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Prevalence and molecular characterization of ESBL/pAmpC producing faecal *Escherichia coli* strains with widespread detection of CTX-M-15 isolated from healthy poultry flocks in Eastern Algeria

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A R T I C L E I N F O

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A B S T R A C T

The intensification of livestock farming has led to the widespread use of massive amounts of antibiotics worldwide. Poultry production, including white meat, eggs and the use of their manure as fertiliser, has been identified as one of the most crucial reservoirs for the emergence and spread of resistant bacteria, including *E. coli* in poultry as an important opportunistic pathogen representing the greatest biological hazard to human and wildlife health. Thus, this study aimed to analyse *E. coli* in the faecal carriage of healthy poultry flocks and to investigate the phenotypic and genotypic characteristics of antimicrobial resistance, including integrons genes and phylogenetic groups. A total of 431 cloacal swabs from apparently healthy poultry from four regions in Eastern Algeria from December 2021 to October 2022. 360 *E. coli* were isolated; from broilers (n = 151), broiler breeders (n = 91), laying hens (n = 72), and breeding hens (n = 46). Among this, 281 isolates exhibited multidrug resistance (MDR) phenotype, 17 of the 360 *E. coli* isolates exhibited ESBL, and one isolate exhibited both ESBL/pAmpC. A representative collection of 183 among 281 MDR *E. coli* was selected for further analysis by PCR to detect genes encoding resistance to different antibiotics, and sequencing was performed on all positive PCR products of $bla_{\text{CTX-M}}$ and $bla_{\text{CMY-2}}$ genes. Phylogenetic groups were determined in 80 *E. coli* isolates (20 from each of the four kinds of poultry). The $bla_{\text{CTX-M}}$ gene was found in 16 (94.11 %) ESBL-producing *E. coli* isolates within 11 strains co-expressing the bla_{SHV} gene and 8 strains co-expressing the bla_{TEM} gene. Sequence analysis showed frequent diversity in CTX-M-group-1, with $bla_{\text{CTX-M-15}}$ being the most predominant (n = 11), followed by $bla_{\text{CTX-M-1}}$ (n = 5). The $bla_{\text{CMY-2}}$ gene was detected only in one ESBL/pAmpC isolate. Among the 183 tested isolates, various antimicrobial resistance genes were found (number of strains) bla_{TEM} (n = 121), bla_{SHV} (n = 12), *tetA* (n = 100), *tetB* (n = 29), *sul1*(n = 67), *sul2* (n = 32), *qnrS* (n = 45), *qnrB* (n = 10), *qnrA* (n = 1), *catA1*(n = 13), *aac-(6′)-Ib* (n = 3). Furthermore, class 1 and class 2 integrons were found in 113 and 2 *E. coli*, respectively. The isolates were classified into multiple phylogroups, including A (35 %), B1 (27.5 %), B2 and D each (18.75 %). The detection of integrons and different classes of resistance genes in the faecal carriage of healthy poultry production indicates that commensal *E. coli* could potentially act as a reservoir for antimicrobial resistance, posing a significant One Health challenge encompassing the interconnected domains of human, animal health and the environment. Here, we present the first investigation to describe the diversity of $bla_{\text{CTX-M}}$ producing *E. coli* isolates with widespread detection of CTX-M-15 and CTX-M-1 in healthy breeders (Broiler and breeding hens) in Eastern Algeria.

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1. Introduction

The poultry sector is a vital component of the global animal food industry, offering affordable products with high-quality protein and meat with low-fat content [1]. In Algeria, the poultry industry produced 430846.2 tons of white meat and 6.08 billion eggs in 2021. However, there was a significant decrease in white meat production (approximately 20.4 %) in 2021 compared to 2019, according to official agricultural statistics and the Algerian Ministry of Agriculture and Rural Development (MARD) [2]. This decline is attributed to fluctuations in feed costs, their rarity, the effects of the COVID-19 pandemic, and animal diseases, especially the H5N8 influenza outbreak.

Disease prevalence, particularly bacterial infections, has led to significant losses and increased antibiotic use in intensive breeding practices, contributing to the rise of resistant strains [3]. In 2020, the global antimicrobial use in food animal production was 99,502 tons, with projections indicating an increase to 107,472 tons by 2030 [4]. *E. coli*, a highly genetically adaptable bacterium commonly found in human gastrointestinal tracts and warm-blooded animals, plays crucial roles in the intestinal microbiota but can also act as an opportunistic pathogen [5].

The presence, appearance, spread and persistence of multidrug-resistant strains and *E. coli* producing ESBL/pAmpC enzymes are global challenges affecting food safety, the environment, animal and human health. Resistant bacteria can colonise the human gut and transfer resistance genes to the indigenous flora [6].

The intestinal microbiota and cloaca can be a source of avian pathogenic *E. coli* (APEC) [7]. Transmission of *E. coli* harbouring resistance genes can spread within the poultry production pyramid through various pathways. These include vertical transmission from parents to offspring [8] and three main horizontal pathways: contamination via eggshells in hatcheries, within breeding farms, and between farms and their environments. ESBL/pAmpC *E. coli* has been identified at all levels of the poultry production pyramid [9].

The intensive use of untreated poultry manure in agricultural soil as an organic, effective, and efficient fertiliser is a growing environmental hazard since it contains feathers, excrement, wasted animal feed, and bedding materials [10] is a major factor behind the emergence and spread of antibiotic resistance. Moreover, poultry manure can be a source of harmful bacteria for humans, including *E.coli*, *Campylobacter jejuni* and *Salmonella* spp [11], which pose a threat of transmitting antibiotic resistance genes (ARGs) through the food chain, water sources and the ecosystems [10]. Furthermore, it can also contaminate fresh vegetables, often linked to foodborne outbreaks [12]. *E. coli* has been associated with multiple outbreaks of infections from food contaminated with poultry manure [13].

The European Food Safety Authority [14] recognises ESBL/pAmpC-producing *E. coli* as a significant concern for chicken meat safety (BIOHAZ, 2013). These resistance mechanisms are commonly associated with the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ groups [15]. Predominant AmpC-family type CMY, $bla_{CMY\text{-}2}$ is the most widespread gene [16].

The initial detection of *E. coli* producer ESBL in animals used for food production in Spain, where $bla_{SHV\text{-}12}$ and $bla_{CTX\text{-}M\text{-}14}$ enzymes were found in chicken faecal samples [17]. In recent years, a decrease in the incidence of ESBL/pAmpC-producing *E. coli* in turkeys and broilers has been reported [18]. Resistance acquisition by *E.coli* via horizontal gene transfer presents an epidemiological threat, particularly when resistance genes are transferred between bacterial species, especially with multidrug-resistant strains [19].

Integrons are key genetic tools identified in various bacteria [20], including *E. coli*, particularly class 1 integrons, which are widespread and significantly influence resistance gene acquisition and the emergence of MDR by acquiring mobile elements [21].

The phylogenetic groups of *E. coli* are defined by the presence or absence of specific gene targets, identifying eight main groups: A, B1, B2, C, D, E, F and *Escherichia* cryptic clades I to V [22,23]. Phylogroups A and B1 typically include commensal bacteria and certain intestinal pathogens, while B2 and D are frequently associated with extra-intestinal pathogens [23]. Classifying *E. coli* into phylogroups aids in studying their ecological niche, community structure, genetic diversity and potential to harbour antimicrobial resistance genes [24], providing valuable epidemiological insights.

In Algeria, as far as we know, research on antibiotic resistance has primarily focused on avian pathogenic *E. coli* (APEC) causal agents of colibacillosis isolates in diseased broilers. There is limited knowledge about multidrug-resistant, ESBL/pAmpC-producing faecal *E. coli* (AFEC) in healthy broilers and no information is available regarding broiler breeders, laying hens, and breeding hens. Understanding the potential connection between antimicrobial resistant bacteria in livestock and humans is crucial for enhancing our knowledge of these bacteria's dynamics within host populations.

This study aims to analyse faecal *E. coli* strains obtained from healthy poultry flocks by (i) determining the incidence of *E. coli* in four types of poultry production (broilers, broiler breeder, laying hens and breeding hens) from different farms in four provinces in Eastern Algeria,(ii) assessing antimicrobial resistance profiles, the occurrence of ESBL/pAmpC producing *E.coli* and the frequency of MDR *E.coli*,(iii) molecular characterisation of antimicrobial genes belonging to different groups, (iv) exploring the MAR index for all isolates and analysing the genetic diversity of these bacteria using Clermont's method and distribution of integrons class 1 and 2.

2. Materials and methods

2.1. Study period and area

This study was conducted from December 2021 to October 2022 in 57 broiler farms located in Eastern Algeria, specifically (Batna, Setif, Biskra and M'Sila) (Fig. 1). Five to ten birds from each farm were randomly chosen for collecting cloacal swab samples.

2.2. Samples collection

A total of 431 fresh cloacal swab samples were aseptically collected in sterile containers from healthy chickens, including broilers (n = 200), laying hens (n = 80), broiler breeders (n = 104), and breeding hens (n = 47). The samples were coded and transported in an isothermal icebox to the Veterinary Microbiology Laboratory (University Batna 1) within 2 hours of collection and immediately processed and examined for the detection of *E. coli*.

2.3. Isolation and identification of E. coli

Cloacal swabs were enriched in nutrient broth (Biokar, France) for 20h at 37 °C, then streaked onto Hektoen enteric agar (HiMedia, Mumbai, India) for 24h at 37 °C. *E.coli* species identification was performed using CHROMagar™ Orientation (France), followed by Indole test and API 20 E system (bioMérieux, Marcy-l'Etoile, France) and finally confirmed by MALDI-TOF MS as described by Seng et al. [25].

2.4. Antimicrobial susceptibility testing and detection of ESBL production

Phenotypic resistance characterisation was carried out using the disc diffusion technique on Mueller Hinton agar (HiMedia, Mumbai, India) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [26]. Fourteen antibiotics (Bioanalyse, Ankara, Türkey) were tested: amoxicillin (25 μg), amoxicillin-clavulanic acid (20/10 μg), aztreonam (30 μg), imipenem (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), nalidixic acid (30 μg), gentamycin (10 μg), enrofloxacin (5 μg), ciprofloxacin (5 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg). Results were interpreted following the Veterinary recommendations of

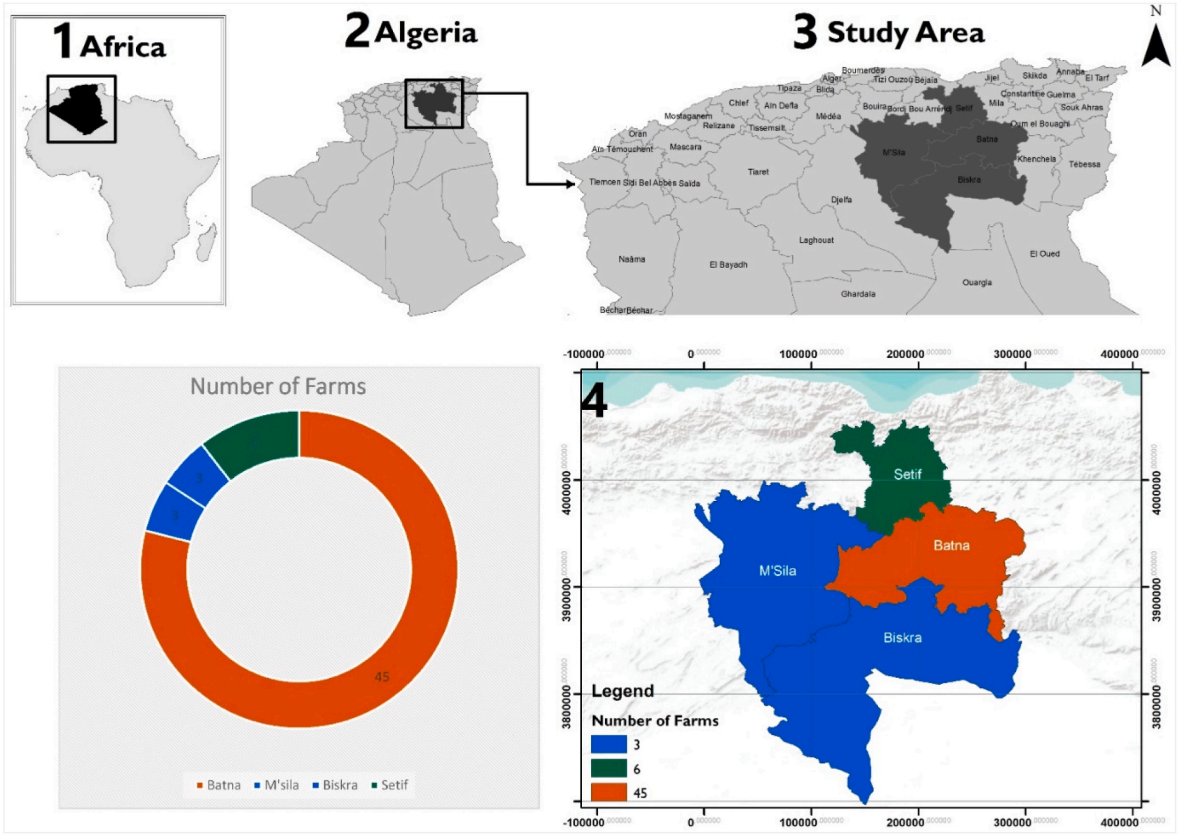


Fig. 1. Map of Algeria showing the designated regions for sample collection (Thematic cartography created using ArcGIS 10.3).

CA-SFM [27] as well as guidelines provided by the CLSI [26]. Double-disk synergy technique (DDST) was used to confirm the ESBL phenotype as previously outlined [28]. *Escherichia coli* ATCC 25922 served as the control strain.

2.5. Calculation of multiple antibiotic resistance index (MARI)

The multiple antibiotic resistance (MAR) index, a quantitative measure of bacterial resistance to multiple antibiotics, was determined as follows: MARI = a/b, where "a" is the number of antibiotics to which *E. coli* is resistant and "b" is the total number of tested antibiotics.

A MAR index exceeding 0.2 indicates a greater level of resistance to multiple antibiotics, reflecting the acquisition of multidrug-resistant genes [29].

2.6. Characterisation of antimicrobial resistance genes

A collection of 183 *E. coli* isolates was selected and cultured overnight on 10 ml LB broth (Lauria & Bertani -India). Chromosomal DNA was isolated following the protocol described by Ghen and Kuo [30] for Gram-negative bacteria and used as template DNA in PCR assays to detect the following resistance genes: four genes associated with beta-lactamases (bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{CMY-2}). Strains positive for the bla_{CTX-M} gene were further screened for bla_{CTX-M} -1-2-8 and 9 groups. Additionally, quinolone resistance genes (*qnrA, qnrB* and *qnrS*), tetracycline resistance genes (*tetA* and *tetB*), aminoglycoside-modifying enzyme (*aac(6′)-Ib*), chloramphenicol resistance genes (*catA1*) and resistance genes to sulfonamides (*sul1* and *sul2*) were detected. The *intI1* and *intI2* genes encoding integrases of class 1 and class 2 integrons were also analysed. Sanger sequencing was used to sequence all positive bla_{CTX-M} groups and bla_{CMY-2} PCR products to identify gene variants [31]. BLAST tools were used to perform similarity queries (http://www.ncbi.nlm.nih.gov). The primers used in the investigation and their characteristics are described in (Table S1 in the supplementary data).

2.7. Clermont's phylogenetic grouping

Eighty *E.coli* isolates (20 from broilers, 20 from laying hens, 20 from broiler breeders and 20 from breeding hens) were subjected to phylogenetic groupings (A, B1, B2 and D) performing multiplex PCR using specific primers for *chuA, yjaA*, and *TspE4.C2* genes [22].

2.8. Statistical analysis

Excel 2016 (Microsoft Office 365) was first used for data collection and organisation. GraphPad Prism 9 for Windows (version 9.5.1, California, USA) was used for statistical analyses following data sorting. All graphs, contingency tables, scatter plots, and matrix were created using GraphPad Prism software. Pearson's correlation coefficient was computed to identify the association among phenotypic and genotypic resistance. A comparison of resistance frequency levels among the tested antibiotics between broilers, broiler breeders, laying and breeding hens was conducted by chi-square test. "Statistical significance" was approved as a *p*-value below 0.05 ($p < 0.05$).

3. Results

3.1. Prevalence of E. coli strains

Out of 431 samples, 83.53 % (n = 360) were confirmed as *E. coli*, with 151 from broilers, 91 from broiler breeders, 72 from laying hens and 46 from breeding hens.

3.2. Antimicrobial susceptibility testing

The findings of antimicrobial susceptibility testing results (Table 1)

Table 1
Frequency of antibiotic resistance (n = 360).

Antibiotic (concentration μg)	Sensitive	Intermediate	Resistant
Tetracycline (30)	42 (11.7 %)	0 (0.00 %)	318 (88.3 %)
Amoxicillin (25)	88 (24.4 %)	0 (0.00 %)	272 (75.6 %)
Ciprofloxacin (5)	160 (44.4 %)	0 (0.00 %)	200 (55.6 %)
Enrofloxacin (5)	156 (43.3 %)	0 (0.00 %)	204 (56.7 %)
Nalidixic acid (30)	90 (25 %)	27 (7.5 %)	243 (67.5 %)
Trimethoprim-sulfamethoxazole (1.25/23.75)	141 (39.2 %)	5 (1.4 %)	214 (59.4 %)
Chloramphenicol (30)	249 (69.2 %)	2 (0.6 %)	109 (30.3 %)
Cefotaxime (30)	343 (95.3 %)	0 (0.00 %)	17 (4.7 %)
Amoxicillin-clavulanic acid (20/10)	198 (55 %)	0 (0.00 %)	162 (45 %)
Cefoxitin (30)	343 (95.3 %)	12 (3.3 %)	5 (1.4 %)
Gentamicin (10)	290 (80.6 %)	17 (4.7 %)	53 (14.7 %)
Aztreonam (30)	341 (94.7 %)	10 (2.8 %)	9 (2.5 %)
Imipenem (10)	360 (100 %)	0 (0.00 %)	0 (0.00 %)
Ceftazidime (30)	351 (97.5 %)	4 (1.1 %)	5 (1.4 %)

indicated that, across the four poultry species, resistance to tetracycline was the highest at (88.3 %), followed by amoxicillin (75.6 %), nalidixic acid (67.5 %), trimethoprim-sulfamethoxazole (59.4 %), enrofloxacin (56.7 %) and ciprofloxacin (55.6 %). Furthermore, resistance to amoxicillin-clavulanic acid was recorded in (45 %) of the isolates, followed by chloramphenicol (30.3 %) and gentamicin (14.7 %). Resistance was also noted in (4.7 %) of the isolates to cefotaxime and in (1.4 %) to ceftazidime and cefoxitin. Resistance to aztreonam was (2.5 %), while no resistance towards imipenem, a member of the carbapenem class.

3.3. MAR index and multidrug-resistant E. coli

Resistance to at least three distinct antibiotic classes was found in 78.06 % (n = 281) of the isolates. The MAR index was greater than 0.2 in 71.67 % (n = 258) of the isolates, while 28.33 % (n = 102) had a MAR index below 0,2. The highest MAR index reported was 0.8 in one isolate, while the lowest MAR index of 0.07 was found in 35 isolates (9.72 %) (Fig. 2).

3.4. Prevalence of ESBL-producing E. coli isolates

The ESBL phenotype was identified in 17 strains, 10 from broilers, 3 from broiler breeders and 4 from breeding hens. One isolate from broilers exhibited both ESBL and pAmpC co-expression. All ESBL-positive isolates demonstrated multidrug-resistant phenotypes, with the predominant MDR profile being resistant to amoxicillin, amoxicillin-clavulanic acid, cefotaxime, enrofloxacin, ciprofloxacin, and nalidixic acid (Table 2).

3.5. Molecular characterisation of beta-lactamases and diversity of ESBL/pAmpC producing E. coli

Based on phenotypic resistance profile data, antimicrobial resistance genes were detected by PCRs in 183 *E. coli* isolates.

Overall, 121 isolates (79.61 %) carried the bla_{TEM}, of which 9 were ESBL/pAmpC producers and 12 (7.89 %) harboured the bla_{SHV}; all being ESBL except one. Furthermore, bla_{CTX-M} was detected in 16 (94.11 %)

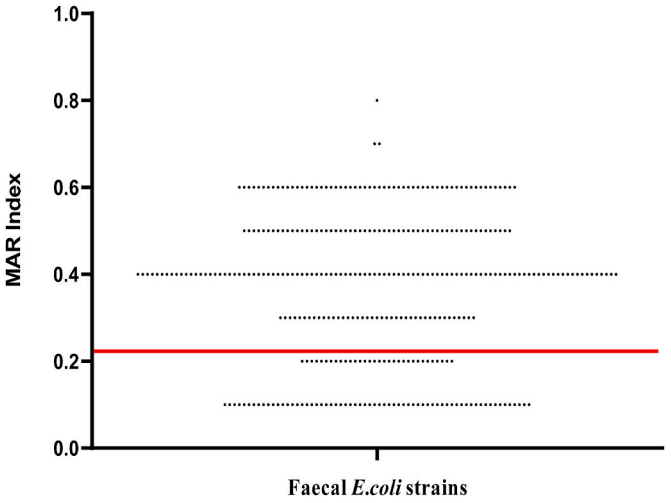


Fig. 2. A scatter plot illustrating the MAR index of *E. coli* strains among different flock categories. The range and distribution of data are shown by the dots representing how each isolate responded. The isolates situated above the red line are referred to as highly resistant isolates.

ESBL isolates. The presence of bla_{CMY-2} was identified in a single isolate ESBL/pAmpC producer. In addition, 5 out of the 17 ESBL isolates possessed a combination of these genes, particularly the bla_{CTX-M}+ bla_{TEM} + bla_{SHV}. (Table 2).

3.6. Frequency of CTX-M groups

Among the 16 positive bla_{CTX-M} isolates, 15 were attributed to the CTX-M group 1, which is considered the prevalent group. These were either independently (n = 5) or combined with other groups: 7 with $bla_{CTX-M-9}$ and 2 with $bla_{CTX-M-2}$. Additionally, a single isolate harboured the $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, and $bla_{CTX-M-9}$ groups.

3.7. Sequence analysis

Sequencing of the bla_{CTX-M} gene revealed diversity within the bla_{CTX-M}-group-1, with $bla_{CTX-M-15}$ emerging as the predominant type at 68.75 %, followed by $bla_{CTX-M-1}$ at 31.25 %. The sequences of the bla_{CTX-M} and bla_{CMY-2} genes were deposited in GenBank on 19 June 2024 under the accession numbers PP909798/PP909799/PP909800/PP909801/PP909802/PP909803/PP909804/PP909805/PP909806/PP909807/PP909808/PP909809/PP909810/PP909811/PP857923.

3.8. PCR detection of resistance genes

AMR genes were identified in the collection of 183 based on the phenotypic resistance profile data. Among the 161 isolates exhibiting resistance to tetracycline, 100 (62.11 %) isolates hosted the *tetA* gene, 29 (18.01 %) isolates contained the *tetB* gene, and 14 (8.69 %) strains carried both genes. Additionally, 120 strains were tested for the genes conferring resistance to trimethoprim-sulfamethoxazole. The *sul1* gene was found in 67 (55.83 %) strains, while the *sul2* gene was found in 32 (26.67 %) isolates.

Regarding the genes conferring resistance to fluoroquinolones, we investigated the *qnrA*, *qnrB*, and *qnrS* in 115 fluoroquinolone-resistant strains. The most common gene was *qnrS*, identified in 45 (39.13 %) strains, followed by the *qnrB* in 10 (8.70 %) isolates. The *qnrA* gene was identified in only one strain. Five *E. coli* strains possessed a combination of *qnrB* and *qnrS* genes. Furthermore, the *catA1* gene was found in 13 out of 54 chloramphenicol-resistant isolates (24.07 %). The *aac-(6′)-Ib* gene was detected in 3 strains (8.82 %) of 34 isolates exhibiting resistance to gentamicin from healthy broilers (Fig. 4).

Table 2
Characterisation of ESBL/pAmpC producing *E. coli* strains from cloacal swabs.

E. coli isolate code	Sampling origin	Area "locality"	Phylogenetic groups	Resistance pattern	MARI	No of AMR class	Phenotype	Beta-lactamase genes	CTX-M variants	Other genes detected	Integron1 and 2 genes
DZEC 1	breeding hens	Biskra	B1	AMX-AMC-CTX-ENR-CIP-NA	0.43	4	ESBL	bla_{CTX-M}	$bla_{CTX-M-15}$	*qnrS*	/
DZEC 3	breeding hens	Biskra	B1	AMX-AMC-CTX-CIP-ENR-NA	0.43	4	ESBL	bla_{CTX-M} – bla_{SHV}	$bla_{CTX-M-15}$	/	/
DZEC 4	breeding hens	Biskra	B2	AMX-CTX-ENR-CIP-NA	0.36	3	ESBL	bla_{CTX-M} – bla_{SHV}	$bla_{CTX-M-15}$	*qnrS*	/
DZEC 5	breeding hens	Biskra	A	AMX-AMC-CTX-ENR-CIP-NA	0.43	4	ESBL	bla_{CTX-M} – bla_{SHV}	$bla_{CTX-M-15}$	/	/
DZEC 10	broiler breeders	Biskra	B2	AMX-CTX-TE-NA-STX	0.36	5	ESBL	bla_{CTX-M} – bla_{SHV}	$bla_{CTX-M-15}$	*tetA -sul1- qnrS- qnrB*	*intI1*
DZEC 11	broiler breeders	Biskra	D	AMX-CTX-CIP-ENR-NA-STX	0.43	4	ESBL	bla_{CTX-M}	$bla_{CTX-M-1}$	*Sul1*	/
DZEC 16	broilers	Batna	A	AMX-CTX-ATM-TE-ENR-CIP-NA-STX	0.57	6	ESBL	bla_{TEM} bla_{CTX-M} bla_{SHV}	$bla_{CTX-M-1}$	*tetA-qnrS-sul2*	*intI1*
E. coli isolate code	Sampling origin	Area "locality"	Phylogenetic groups	Resistance pattern	MARI	No of AMR class	phenotype	Beta-lactamase genes	CTX-M variants	Other genes detected	Integron1 and 2 genes
DZEC 50	broilers	Batna	B2	AMX-AMC-CTX-CAZ-ATM-TE-ENR-CIP-GEN-NA-STX	0.79	8	ESBL	bla_{TEM} bla_{CTX-M} bla_{SHV}	$bla_{CTX-M-15}$	*tetA -qnrS -sul1*	*intI1*
DZEC 107	broilers	Batna	B1	AMX-AMC-CTX-TE-ENR-CIP-CHL-NA-STX	0.64	7	ESBL	bla_{CTX-M} bla_{SHV}	$bla_{CTX-M-1}$	*qnrB -qnrS- sul1- sul2- CatA*	/
DZEC 139	broilers breeders	Barika	B2	AMX-AMC-CTX-CAZ- TE-ENR-CIP-CHL-NA-STX	0.71	7	ESBL	bla_{CTX-M} - bla_{TEM}	$bla_{CTX-M-1}$	*tetA- tetB- qnrS sul1- sul2- CatA*	*intI1*
DZEC 168	broilers	Batna	D	AMX-AMC-CTX-TE-ENR-CIP-NA-STX	0.57	6	ESBL	bla_{CTX-M} bla_{TEM} bla_{SHV}	$bla_{CTX-M-15}$	*tetA-qnrS-sul1*	/
DZEC 169	broilers	Batna	D	AMX-AMC-CTX-CAZ-FOX-TE-CHL- STX	0.6	7	ESBL + AmpC	bla_{TEM}- bla_{CMY-2}	/	*tetB-sul2*	*intI2*
E. coli isolate code	Sampling origin	Area "locality"	Phylogenetic groups	Resistance pattern	MARI	No of AMR class	phenotype	Beta-lactamase genes	CTX-M variants	Other genes detected	Integron1 and 2 genes
DZEC 208	broilers	Batna	D	AMX-CTX-CAZ-ATM-TE	0.36	4	ESBL	bla_{CTX-M} - bla_{TEM}	$bla_{CTX-M-15}$	*tetA- tetB*	*intI1*
DZEC 242	broilers	Batna	D	AMX-CTX-CAZ-ATM-TE-ENR-CIP-CHL-NA-STX	0.71	7	ESBL	bla_{CTX-M} - bla_{SHV}	$bla_{CTX-M-15}$	*qnrS- sul1*	*intI1*
DZEC 323	broilers	Batna	D	AMX-CTX-TE-ENR-CIP-NA-STX	0.50	5	ESBL	bla_{CTX-M} - bla_{TEM}	$bla_{CTX-M-15}$	*qnrS- sul1- sul2*	*intI1*
DZEC 359	broilers	Batna	D	AMX-CTX-ATM-TE-ENR-CIP-NA-STX	0.57	6	ESBL	bla_{CTX-M} - bla_{TEM} bla_{SHV}	$bla_{CTX-M-15}$	*qnrS- sul1- sul2*	*intI1*
DZEC 360	broilers	Batna	D	AMX-CTX-ATM-TE-ENR-CIP-NA-STX	0.57	6	ESBL	bla_{CTX-M} - bla_{TEM} bla_{SHV}	$bla_{CTX-M-1}$	*tetA- tetB- qnrS sul1- sul2*	*intI1*

(TE) tetracycline; (AMX) amoxicillin; (CIP) ciprofloxacin; (ENR) enrofloxacin; (NA) nalidixic acid; (STX) sulfamethoxazole; (CHL) chloramphenicol; (CTX) cefotaxime; (AMC) amoxicillin/clavulanic acid; (FOX) cefoxitin; (GN) gentamicin; (ATM) aztreonam; (CAZ) ceftazidime.MARI, multiple antibiotic resistance index;/: no genes detected.

Out of the 17 ESBL/pAmpC producing *E. coli* strains, 15 (88.23 %) carried additional genes including *sul1* and *sul2*, *tetA* and *tetB*, *qnrB* and *qnrS* and *catA1* (Table 2).

3.9. Detection of integrons

Among 183 *E. coli* isolates tested for the presence of class I and II integrons, the *intI-1* gene was detected in 113 (61.75 %) isolates. The distribution of these isolates was as follows: 74 from broilers, 17 from broiler breeders, 12 from laying hens, and 10 from breeding hens. The *intI-2* gene was determined in 2 isolates from broilers and both genes were present in a single *E. coli* isolate.

3.10. Phylogenetic analysis

Analysis of the 80 *E. coli* strains revealed four distinct phylogroups: A, B1, B2 and D. The distribution of these phylogroups was as follows: phylogroup A (n = 28), B1 (n = 22), B2 (n = 15) and D (n = 15) (Fig. 5). Phylogroup A was the most common, including more than 70 % of strains from breeding and laying hens. Phylogroup B1 accounted for 45.5 % of the isolates from laying hens, phylogroup B2 for 46.7 % of the isolates from broiler breeders and phylogroup D for 80 % of the isolates from broilers.

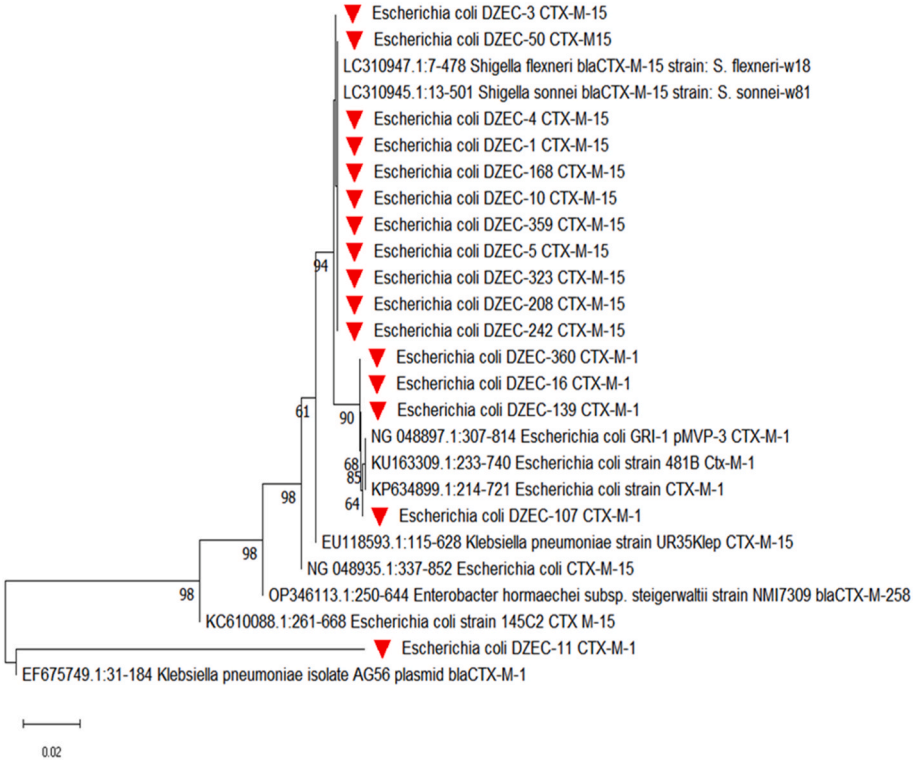


Fig. 3. A phylogenetic tree among ESBL-producing *Escherichia coli* constructed using partial $bla_{\text{CTX-M}}$ nucleotide sequences. A red triangle (n = 16) highlights the strains of this investigation.

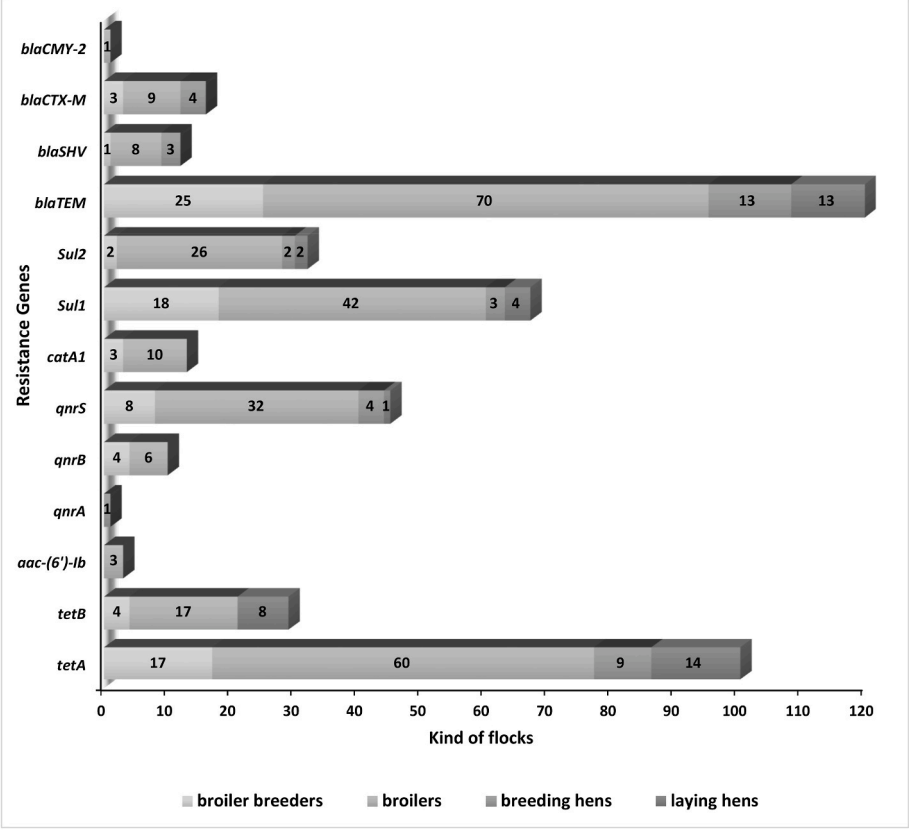


Fig. 4. Prevalence of antimicrobial resistance genes (ARGs) identified for each flock category.

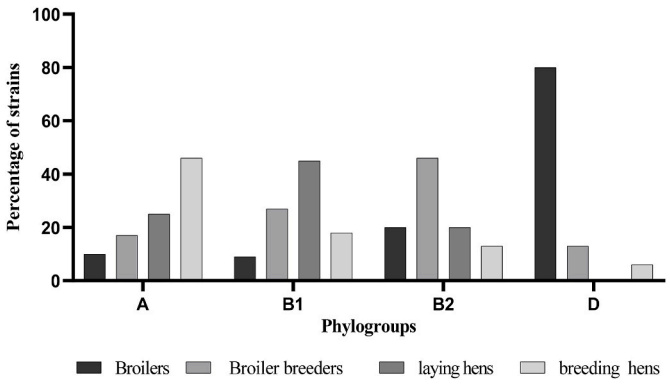


Fig. 5. Distribution of different phylogenetic groups in 80 *E. coli* strains recovered from four types of poultry flocks.

3.11. Correlation between the phenotypic and genotypic resistance profiles

Correlation analysis was conducted to investigate the connection between genotypic and phenotypic resistance within 183 isolates using Pearson's correlation coefficient (r). Significant positive correlations were observed among resistance genes and antibiotics within the same class. The beta-lactamase genes showed strong positive correlation, i.e., AMX and bla_{TEM} (r = 0.63); CTX and bla_{SHV} (r = 0.75); CTX and $bla_{CTX\text{-}M}$ (r = 0.97); CAZ and bla_{SHV} (r = 0.45); CAZ and $bla_{CTX\text{-}M}$ (r = 0.65); FOX and $bla_{CMY\text{-}2}$ (r = 1). Additionally, positive correlations were noted between other antimicrobials and their respective resistant genes, i.e., TE and *tetA* (r = 0.41); TE and *tetB* (r = 0.16); ENR and *qnrA*, *qnrB* and *qnrS* with a correlation coefficient of 0.05, 0.14 and 0.40 respectively; SXT and *sul1* (r = 0.53); SXT and *sul2* (r = 0.32); CHL and *catA1*(r = 0.43); GEN with *aac-(6′)-Ib* (r = 0.25) (Fig. 6).

4. Discussion

Antimicrobial resistance is an increasingly global issue in human and veterinary medicine. It is well-documented that the rising frequency of antibiotic resistance in gram-negative bacteria is primarily due to the widespread use of antibiotics in agriculture and medicine [32]. *Escherichia coli* is the most common pathogen globally, affects birds of all ages and has a significant economic impact on poultry production, causing increased mortality rates and reduced bird output.

This study focused on *E. coli* isolated from the intestinal microbiota of apparently healthy chickens. According to our knowledge, all previous studies in Algeria have examined pathogenic *E. coli* (APEC) from diseased broilers with colibacillosis. Although there have been limited studies on the faecal carriage of *E. coli* in broilers, no research has been conducted on broiler breeders, laying or breeding hens. Thus, this is the first report on the prevalence, antimicrobial resistance (AMR), multidrug resistance (MDR), integrons genes of *E. coli* collected from cloacal samples of healthy poultry (broiler breeders, laying and breeding hens) in Eastern Algeria.

We identified 360 (83.53 %) *E. coli* strains from 431 cloacal swabs, a significantly higher rate compared to previous reports from Algeria. Amairi et al. [33] reported the lowest rate of 30.86 % conducted in 3 cities (Batna, Biskra and Khenchla), while Barour et al. [34] found a 65.33 % isolation rate in Souk Ahras, Tebessa, Khenchla, and Constantine provinces. In Western Algeria, a rate of 56.25 % isolation was reported [35].

Variations in research findings can be attributed to factors such as geographical differences, breeding conditions, flock size, level of immunity and the type of poultry production, which can contribute to differences in each research study [36]. Besides *E. coli*, our samples revealed a diversity of isolated bacteria, including *Klebsiella pneumonia*, *Enterobacter cloacae, Pseudomonas aeruginosa* and *Proteus mirabilis*.

The widespread use of antibiotics has resulted in a substantial increase in the prevalence of antimicrobial resistance within gram-negative bacteria [32]. Out of the fourteen antibiotics tested for *E. coli*

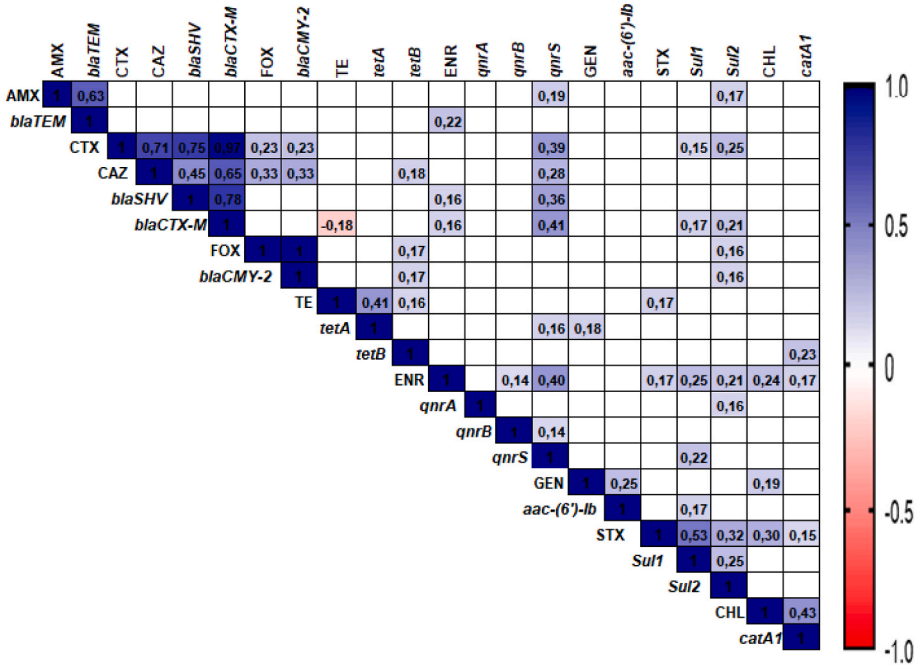


Fig. 6. The correlation matrix represented the association between phenotypic and genotypic antimicrobial resistance within 183 *E. coli* isolates. Red cells reveal negative correlations, while blue cells reveal positive correlations. The size and intensity of the colour represent the value of the correlation coefficient (r = 1 "positive correlation"; r = 0 "no correlation"; r = −1 "negative correlation"). Statistical significance was assessed at a *p*-value <0.05; cells without significant correlations were left empty. (TE) tetracycline; (AMX) amoxicillin; (ENR) enrofloxacin; (NA) nalidixic acid; (STX) sulfamethoxazole; (CHL) chloramphenicol; (CTX) cefotaxime; (FOX) cefoxitin; (GN) gentamicin; (CAZ) ceftazidime.

strains, tetracycline resistance was the highest at 88.3 %. This is consistent with research conducted in China [37], where 89.3 % of *E.coli* strains were resistant to tetracycline. The high resistance levels may be linked to the extensive use of tetracycline in poultry, as these antibiotics have been widely used in animal farming, particularly in the poultry industry, for many years [38].

Our study showed high resistance rates to amoxicillin, enrofloxacin, and trimethoprim-sulfamethoxazole. These findings are consistent with a previous Algerian study [35], where *E. coli* isolates exhibited high resistance to these antibiotics (rates ranging from 50 % to 100 %). The absence of legislative restrictions on antibiotic use in the poultry industry contributes to these high resistance rates [39].

In contrast, no resistance to imipenem was observed, which aligns with the results of Belmahdi et al. [40].

Notably, our isolates showed relatively high resistance to chloramphenicol (30.3 %) despite its ban on use in animals in Algeria. Two recent Algerian studies reported even higher resistance rates of 43.33 % and 47.86 %, respectively [33,34]. We also found that 14.7 % of our isolates were resistant to gentamicin, comparable to the 13.10 % reported by Benklaouz et al. [41].

Our data indicates that 78.06 % of *E. coli* strains exhibited MDR profiles, with approximately 49.82 % of these strains coming from broilers. Similar high incidence of MDR was documented in previous Algerian and international studies ranging from 64.6 % to 100 %; in Algeria [34,42], Zambia [43], Naples [44], Türkiye [45] and Spain [46]. The predominant MDR phenotype was associated with resistance to amoxicillin, tetracycline, nalidixic acid, ciprofloxacin, enrofloxacin, and sulfamethoxazole, highlighting the extensive use of antimicrobial drugs in poultry production, whether prophylactic purposes or growth promotion [47].

Our findings reveal that *E. coli* strains from broilers exhibited a higher frequency of AMR and MDR compared to those from broiler breeders, laying hens, and breeding hens. Resistance levels for all tested antibiotics displayed notable differences among the four poultry types ($p < 0.05$) except for cefotaxime, ceftazidime and cefoxitin (Fig. 7).

The key aspect of this investigation was assessing potential health risks associated with antibiotic resistance in the ecosystem using the MARI analysis [48]. Results showed that 258 of 360 (71.67 %) *E. coli* isolates had a MAR index above 0.2, ranging from 0.07 to 0.8. Compared to previous reports, the MAR indexes varied from 0.27 to 0.72 in Algeria [42], 0.6 to 0.9 in Nigeria [48] and 0.29 to 0.7 in Mexico [49]. The highest MAR index of 0.8 was recorded in phylogenetic group B2, followed by phylogroup D. These findings confirm significant antibiotic exposure in chickens, indicating a high contamination risk and underscoring the need for monitoring and legislation to prevent the spread of antibiotic-resistant bacteria within poultry products.

Integrons are critical components in the evolution of antibiotic resistance, playing a key role in the widespread development, acquisition, and propagation of antimicrobial resistance, as well as the evolution and adaption of bacteria [21]. In our study, 61.75 % of *E. coli* strains harboured class 1 integrons, mostly from broilers (40.43 %). This prevalence is higher than previously documented in Algeria [42] and Tunisia [50]. In contrast, only 2 strains (1.09 %) carried class 2 integrons, which is less than the prevalence reported in Tunisia [50]. One isolate carried both classes. According to the literature, class 1 integrons are the predominant type detected in gram-negative bacteria, while class 2 integrons are less commonly found in *E. coli* [51]. Further investigation is needed to understand the distribution and role of integrons and cassette arrays in spreading antimicrobial resistance. Understanding their prevalence is crucial for effective treatment and appropriate antibiotic use.

The widespread emergence of *E. coli* producers ESBL in poultry is increasingly recognised as a significant public health issue due to the potential for zoonotic transmission via the food chain and close contact with birds and environmental exposure [52]. Seventeen *E. coli* strains (4.7 %) were ESBL producers and carried several beta-lactamase genes, specifically bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$ and $bla_{CMY\text{-}2}$ genes (Table 2). This contrasts with 10.8 % of ESBL *E. coli* in Nigeria harbouring bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ [53].

The genes bla_{TEM} and bla_{SHV} were detected in 79.61 % and 7.89 % of amoxicillin-resistant isolates, respectively. Nine bla_{TEM} isolates were ESBL/pAmpC, and all bla_{SHV} were ESBL except one. According to Langata et al. [54], amoxicillin-resistant *E. coli* displayed bla_{TEM} (46 %) and bla_{SHV} was not detected. The presence of these ARGs in reared chicken is a major threat to infections. In Spain, *E. coli* identified in poultry products carried 100 % bla_{TEM} genes [55]. The $bla_{CTX\text{-}M}$ gene was found in 94.11 % of the third-generation-cephalosporin-resistant strains, while one cefoxitin-resistant isolate carried the $bla_{CMY\text{-}2}$ gene. This is higher

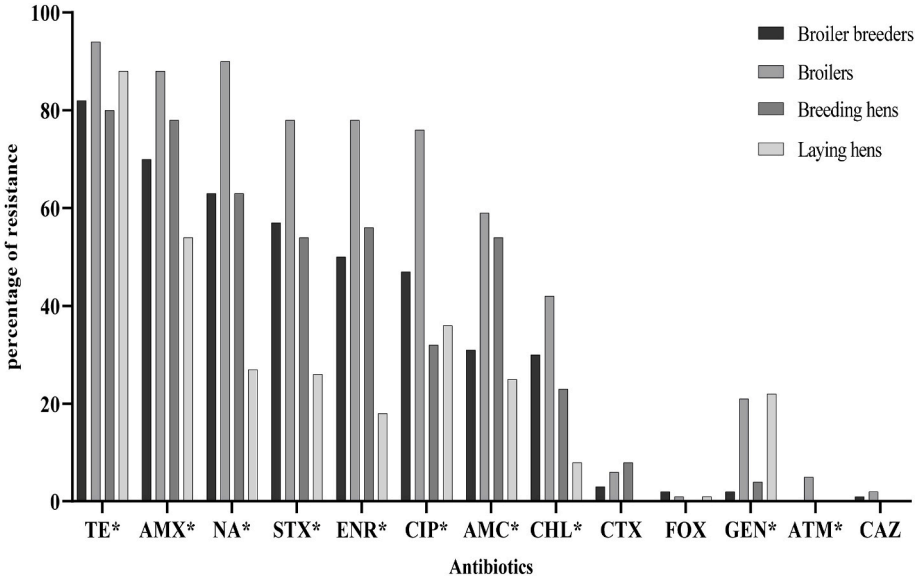


Fig. 7. Percentage of antibiotic resistance in faecal *E. coli* strains from broilers (n = 151), broiler breeders(n = 91), laying hens (n = 72) and breeding hens (n = 46). Abbreviation: TE-tetracycline; AMX-amoxicillin; CIP-ciprofloxacin; ENR-enrofloxacin; NA-nalidixic acid; STX-sulfamethoxazole; CHL-chloramphenicol; CTX-cefotaxime; AMC- amoxicillin/clavulanic acid; FOX-cefoxitin; GN-gentamicin; ATM-aztreonam; CAZ-ceftazidime. No resistance for imipenem was identified among the isolates. (*): *p*-value <0.05.

than the findings of Vogt et al. [56], who reported 79.4 % bla_{CTX-M} in 3 GC-R-Ec from chicken retail meat in Switzerland, with 19.2 % carrying bla_{CMY} and 1.5 % bla_{SHV}.

The bla_{CTX-M} gene had the highest detection frequency among ESBL isolates. Sequence analysis showed diversity within the $bla_{CTX-M-1}$ group, with the $bla_{CTX-M-15}$ type being the most prevalent at 68.75 %, compared to the $bla_{CTX-M-1}$ type at 31.25 %. This resistance was observed at various levels of the poultry production pyramid, including broiler breeders, broilers, and breeding hens, suggesting potential vertical transmission of resistance through contaminated eggs [57] and raising concerns about possible food chain contamination during the slaughtering process. No ESBL/pAmpC-producing *E. coli* were detected in laying hens samples. The high occurrence of the $bla_{CTX-M-15}$ gene aligns with the findings by Nossair et al. [58] in Egypt. The bla_{CTX-M} variant was previously identified in clinical *E. coli* samples from Algeria; the $bla_{CTX-M-15}$ from turkey [59], $bla_{CTX-M-1}$ from broilers [35,40,42] and $bla_{CTX-M-1-15-55}$ in retail chicken liver [60]. This study is the first to report $bla_{CTX-M-15}$ and $bla_{CTX-M-1}$ in faecal *E. coli* from broiler breeders and breeding hens in Eastern Algeria. The CTX-M-15 and CTX-M-1 are widely reported CTX-M enzymes found in different environments worldwide [61]. In Asia, CTX-M-15 and CTX-M-14 are the most commonly found ESBLs in poultry [15]. In North Africa and the Middle East, CTX-M-15 is the predominant ESBL type [62], which could contribute to the spread of this gene to humans. The phylogenetic tree (Fig. 3) illustrates the genetic relationships among current CTX-M types from this investigation and other previously published CTX-M sequences, showing a high degree of genetic similarities with 99 % identity.

The bla_{CMY-2} gene was identified in one *E.coli* isolate, which produced ESBL/pAmpC and was isolated from broilers, similar to the findings of Saidani et al. [63] in Tunisia. In both human and veterinary medicine, bla_{CMY-2} has been identified in the literature as the most common pAmpC gene [15].

Additional genes encoding antibiotic resistance, mainly sulfonamide resistance genes (*sul1* and *sul2*), tetracycline resistance genes (*tetA* and *tetB*) and quinolone resistance genes (*qnrB* and *qnrS*) were identified in 15 ESBL strains. Among these, one isolate was ESBL/pAmpC. The presence of these additional resistance genes leading to multi-antibiotic resistance was also confirmed by others as well [40,42,59,60]. The bla_{CTX-M} was found to coexist with 13 *qnrS*-positive isolates. Moreover, nine *E. coli* strains producing ESBL carried class 1 integrons, while one ESBL/pAmpC *E. coli* isolate carried class 2 integrons.

Escherichia coli has been demonstrated as a significant source for genes encoding antimicrobial drug resistance, as our strains exhibited a diversity of ARGs. The *tetA* gene was present in 62.11 % and *tetB* in 18.01 % of tetracycline-resistant strains. Our results confirmed that the *tetA* gene occurred more frequently than other tetracycline determinants among *E. coli* strains, consistent with the previous reports from Algeria [59,60] and in other countries [39]. In Iran Seifi and Khoshbakht [64], reported a 73 % resistance rate to tetracycline in *E. coli* from broiler flocks, with 46 % and 41 % containing *tetA* and *tetB* genes, respectively.

Sulfonamide resistance is common in bacteria from farm animals, typically due to the acquisition of genes *sul1*, *sul2*, and *sul3* [65]. Consistent with our result, the *sul1* gene (55.83 %) was more widely distributed in avian *E. coli* than *sul2* (26.67 %). These findings align with [36], who reported *sul1* as the most prevalent at 42.85 %, followed by *sul2* at 38.09 %. However, these results differ from those of [42,66], who reported a wider dissemination of the *sul2* gene in China and Algeria, respectively.

The *catA1* gene was detected in 13 (24.07 %) resistant isolates, two of which were ESBL. The study conducted in Djelfa by Chenouf et al. [60] was the only investigation to our knowledge conducted in broiler livers destined for human consumption, where they detected the chloramphenicol acetyltransferase resistance gene in three strains.

The aminoglycoside modifying enzyme *aac(6′)-Ib* gene was detected in three broilers strains in combination with other resistance genes (*tetA*, *qnrS*, *sul1*, *sul2,* bla_{TEM} and *intI1*). No *aac(6')-Ib* gene was identified by Belmahdi et al. [40] in broilers in Bejaia City (East of Algeria) or by Messaili et al. [42] in central Algeria.

The most common PMQR gene in this investigation was *qnrS*, similar to findings by Zou et al. [37] in China, which differed from those found in humans [67]. The *qnrA* gene was not detected in any previous Algerian investigations involving *E. coli* collected from laying hens, making it the first detection in this study.

In some resistant *E. coli* isolates, AMR genes were not detected, likely due to the limited number of genes investigated. These strains may contain genes not examined in our study. Further investigation is required.

Phylogenetic analysis of the 80 *E. coli* isolates showed that 60 % corresponded to phylogroups A and B1, consistent with previous research [42,63], mostly from laying and breeding hens. As previously reported, Phylogroups A and B1 are primarily composed of commensal *E. coli* strains [22], followed by phylogroups B2 and D, which comprised 40 %, predominantly found in broilers and broiler breeders. Most ESBL/pAmpC-producing *E. coli* strains were associated with phylogroups D (n = 8), followed by B2 (n = 4), B1 (n = 3) and A (n = 2). Literature indicates that phylotypes B2 and D are pathogenic *E. coli* [23] and most commonly found in human urinary tract infections [68]. It was proposed that poultry serves as one of the main reservoirs for antimicrobial-resistant *E. coli* associated with human urinary tract infections "UTIs".

5. Conclusion

This research, the first in Algeria, provides detailed information about the faecal carriage of healthy poultry, including broilers, broiler breeders, laying and breeding hens. Our findings suggest that commensal *E. coli* isolated from the cloaca of apparently healthy poultry may represent zoonotic transmission routes of antimicrobial resistance to humans, potentially mediated by integrons. Additionally, poultry manure used as a fertiliser represents a potential reservoir and transmission pathway for multidrug-resistant ESBL/pAmpC strains, posing a critical One Health challenge that intersects human, animal health and the environment. Finally, we report that 78.06 % of *E. coli* isolates were MDR, with 71.67 % having a MAR index above 0.2. All ESBL-producing *E. coli* contained at least one β-lactamase resistance gene, with most harbouring the $bla_{CTX-M-15}$ and $bla_{CTX-M-1}$ genes.

A rigorous epidemiological study is needed in Algeria to evaluate the diversity of *E. coli* across all poultry pyramids, from grandparents stocks to the final poultry product (eggs/white meat), as well as poultry manure used by agriculture as an excellent fertiliser. This will help analyse the occurrence of AMR and MDR genes in the chicken microbiota and develop alternatives to antibiotics and breeding strategies for poultry with zero antibiotic use in the future.

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CRediT authorship contribution statement

Hafsa Akkari: Writing – review & editing, Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Nouzha Heleili:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Osman Birol Ozgumus:** Validation, Supervision, Methodology, Data curation, Conceptualization. **Manel Merradi:** Visualization. **Ahu Reis:** Data curation. **Ammar Ayachi:** Supervision, Methodology. **Neslihan Akarsu:** Data curation. **Enis Fuat Tufekci:** Software, Formal analysis, Validation. **Ali Osman Kiliç:** Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2024.106973.

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