. Fermented products selected included the part of the animal of interest to follow the entire food chain.

The total number of samples collected were 245 fecal swabs, 225 carcass sponges, 62 fresh meat samples, 15 seasoned products and 7 fermented products.

All the samples were sent to the laboratory of Food Hygiene and Inspection of the Veterinary Science Department, University of Parma. The timeline of the study is represented in Fig. 1.

2.1. *Escherichia coli isolation*

E. coli isolation followed UNI EN ISO 16649-2:2001 [19]. After the enrichment phase, Triptone Bile X-gluc agar (TBX; Biolife Italiana, Milan, Italy) was used as selective medium for the isolation and identification phases. The miniaturized API 20E system (bioMérieux, France) was used for biochemical confirmation.

2.2. *β-lactams antimicrobial resistance evaluation- ESBL, AmpC*

2.2.1. *Phenotypic analyses*

All the *E. coli* isolated were tested for the ability to produce ESBL and AmpC, using the disk diffusion test, following the protocol defined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) [20].

ESBL and AmpC bacteria detection includes a screening followed by a confirmation disk test.

Screening was performed using two cephalosporins: cefotaxime 5 μg (CTX05) and ceftazidime 10 μg (CAZ10). Resistant and intermediate *E. coli* were phenotypically confirmed as ESBL and/or AmpC with the combination disk test (CDT) as described in Table 1 [20].

2.2.2. *Genotypic analyses*

DNA from phenotypically confirmed ESBL and AmpC *E. coli* isolates was extracted by heating. In addition, *E. coli* isolates that resulted resistant and intermediate at the screening with cefotaxime and ceftazidime were selected and analysed for the presence of ESBL and AmpC genes.

A real-time PCR was applied in order to verify the presence of ESBL-associated genes: $bla_{\text{CTX-M1}}$, $bla_{\text{CTX-M2}}$, bla_{TEM} and bla_{SHV}, as described by Roschanski et al. [21] and showed in Table 2.

In each reaction positive (*K. pneumoniae* NCTC 13368 for bla_{SHV}, *E. coli* NCTC 13351 for bla_{TEM} and *E. coli* NCTC 13353 for $bla_{\text{CTX-M}}$) and

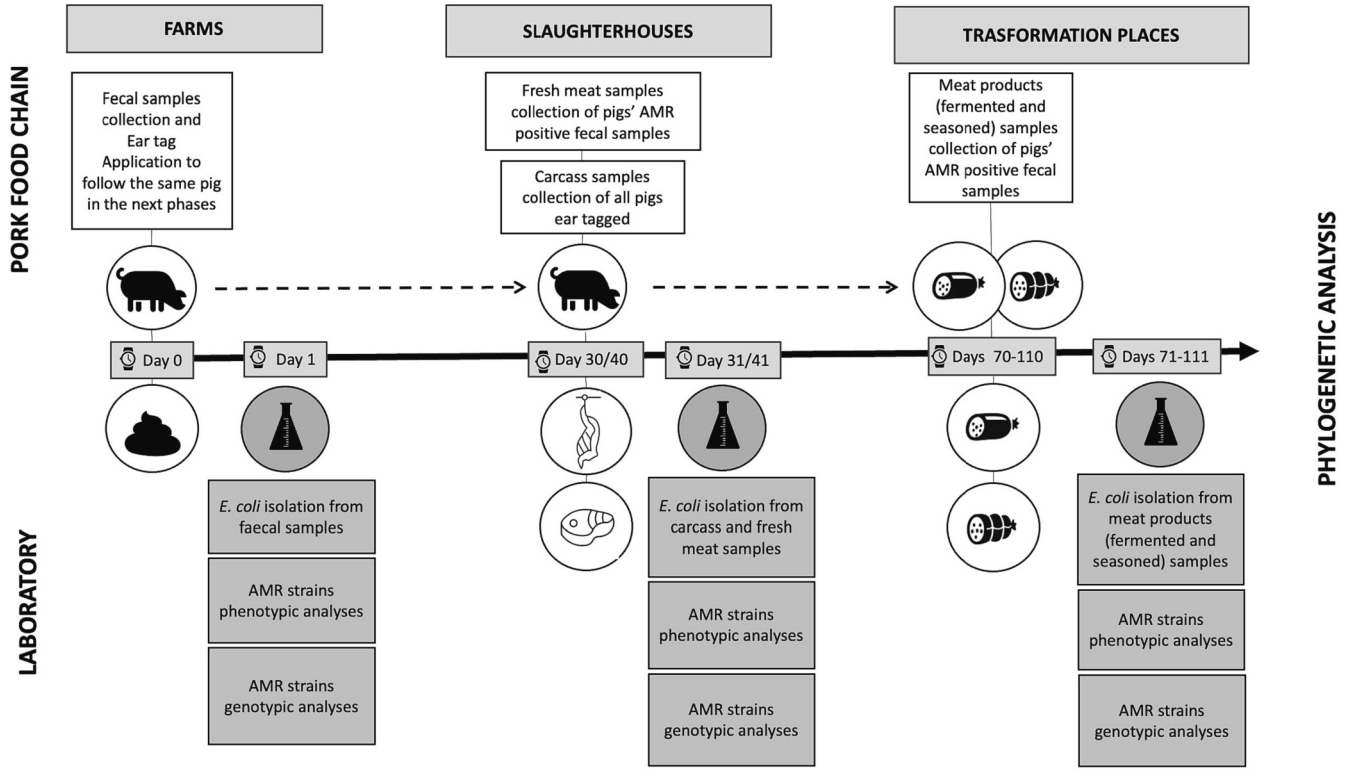

Fig. 1. Time line of the study representing samples and analysis organization.

Table 1
Description of screening and combination disk test (CDT) for the detection of ESBL and AmpC *E. coli*. The CDT was considered positive if the growth inhibition zone around the CTX or the CAZ disks with clavulanate or cloxacillin was 5 mm wider than the diameter around the disks containing CTX or CAZ alone.

	ESBL		AmpC	
	Disk	Growth inhibition diameter	Disk	Growth inhibition diameter
Screening	CTX05	CTX: S ≥ 20 mm, *R* > 17 mm	CTX05	CTX: S ≥ 20 mm, R > 17 mm
	CAZ10	CAZ: S ≥ 22, *R* < 19 mm	CAZ10	CAZ: S ≥ 22, R < 19 mm
CDT	CTX30 and CTX30 + C	ID CTX30 + C > 5 mm ID CTX 30	CTX30 and CTX30 + CX	ID CTX30 + Cx > 5 mm ID CTX 30
	CAZ30 and CAZ30 + C	ID CAZ30 + C > 5 mm ID CAZ 30	CAZ30 and CAZ30 + CX	ID CAZ30 + Cx > 5 mm ID CAZ 30

CTX05 = cefotaxime 5 μg, CAZ10 = ceftazidime 10 μg, CTX + C = cefotaxime 30 μg + clavulanic acid 10 μg CTX + CX = cefotaxime 30 μg + cloxacillin 10 μg CAZ + C = ceftazidime 30 μg + clavulanic acid 10 μg CAZ + CX = ceftazidime 30 μg + cloxacillin 10 μg, ID = inhibition diameter.

negative controls were added.

The presence of AmpC genes was verified using the multiplex PCR protocol described by Pérez-Pérez and Hanson [22], with some modifications reported in Table 2.

2.2.3. Phylogenetic analyses

The isolates that showed the same phenotypic and genotypic AMR profiles at in least two stages of the food chain were considered for phylogenetic analyses. Moreover, if the resistant strains were found only in pork products, the entire related food chain was analysed. Determination of the *E. coli* isolates' phylogenetic relatedness was performed by Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) as described by Ventura et al. [23] and showed in Table 2.

2.3. Comparative genomic analysis - average nucleotide identity

Bacterial strains that showed phylogenetic similarities were analysed for comparative genomic analyses, according to Alessandri et al. [24]. After overnight culture of cells, 10 mL were centrifugated at 6000 rpm for 8 min and the pellet was used for DNA extraction using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, Darmstadt, Germany) following the manufacturer's guidelines.

Bacterial chromosomal DNA was decoded through a MiSeq platform (Illumina, San Diego, CA, USA) according to the manufacturer's protocol by using the Nextera XT DNA Library Prep kit (Illumina). The library samples obtained were then pooled into a Flow Cell V3 600 cycle (Illumina). Subsequently, the .fastq files of paired-end reads generated from each genome sequences were employed as input for the genome assembly by using the MEGAannotator pipeline [25]. Then, to predict protein-coding open reading frame (ORFs), MEGAnnotator employed contigs longer than 1000 bp, through Prodigal [26].

A value of average nucleotide identity (ANI) was calculated through the program fastANI, using each genome pair as the input [24,27]. Subsequently the generated ANI matrix was reordered by using a Hierarchical Clustering Analysis (HCL) performed through OriginPro 2021.

3. Results

3.1. Escherichia coli isolation

E. coli were isolated from all carcasses (225/225), fresh meat (62/62), and fermented meat product samples (7/7). In fecal and seasoned meat product samples, 243/245 and 8/15 *E. coli* were isolated,

Table 2
Oligonucleotide primers and PCR conditions used in this study.

Genes	Sequences	Size bp	PCR conditions		Reference
ESBL resistance genes- Real Time PCR					
bla_{CTX-M1}	**F** 5'-CGGGCRATGGCGCARAC-3′ **R** 5'-TGCRCCGGTSGTATTGCC-3'	–	Denaturation 95 °C for 3 mins, 39 cycles 95 °C for 15 s, 50 °C for 15 s, 72 °C for 20 s	Final volume 20 μL: 1× Gotaq qPCR Mix, primers at 0.3 μM,1 μL of sample lysate, Nuclease Free Water to final volume.	[21]
bla_{CTX-M2}	**F** 5'-ACCGAGCCSACGCTCAA-3′ **R** 5'-CCGCTGCCGGTTTTATC-3'				
bla_{TEM}	**F** 5'- GCATCTTACGGATGGCATG-3′ **R** 5'-GTCCTCCGATCGTTGTCAG-3′				
bla_{SHV}	**F** 5'-TCCCATGATGAGCACCTTT-3′ **R** 5'-TCCTGCTGGCGATAGTGGA-3′				
AmpC resistance genes- End Point PCR					
bla_{MOX}	F: 5′-GCTGCTCAAGGAGCACAGGAT-3′ R: 5'-CACATTGACATAGGTGTGGTGC-3'	520	Denaturation 95 °C for 5 mins, 25 cycles 95 °C for 45 s, 62 °C for 30 s, 72 °C for 1 min, final extension 72 °C for 8 mins	Final volume 50 μL: 1× Green GoTaq Flexi Buffer, 2 mM of $MgCl_2$, 0.2 mM of dNTPs,1.25 U of GoTaq G2 Flexi DNA Polymerase, primers bla_{MOX}, bla_{CIT}, bla_{DHA} at 0.6 μM, bla_{ACC}, bla_{EBC} at 0.5 μM, primers bla_{FOX} at 0.4 μM, 2 μL of sample lysate, Nuclease Free Water to final volume.	[22]
bla_{CIT}	F: 5'-TGGCCAGAACTGACAGGCAAA- 3′ R: 5'-GTACGTTTCAAGAGTGATGC-3'	462			
bla_{DHA}	F: 5'-AACTTTCACAGGTGTGCTGGGT-3′ R: 5'-CCGTACGCATACTGGCTTTGC-3′	405			
bla_{ACC}	F: 5'-AACAGCCTCAGCAGCCGGTTA-3′ R: 5'-TTCGCCGCAATCATCCCTAGC-3'	346			
bla_{EBC}	F:5'-TCGGTAAAGCCGATGTTGCGG-3′ R:5'-CTTCCACTGCGGCTGCCAGTT-3'	302			
bla_{FOX}	**F** 5'-AACATGGGGTATCAGGGAGATG-3′ **R** 5′-CAAAGCGCGTAACCGGATTGG-3'	190			
Phylogenetic analysis- ERIC PCR					
ERIC-1	5'-ATGTAAGCTCCTGGGGATTCAC -3′		Denaturation 94 °C for 3 mins, 35 cycles 94 °C for 20 s, 48 °C for 30 s, 72 °C for 4 min, final extension 72 °C for 6 mins	Final volume 25 μL: 1× Green GoTaq Flexi Buffer, 3 mM of $MgCl_2$, 0.2 mM of dNTPs,2.5 U of GoTaq G2 Flexi DNA Polymerase, primers at 1 μM, 3 μL of sample lysate, Nuclease Free Water to final volume.	[23]
ERIC-2	5′-AAGTAAGTGACTGGGGTGAGCG −3′				

respectively. The *E. coli* prevalence in processed meat products is significantly reduced from the first year of sampling compared to the second. It should be noted that during the 2019/2020 sampling period, some of the meat products delivered had not perfectly completed the transformation process.

3.2. β-lactams antimicrobial resistance evaluation- ESBL, AmpC

3.2.1. Phenotypic analyses

All the isolates were tested for their ability to produce ESBL and AmpC β-lactamases through disk diffusion method. Data are reported in Table 3.

The analysis of information on pharmacological treatments administered to pigs highlighted that no β-lactams were used in the last six months before slaughter. Data on previous treatments were not collected.

3.2.2. Genotypic analyses

Resistant strains were tested for the presence of the most common plasmidic resistance genes. Not all phenotypically ESBL or AmpC strains harboured resistance genes. In 2019/2020, 40% (2/5) of fecal ESBL *E. coli* and 57.1% (4/7) of carcass ESBL *E. coli* were genotypically confirmed. All fresh meat ESBL *E. coli* (3/3), 50% (1/2) of ESBL seasoned meat product isolates and 100% (1/1) of fermented meat product ESBL *E. coli* were genotypically confirmed. In AmpC isolates, ESBL resistance genes were detected in 25% (1/4) of fecal *E. coli* and 33.3% (1/3) of carcass *E. coli*. No AmpC resistance genes were found.

Intermediate *E. coli* were also genotypically tested. Approximately 84% (16/19) of intermediate fecal *E. coli*, 69.2% (9/13) of intermediate carcass isolates and 100% of pork product isolates, with the sole exception of fermented meat products (33.3% - 1/3) harboured ESBL resistance genes (data not shown).

In the second sampling period, 83.3% (10/12) of fecal ESBL *E. coli*, 87.5% (14/16) of carcass ESBL isolates and 100% (4/4) of fresh meat ESBL isolates harboured ESBL resistant genes. AmpC resistant isolates

Table 3
Prevalence of ESBL, AmpC and Intermediate *E. coli* isolated in the entire pork food chain. (MP = meat product) with graphical representation.

	2019/2020			2020/2022		
ESBL	**AMR *E. coli***	***E. coli* isolates**	**%**	**AMR *E. coli***	***E. coli* isolates**	**%**
Feces	5	122	**4,10**	12	121	**9,92**
Carcasses	7	103	**6,80**	16	122	**13,11**
Fresh MP	3	19	**15,79**	4	43	**9,30**
Seasoned MP	2	7	**28,57**	0	0	–
Fermented MP	1	5	**20,00**	0	0	–
AmpC						
Feces	4	122	**3,28**	7	121	**5,79**
Carcasses	3	103	**2,91**	7	122	**5,74**
Fresh MP	0	19	**0,00**	2	42	**4,76**
Seasoned MP	0	7	**0,00**	0	0	–
Fermented MP	0	5	**0,00**	0	0	–
Intermediate						
Feces	19	122	**15,57**	15	121	**12,40**
Carcasses	13	103	**12,62**	18	122	**14,75**
Fresh MP	3	19	**15,79**	3	43	**6,98**
Seasoned MP	2	7	**28,57**	0	0	–
Fermented MP	3	5	**60,00**	0	0	–

harboured ESBL genes in the most of resistant isolates: 85.7% (6/7) of fecal AmpC *E. coli*, 71.4% (5/7) of carcass AmpC *E. coli* and 100% (2/2) of fresh meat AmpC *E. coli*. No AmpC related resistance genes were found. Isolates with an intermediate profile harboured ESBL mobile genetic elements in most of the cases: 93.3% (14/15) of fecal intermediate *E. coli*, 94.4% (17/18) of carcass intermediate *E. coli* and the 100% (3/3) of fresh meat intermediate isolates. The most frequently mobile genetic element was bla_{TEM} followed by $bla_{CTX\text{-}M1}$, which were frequently found in association in isolates from all food chain phases.

$Bla_{CTX\text{-}M2}$ was detected in resistant isolates from carcass and fresh meat and bla_{SHV} was found only in fecal resistant isolates. Considering both sampling periods, the resistant and intermediate *E. coli* harboured $bla_{CTX\text{-}M1}$ in 8.2%, 24.4% and 33.3% of the fecal, carcass and fresh meat resistant *E. coli,* respectively. $Bla_{CTX\text{-}M2}$ was harboured by the 16.3%, 26% and 50% of fecal, carcass and fresh meat resistant isolates, respectively. Bla_{TEM} was harboured by the 53%, 28%, 66.7% of fecal, carcass and fresh meat resistant isolates, respectively. bla_{SHV} was found only in fecal resistant isolates with a 20.4% prevalence. Resistant or intermediate strains isolated from processed (seasoned and fermented) meat products harboured bla_{TEM} in 100% of isolates (3/3 *E. coli* for seasoned meat product and 2/2 *E. coli* for fermented meat product). $bla_{CTX\text{-}M1}$ was detected in the 33.3% of seasoned meat products and in 100% of fermented meat product, in combination with bla_{TEM}.

3.2.3. Phylogenetic analysis

Phylogenetical analyses were performed on pork food chain isolates based on AMR data. Nine pork food chains and 19 pork food chains were chosen for the analysis in the first and second sampling periods, respectively (Table 1). Selection criteria are described as follows. The bacteria isolated from Pig 3 of Farm A, in the first sampling period, showed an intermediate profile both in fecal isolates and fermented meat product isolates: all the isolates along the food chain were considered for phylogenetical analysis. At the same time, in Pig 9 from Farm A (2019/2020) all the strains isolated from feces to seasoned meat product showed an intermediate profile, and they were chosen for further analysis. In Pig 34 from Farm B (2020/2022) only the isolates from meat products showed a resistant profile, but all the isolates of the same food chain were included. The same criteria were applied to all the isolates of the pork food chains, as reported in Table 1.

Phylogenetic similarities were found in Pig 3 (Farm A), including isolates from feces, carcasses, and fresh meat. The same results were found in Pig 24, belonging to farm F, that showed similar phenotypic and genotypic profiles. Similarities between *E. coli* isolated from carcasses and fresh meat were observed in Pig 14 (Farm A), Pig 57 (Farm A) and Pig 9 (Farm H), even if phenotypical and genotypical analyses did not show the same resistance profile. Similarities were detected in *E. coli* isolated from carcasses and fermented meat products in Pig 34 (Farm F), and between isolates from fresh meat and seasoned products in Pig 40 (Farm C). All data are reported in Table 4.

3.3. Comparative genomic analysis - average nucleotide identity

The strains that showed phylogenetic similarities were subjected to ANI sequencing technique. Phylogenetic relations observed in Pig 3, were confirmed by sequencing analysis only for feces and fresh meat isolates, with 99.1% of similarities. The relations between isolates, obtained from Pig 24, were confirmed by ANI with 99.1% of similarities in feces, carcasses, and fresh meat *E. coli*. Strong similarities were found between *E. coli* strains isolated from carcasses and fresh meat, confirmed at 99.9% in Pig 14 and Pig 57, while in Pig 9 no were detected. Similarities were highlighted by sequencing analysis in *E. coli* isolated from carcass and fermented meat products of Pig 34 (99.9%) and in fresh meat and seasoned meat products of Pig 40 (99.8%). All data are reported in Fig. 2.

4. Discussion

Monitoring ESBL, AmpC and carbapenemases producing *Salmonella* and indicator *E. coli* is mandatory in Europe since 2013 (According to [4]) in major food producing animals, including pigs. Pig farming is the most diffused animal industry worldwide and pork products have a high economic impact [28]. With regulatory differences between countries, the extensive use of antimicrobials in pig farming impact the phenomenon of AMR [29].

Humans can be exposed to pig-derived AMR microorganisms and ARGs during food production and consumption [15,30]. All pig tissues are considered sterile in healthy animals (with the sole exception of lymph nodes), but during carcass dressing and retail, meat can be contaminated by microorganisms deriving from the animal, workers, and/or from slaughter and market environments [31–33].

Worldwide, the monitoring of *E. coli* resistance prevalence in the different phases of the food producing chain showed that resistance prevalence is higher in slaughterhouses than in farms, while low prevalence is detected in meat products handled at market. It has been reported that AMR prevalence is higher in raw meat than in processed meat, even though AMR can be a risk if the production process is not correctly performed [34].

The prevalence of ESBL and AmpC is reported as low in commensal *E. coli* isolated across Europe. In fact, only 0.6% are phenotypically resistant to 3rd and 4th generation cephalosporins. Despite these comforting data, AMR in isolates from food producing animals vary among European countries and different factors can influence resistance at all levels of the food producing chain [35].

The most common genes encoding ESBL in animals are $bla_{CTX\text{-}M1}$ and $bla_{CTX\text{-}M14}$, followed by $bla_{TEM\text{-}52}$ and $bla_{SHV\text{-}12}$ while the gene mainly associated with resistance in AmpC-type β-lactamases is $bla_{CMY\text{-}2}$ [5]. $bla_{CTX\text{-}M1}$ is one of the most prevalent ESBL genes in enteric bacteria of domestic animals and large game animals in Europe [36–38] and is also frequently found among *Enterobacteriaceae* from meat of farm animals [39,40].

Data highlighted in this study showed that phenotypical resistant profiles were more frequent than the data reported by EFSA [41]. In fact, the percentage of ESBL strains in fecal *E. coli* was approximately 7% considering the two-sampling periods. The prevalence of phenotypically resistant bacteria increased slightly in the pork food chain: the 10% of ESBL *E. coli* isolated from carcasses and the 12.5% of isolates from fresh meat products. Resistance and intermediate profile prevalence rates

Table 4
Pork food chain selected for ERIC analysis. Phylogenetic similarities are highlighted in the table (bold and underlined). The enumeration of "Pig" is independent and related to each farm. (Int = Intermediate AMR profile).

Sampling period	Farm	Feces	AMR	AMR Genes	Carcasses	AMR	AMR Genes	Fresh Meat	AMR	AMR Genes	Seasoned MP	AMR	AMR Genes	Fermented MP	AMR	AMR Genes
2019/2020	A	**Pig 3**	**Int**		**Pig 3**			**Pig 3**			Pig 3			Pig 3	Int	
		Pig 8	ESBL		Pig 8			Pig 8			Pig 8	Int	bla_{CTXM1} bla_{TEM}			
		Pig 9	Int	bla_{TEM}	Pig 9	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 9	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 9	Int	bla_{CTXM1} bla_{TEM}			
		Pig 14	Int	bla_{TEM}	**Pig 14**			**Pig 14**	**ESBL**	**bla_{CTXM1} bla_{TEM}**						
	C	Pig 36	AmpC		Pig 36			Pig 36						Pig 36	Int	bla_{CTXM1} bla_{TEM}
		Pig 40	Int		Pig 40	Int		**Pig 40**			**Pig 40**	**ESBL**				
		Pig 46	Int	bla_{CTXM2} bla_{SHV}	Pig 46	Int		Pig 46			Pig 46	ESBL	bla_{TEM}			
	F	**Pig 24**	**ESBL**	**bla_{TEM}**	**Pig 24**	**ESBL**	**bla_{TEM}**	**Pig 24**	**ESBL**	**bla_{TEM}**				Pig 24	ESBL	bla_{TEM}
	G	Pig 34			Pig 34			Pig 34	Int	bla_{CTXM2}				Pig 34	Int	
	A	Pig 47	Int	bla_{CTXM1} bla_{TEM}	Pig 47	ESBL+AmpC	bla_{CTXM1} bla_{CTXM2} bla_{TEM} bla_{SHV}	Pig 47								
		Pig 57	AmpC	bla_{TEM}	**Pig 57**			**Pig 57**	**ESBL**	**bla_{CTXM1} bla_{CTXM2} bla_{TEM}**						
		Pig 58	ESBL+AmpC	bla_{CTXM1} bla_{TEM}	Pig 58	ESBL	bla_{TEM}	Pig 58								
		Pig 59	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 59			Pig 59	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}						
2020/2022	B	Pig 34			Pig 34			Pig 34	ESBL	bla_{CTXM1} bla_{CTXM2} bla_{TEM}						
		Pig 35	ESBL+AmpC	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 35	ESBL+AmpC	bla_{CTXM1} bla_{CTXM2} bla_{TEM} bla_{SHV}	Pig 35								
	C	Pig 17	ESBL	bla_{CTXM2}	Pig 17	Int	bla_{CTXM1} bla_{TEM}	Pig 17								
		Pig 21	Int	bla_{CTXM2} bla_{TEM}	Pig 21	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 21								
		Pig 27	ESBL		Pig 27	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 27	AmpC	bla_{CTXM1} bla_{CTXM2} bla_{TEM}						
		Pig 29	Int	bla_{CTXM2} bla_{TEM}	Pig 29	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 29								
	D	Pig 1	ESBL	bla_{TEM}	Pig 1	Int	bla_{TEM}	Pig 1	AmpC	bla_{CTXM2} bla_{TEM}						
		Pig 3			Pig 3	ESBL		Pig 3	Int	bla_{TEM}						
		Pig 13	Int	bla_{CTXM2}	Pig 13	AmpC	bla_{TEM}	Pig 13								

(*continued on next page*)

Table 4 (*continued*)

Sampling period	Farm	Feces	AMR	AMR Genes	Carcasses	AMR	AMR Genes	Fresh Meat	AMR	AMR Genes	Seasoned MP	AMR	AMR Genes	Fermented MP	AMR	AMR Genes
	E	Pig 30	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 30	ESBL	bla_{TEM}	Pig 30	Int	bla_{TEM}				Pig 30		
	F	Pig 34	Int	bla_{CTXM2} bla_{TEM}	**Pig 34**	**ESBL**	***bla*$_{CTXM1}$** ***bla*$_{CTXM2}$** ***bla*$_{TEM}$**	Pig 34						**Pig 34**		
		Pig 38	ESBL+AmpC	bla_{CTXM2} bla_{TEM}	Pig 38			Pig 38	ESBL	bla_{CTXM1} bla_{CTXM2} bla_{TEM}						
	G	Pig 48	Int		Pig 48			Pig 48			Pig 48			Pig 48		
	H	Pig 9			**Pig 9**	**Int**		**Pig 9**			Pig 9					
		Pig 13	Int	bla_{CTXM1} bla_{CTXM2} bla_{TEM}	Pig 13	Int	bla_{CTXM1} bla_{TEM}	Pig 13								

were higher in the first (25% and 41.7%, respectively) than in the second (0%) sampling period in both seasoned and fermented meat products. This can be due to an incomplete transformation process that led to an increased bacterial population in the products, and consequently a greater possibility of AMR diffusion.

In fact, samples collected after proper seasoning and fermentation process showed different results, confirming the processing meat techniques role to reduce bacterial load, and the AMR transmission possibility as reported by Sacher-Pirklbauer et al., 2021, Rega et al., 2022 [42,43]. AmpC *E. coli* were found only in feces, carcasses, and fresh meat and with a low prevalence. Despite this, percentages are higher than those reported by other European countries [41]. In the present study, *E. coli* isolated from feces, carcasses, and pork meat products frequently harboured bla_{TEM} followed by $bla_{CTX\text{-}M1}$ and $bla_{CTX\text{-}M2}$. No bacteria strains were genotypically identified as AmpC.

Currently, the role of food in the transmission of AMR bacteria is still unclear [44,45], although genetic fingerprint similarities between nosocomial human infection and pig carcasses have been identified [46,47]. Studies reported the possibility of transmission of ESBL and AmpC β-lactamases plasmid related genes harboured by *E. coli* strains from livestock and/or retail meat to humans [48].

The present study evaluated the role of the pork food chain in the spread of β-lactams resistance, based on sample traceability. To the authors knowledge, the present study is the first to report food chain analysis following the same animals directly from farm to meat product. Results showed that out of 243 fully or partially analysed pork food chains (feces, carcasses, fresh meat, fermented and seasoned meat product), in only one case β-lactams resistant bacteria were genotypically similar from farm-to-fork (feces, carcasses, and fresh meat). Frequent similarities were shown in β-lactams resistant *E. coli* isolates in the combination "carcasses and fresh meat" or "carcasses and fermented product" (3 food chains). Bacteria were similar in the combination "feces and fresh meat" (1 food chain) and "fresh meat and seasoned product" (1 food chain). Moreover, food safety systems such as Hazard Analysis and Critical Control Points (HACCP) must be implemented to include foodborne AMR control in management measures [49].

5. Conclusion

In conclusion, direct transmission of β-lactams resistance from farm-to-fork is possible but not frequent. Certainly, food processing techniques are used to extend the shelf life of food products reducing bacterial load. The contribution of raw or minimally processed food to the spread of AMR is visibly higher.

Consequently, the prudent use of antimicrobials in pig farms, the correct management of food production stages and of the environment continue to play a strategic role in the spread of antimicrobial resistant microorganisms.

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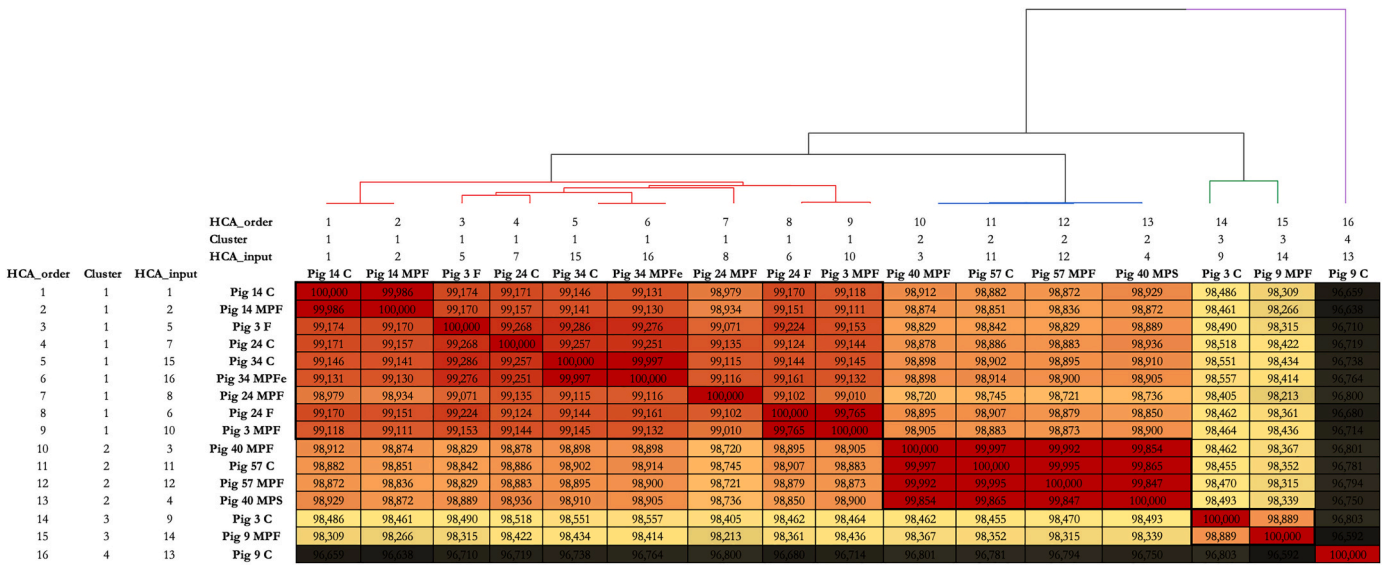
Institutional Review Board statement

Ethical review and approval were waived for this study due to the fact that no live animal manipulation or injury was performed to collect samples needed.

Informed consent statement

Not applicable.



			HCA_order	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
			Cluster	1	1	1	1	1	1	1	1	1	2	2	2	2	3	3	4
			HCA_input	1	2	5	7	15	16	8	6	10	3	11	12	4	9	14	13
HCA_order	Cluster	HCA_input		Pig 14 C	Pig 14 MPF	Pig 3 F	Pig 24 C	Pig 34 C	Pig 34 MPFe	Pig 24 MPF	Pig 24 F	Pig 3 MPF	Pig 40 MPF	Pig 57 C	Pig 57 MPF	Pig 40 MPS	Pig 3 C	Pig 9 MPF	Pig 9 C
1	1	1	Pig 14 C	100,000	99,986	99,174	99,171	99,146	99,131	98,979	99,170	99,118	98,912	98,882	98,872	98,929	98,486	98,309	96,659
2	1	2	Pig 14 MPF	99,986	100,000	99,170	99,157	99,141	99,130	98,934	99,151	99,111	98,874	98,851	98,836	98,872	98,461	98,266	96,638
3	1	5	Pig 3 F	99,174	99,170	100,000	99,268	99,286	99,276	99,071	99,224	99,153	98,829	98,842	98,829	98,889	98,490	98,315	96,710
4	1	7	Pig 24 C	99,171	99,157	99,268	100,000	99,257	99,251	99,135	99,124	99,144	98,878	98,886	98,883	98,936	98,518	98,422	96,719
5	1	15	Pig 34 C	99,146	99,141	99,286	99,257	100,000	99,997	99,115	99,144	99,145	98,898	98,902	98,895	98,910	98,551	98,434	96,738
6	1	16	Pig 34 MPFe	99,131	99,130	99,276	99,251	99,997	100,000	99,116	99,161	99,132	98,898	98,914	98,900	98,905	98,557	98,414	96,764
7	1	8	Pig 24 MPF	98,979	98,934	99,071	99,135	99,115	99,116	100,000	99,102	99,010	98,720	98,745	98,721	98,736	98,405	98,213	96,800
8	1	6	Pig 24 F	99,170	99,151	99,224	99,124	99,144	99,161	99,102	100,000	99,765	98,895	98,907	98,879	98,850	98,462	98,361	96,680
9	1	10	Pig 3 MPF	99,118	99,111	99,153	99,144	99,145	99,132	99,010	99,765	100,000	98,905	98,883	98,873	98,900	98,464	98,436	96,714
10	2	3	Pig 40 MPF	98,912	98,874	98,829	98,878	98,898	98,898	98,720	98,895	98,905	100,000	99,997	99,992	99,854	98,462	98,367	96,801
11	2	11	Pig 57 C	98,882	98,851	98,842	98,886	98,902	98,914	98,745	98,907	98,883	99,997	100,000	99,995	99,865	98,455	98,352	96,781
12	2	12	Pig 57 MPF	98,872	98,836	98,829	98,883	98,895	98,900	98,721	98,879	98,873	99,992	99,995	100,000	99,847	98,470	98,315	96,794
13	2	4	Pig 40 MPS	98,929	98,872	98,889	98,936	98,910	98,905	98,736	98,850	98,900	99,854	99,865	99,847	100,000	98,493	98,339	96,750
14	3	9	Pig 3 C	98,486	98,461	98,490	98,518	98,551	98,557	98,405	98,462	98,464	98,462	98,455	98,470	98,493	100,000	98,889	96,803
15	3	14	Pig 9 MPF	98,309	98,266	98,315	98,422	98,434	98,414	98,213	98,361	98,436	98,367	98,352	98,315	98,339	98,889	100,000	96,592
16	4	13	Pig 9 C	96,659	96,638	96,710	96,719	96,738	96,764	96,800	96,680	96,714	96,801	96,781	96,794	96,750	96,803	96,592	100,000

Fig. 2. ANI results. The isolates subjected to the analysis are those phylogenetically related using ERIC. Colors reflect the abundance of the identified genes, starting from zero (white) to 100% (red). Hierarchical clustering is reported. (F = feces, C = carcasses, MPF = fresh meat product, MPFe = fermented meat product, MFS = seasoned meat product). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Author contributions

Conceptualization, C.B., C.C., G.D. and M.R.; methodology, L.A.; software, M.R.; validation, M.R.; formal analysis, M.R.; investigation, M. R. and L.A.; resources, A.P.; data curation, M.R. writing original draft preparation, M.R.; writing-review and editing, M.C.; visualization, C.B.; supervision, C.B. and S.B.; project administration, C.B., C.C., G.D.; funding acquisition, C.B. All authors have read and agreed to the published version of the manuscript.

Relationships

There are no additional relationships to disclose.

Patents and Intellectual property

There are no patents to disclose.

Other activities

There are no additional activities to disclose.

Declaration of Competing Interest

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Data availability

The data that has been used is confidential.

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