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Molecular characterization and prevalence of β-lactamase-producing *Enterobacterales* in livestock and poultry slaughterhouses wastewater in Iran

Mehran Sardari[a], Meysam Manouchehrifar[b], Kamal Hasani[c], Nasrin Habibzadeh[a], Hadi Peeri Doghaheh[a], Taher Azimi[d] and Mohsen Arzanlou [a,e,*]

[a] Department of Microbiology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran
[b] Deputy of Food and Drug, Ardabil University of Medical Sciences, Ardabil, Iran
[c] Department of Environmental Health Engineering, School of Public Health, Ardabil University of Medical Sciences, Ardabil, Iran
[d] Department of Bacteriology and Virology, Shiraz University of Medical Sciences, Shiraz, Iran
[e] Zoonoses Research Center, Ardabil University of Medical Sciences, Ardabil, Iran
*Corresponding author. E-mail: m.arzanlou@arums.ac.ir; arzanlo53@gmail.com

MA, 0000-0002-4233-9497

ABSTRACT

Beta-lactamase-producing *Enterobacterales* bacteria cause severe hard-to-treat infections. Currently, they are spreading beyond hospitals and becoming a serious global health concern. This study investigated the prevalence and molecular characterization of extended-spectrum β-lactamase and AmpC-type β-lactamase-producing *Enterobacterales* (ESBL-PE, AmpC-PE) in wastewater from livestock and poultry slaughterhouses in Ardabil, Iran. A total of 80 *Enterobacterales* bacteria belonging to 9 species were identified. Among the isolates, *Escherichia coli* ($n = 21/80$; 26.2%) and *Citrobacter* spp. ($n = 18/80$; 22.5%) exhibited the highest frequency. Overall, 18.7% ($n = 15/80$) and 2.5% ($n = 2/80$) of *Enterobacterales* were found to be ESBL and AmpC producers, respectively. The most common ESBL producer isolates were *E. coli* ($n = 9/21$; 42.8%) and *Klebsiella pneumoniae* ($n = 6/7$; 85.7%). All AmpC-PE isolates belonged to *E. coli* strains ($n = 2/21$; 9.5%). In this study, 80% of ESBL-PE and 100% of AmpC-PE isolates were recovered from poultry slaughterhouse wastewater. All ESBL-PE and AmpC-PE isolates were multidrug-resistant. In total, 93.3% of ESBL-PE isolates harbored the $bla_{CTX\text{-}M}$ gene, with the $bla_{CTX\text{-}M\text{-}15}$ being the most common subgroup. The emergence of ESBL-PE and AmpC-PE in wastewater of food-producing animals allows for zoonotic transmission to humans through contaminated food products and contaminations of the environment.

Key words: *Enterobacterales*, antibiotic resistance, extended-spectrum β-lactamases, farm animals, wastewater

HIGHLIGHTS

- Epidemiology of ESBL and AmpC-producing *Enterobacterales* in Iranian slaughterhouses wastewater was investigated.
- 18.7% of *Enterobacterales* were ESBL producers, mostly from poultry wastewater.
- 2.5% of *Enterobacterales* were AmpC producers, all from poultry wastewater.
- *E. coli* and *Klebsiella pneumoniae* were the predominant ESBL producer species.
- $bla_{CTX\text{-}M\text{-}15}$ was the most prevalent ESBL-encoding gene.

1. BACKGROUND

Enterobacterales constitute the main group of Gram-negative bacteria (GNB) that cause several different nosocomial and community-acquired diseases (González 2022). The bacteria included in the *Enterobacterales* cause severe infections, such as pneumonia, bloodstream infections, wound infections, urinary tract infections, gastroenteritis, and meningitis (González 2022). The use of various antibiotics especially β-lactam agents is the mainstay treatment of these infections (Arzanlou *et al.* 2017). However, in recent years, the emergence of antibiotic resistance among *Enterobacterales* has complicated the treatment of infections all over the world (Lavakhamseh *et al.* 2016; De Angelis *et al.* 2020). It is demonstrated that overuse or misuse of different antibiotics in medical clinics and animal husbandry has led to the emergence and dissemination of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), which can be transmitted in animals and humans (Nabovati *et al.* 2021; Rahman & Hollis 2023).

Given that various β-lactam agents, especially extended-spectrum cephalosporins and carbapenems are used in human and veterinary medicine; therefore, it is predictable that high-level β-lactam-resistant *Enterobacterales* have emerged (Arzanlou *et al.* 2017; Habibzadeh *et al.* 2022). β-lactam agents bind to the penicillin-binding proteins of the bacterial cell wall and inhibit the synthesis of peptidoglycan, which leads to the lysis of the bacteria cells (Arzanlou *et al.* 2017). The GNB belonging to the *Enterobacterales* become resistant against β-lactams through multiple mechanisms including the production of various β-lactamase enzymes such as plasmid-mediated AmpC (pAmpC), extended-spectrum β-lactamases (ESBLs), and metallo-β-lactamase (MBL) (Dantas Palmeira & Ferreira 2020). Based on the World Health Organization (WHO) reports, emerging ESBL-producing *Enterobacterales (ESBL-PE)* are considered the most serious and life-threatening threats of the 21st century (Tacconelli *et al.* 2018). AmpC β-lactamases are enzymes encoded on the chromosomes or plasmids and mediate resistance to cephalosporins, penicillins, β-lactamase inhibitor combinations, and cephamycins (Tamma *et al.* 2023). These two enzymes are the most important resistance mechanisms against β-lactams among *Enterobacterales* members that can hydrolyze various β-lactam antibiotics (Sheng *et al.* 2013). During the last 20 years, ESBL-PE and AmpC-producing *Enterobacterales* (AmpC-PE) have emerged in human health care and animals worldwide. Depending on geographical regions, the global prevalence of ESBL-PE isolates varies widely (2–70%) (Telling *et al.* 2020). It is revealed that the meat, digestive tract of domestic livestock and poultry, and the wastewater of slaughterhouses are important reservoirs of ESBL-/AmpC-PE isolates (Pormohammad *et al.* 2019; Gregova & Kmet 2020). These resources may lead to the dissemination of ABR *Enterobacterales* into the environment and serve as a possible source of human colonization (Wu *et al.* 2009). Therefore, surveillance for the presence of β-lactamase-producing *Enterobacterales* in the wastewater of livestock and poultry slaughterhouses is important. However, there is limited data on antibiotic resistance profiles of *Enterobacterales* in wastewater samples from livestock and poultry slaughterhouses wastewater in Iran.

This research aimed to achieve an understanding of the epidemiology and molecular characterization of ESBL-PE and AmpC-PE isolates from livestock and poultry slaughterhouse wastewater in Iran.

2. METHODS

2.1. Sample collection, processing, and bacterial isolates

In the current study, a total of 12 raw sewage samples were collected in 500 mL sterile bottles from livestock and poultry slaughterhouses in Ardabil, Iran between June 2020 and June 2021. The samples were transported to the microbiology laboratory in ice-cold containers and kept at 4 °C until microbiological analysis. The analysis was carried out within 2 h after sample collection.

For isolation of *Enterobacterales*, 5 mL of raw sewage samples were cultured into 5 mL double concentration *Enterobacterales* enrichment (EE) broth (Merck, Germany). The cultures were incubated at 37 °C for 24 h, then 100 µL of each enriched EE-broth media was subcultured onto MacConkey agar (Merck, Germany) plates and additionally incubated at 37 °C overnight. Raised colonies were examined based on Gram staining and colonial characteristics. Three colonies with the same appearance per colony morphology in each sample were selected and isolated on nutrient agar (Merck, Germany) plates (Ojer-Usoz *et al.* 2014; Miyagi & Hirai 2019). Definitive identification of the genus and species of *Enterobacterales* members was conducted using conventional biochemical tests. Selected bacteria were isolated on Trypticase Soy Agar (Merck, Germany) and initially screened using three tests: the oxidase test, the oxidative/fermentative glucose test (OF medium), and the nitrate reduction test (Nitrate Broth). Bacteria identified as oxidase-negative, nitrate-positive, and glucose fermenters underwent further characterization to the species level using a battery of biochemical tests based on standard manuals (Hasani *et al.* 2023; Mahon *et al.* 2014). This battery included tests for carbohydrate fermentation (lactose fermentation, H_2S, and gas production on Triple Sugar Iron Agar), enzyme and metabolite production (indole and motility on Sulfide Indole Motility medium, urease on urease agar, citrate utilization on Simmons Citrate Agar, Voges-Proskauer and methyl red tests on MR/VP medium), amino acids (lysine, arginine, and ornithine) decarboxylase test, and additional tests like phenylalanine deaminase on Phenylalanine Agar. All differential culture media were purchased from Himedia Laboratories Pvt. Ltd, India. The validity of the biochemical tests was ensured by incorporating established reference strains: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Enterobacter aerogenes* ATCC 13049, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, and *Proteus mirabilis* ATCC 43071.

To prevent biases that may have been introduced by the enrichment process, duplicate isolates of each species and each sample showing the same phenotypic and genotypic antimicrobial resistance characteristics were excluded from the study.

The isolates were stored in Tryptic Soy Broth (TSB; Merck, Germany) with 15% glycerol in the deep freezer (−70 °C) until use.

2.2. Antimicrobial susceptibility testing

Antibiotic susceptibility of the isolates was determined by the Kirby–Bauer disk diffusion method (DDM) on Mueller Hinton agar (Merck, Germany). Ampicillin (AM,10 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), cefepime (FEP, 30 μg), imipenem (IMP, 10 μg), gentamicin (GM,10 μg), amikacin (AN, 30 μg), nitrofurantoin (FM, 300 μg), ciprofloxacin (CP, 5 μg), nalidixic acid (NA, 30 μg), cephalexin (CN, 30 μg), trimethoprim-sulfamethoxazole (SXT, 1.25 + 23.75 μg), and tetracycline (TET, 30 μg) were used. The antibiotic susceptibility testing was carried out and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI 2023). The *E. coli* ATCC 25922 was used as a quality control strain for DDM. The bacteria that were resistant to at least one antibiotic among at least three or more drug categories were considered multidrug-resistant (MDR).

2.3. Phenotypic screening for ESBL-PE

Initially, all the *Enterobacterales* isolates were subjected to susceptibility testing using CTX (30 μg) and CAZ (30 μg) disks. The isolates with reduced inhibition zone diameter for CTX (≤27 mm) and/or CAZ (≤22 mm) were considered as suspected ESBL-producing isolates. For definitive identification of ESBL-producing isolates, double-disk synergy test (DDST) was used as described with CLSI (CLSI 2023). The CAZ and CAZ/clavulanic acid (30 μg/10 μg, PadtanTeb, Iran), CTX, and CTX/clavulanic acid (30 μg/10 μg, PadtanTeb, Iran) disks were placed on the cultured Muller–Hinton agar plate with 30 mm apart from each other and incubated at 37 °C overnight. Isolates were considered ESBL producers when the inhibition zone diameter produced by the combined effects of either CAZ- or CTX-plus clavulanic acid disks was at least 5 mm larger than that produced by either CAZ or CTX disks individually. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were used as the ESBL positive and negative controls, respectively.

2.4. Phenotypic screening for AmpC-PE

The *Enterobacterales isolates* with the following characteristics in the DDM test were selected for phenotypic screening for AmpC β-lactamases production: (i) the isolates that were resistant to CAZ (30 μg) and/or CTX (30 μg), (ii) the isolates which were susceptible to CPM (30 μg), and (iii) the isolates showing zone of inhibition less than or equal to 18 mm to cefoxitin (30 μg). AmpC production was confirmed by disk potentiation test (DPT) using cefoxitin (30 μg) and cefoxitin/boronic acid (30 μg/400 μg) disks as described by others (Pitout *et al.* 2010). Isolates were considered AmpC β lactamase producers when the diameter of the inhibition zone produced by the combined effects of cefoxitin plus boronic acid was at least 5 mm larger than that produced by the cefoxitin disk alone.

2.5. Molecular detection of AmpC β-lactamases- and ESBL-encoding genes

Genomic DNA was extracted using the boiling method (Habibzadeh *et al.* 2022; Hasani *et al.* 2023), and extracted DNA was preserved at −80 °C until further use.

ESBL-encoding genes including bla_{TEM}, bla_{SHV}, bla_{OXA-1}, and bla_{CTX-M} ($bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-3}$, $bla_{CTX-M-8}$, $bla_{CTX-M-9}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, $bla_{CTX-M-25}$) were detected in all ESBL-producing isolates using a conventional PCR assay. The sequence of all specific primers (Ruppé *et al.* 2009; Afzali *et al.* 2015) used for PCR reaction is shown in Table 1. The PCR reaction was performed in a final volume of 25 μL using 12.5 μL of Premix Taq® mix (CinnaGen, Tehran, Iran), 1 μL (5 μg) of template DNA, l μL (10 pmol) of each forward and reverse primers, and 9.5 μL of nuclease-free water. Amplification was performed using a thermocycler (Bio-Rad, Malaysia) with the following conditions: one cycle of 95 °C for 5 min, followed by 35 cycles of 94 °C for 55 s, 52–60 °C (Table 1) for 1 min, and 72 °C for 55 s with a final extension at 72 °C for 8 min. ESBL variants of bla_{TEM} and bla_{SHV} genes were identified by sequencing and alignment of the PCR products (Microsynth Company, Switzerland). Sequences were aligned and analyzed using the BLAST program available at the National Center for Biotechnology Information (NCBI).

The frequency of AmpC β-lactamase encoding genes (bla_{FOX}, bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC}, and bla_{CMY}) among AmpC-PE isolates was determined by multiplex PCR method using specific primers (Table 1). DNA amplification was performed following a previously published protocol with some modifications (Pérez-Pérez & Hanson 2002). Briefly, 25 μL of Premix Taq® mix (CinnaGen, Tehran, Iran) was combined with 1 μL (5 μg) of template DNA, 1 μL each of forward and reverse primers (10 pmol each), and nuclease-free water to reach a final volume of 50 μL. Amplification was carried out

Table 1 | PCR primers used for amplification of ampC β-lactamases- and ESBL-encoding genes

Primer name	Sequence (5′ → 3′)		PCR product size (bp)
bla_{TEM}	F	TCGGGGAAATGTGCGCG	1,100
	R	TGCTTAATCAGTGAGGCACC	
bla_{SHV}	F	TTATCTCCCTGTTAGCCACC	1,100
	R	GATTTGCTGATTTCGCTCGG	
$bla_{CTX\text{-}M}$	F	ATGTGCAGYACCAGTAA	605
	R	CCGCRATATGRTTGGTGGTG	
$bla_{OXA\text{-}1}$	F	TATCAACTTCGCTATTTTTTTA	700
	R	TTTAGTGTGTTTAGAATGGTGAC	
$bla_{CTX\text{-}M\text{-}1}$	F	GGTTAAAAAATCACTGCGTC	864
	R	TTGGTGACGATTTTAGCCGC	
$bla_{CTX\text{-}M\text{-}2}$	F	ATGATGACTCAGAGCATTCG	390
	R	TGGGTTACGATTTTCGCCGC	
$bla_{CTX\text{-}M\text{-}3}$	F	AATCACTGCGCCAGTTCACGCT	690
	R	GAACGTTTCGTCTCCCAGCTGT	
$bla_{CTX\text{-}M\text{-}8}$	F	CACACGAATTGAATGTTCAG	600
	R	TCACTCCACATGGTGAGT	
$bla_{CTX\text{-}M\text{-}9}$	F	ATGGTGACAAAGAGAGTGCA	520
	R	CCCTTCGGCGATGATTCTC	
$bla_{CTX\text{-}M\text{-}14}$	F	TACCGCAGATAATACGCAGGTG	430
	R	CAGCGTAGGTTCAGTGCGATCC	
$bla_{CTX\text{-}M\text{-}15}$	F	AGAATAAGGAATCCCATGGTT	290
	R	ACCGTCGGTGACGATTTTAG	
$bla_{CTX\text{-}M\text{-}25}$	F	CCAGCGTCAGATTTTTCAGG	340
	R	ACGCTCAACACCGCGATC	
bla_{FOX}	F	AACATGGGGTATCAGGGAGATG	190
	R	CAAAGCGCGTAACCGGATTGG	
bla_{MOX}	F	GCTGCTCAAGGAGCACAGGAT	520
	R	CACATTGACATAGGTGTGGTGC	
bla_{CIT}	F	TGGCCAGAACTGACAGGCAAA	462
	R	TTTCTCCTGAACGTGGCTGGC	
bla_{DHA}	F	AACTTTCACAGGTGTGCTGGGT	405
	R	CCGTACGCATACTGGCTTTGC	
bla_{ACC}	F	AACAGCCTCAGCAGCCGGTTA	346
	R	TTCGCCGCAATCATCCCTAGC	
bla_{EBC}	F	TCGGTAAAGCCGATGTTGCGG	302
	R	CTTCCACTGCGGCTGCCAGTT	
bla_{CMY}	F	ATGATGAAAAAATCGTTATGCTGC	1,030
	R	GCTTTTCAAGAATGCGCCAGG	

under the following conditions: initial denaturation at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 1 min. A final extension step at 72 °C for 7 min was included. PCR products were separated on a 1.5% agarose gel, stained with DNA-safe stain (Sinaclon Co., Iran), and photographed under UV light using a gel documentation system (UVitec-England). Genomic DNA from ESBL-PE and AmpC-PE isolates from the authors' previous studies containing target genes were used as the positive control (Habibzadeh *et al.* 2022; Hasani *et al.* 2023) and *Staphylococcus aureus* ATCC 25212 was used as a negative control for PCR assays. Additionally, the 16 s rRNA gene amplification was used as a positive control in PCR testing to ensure that any amplification failure with the specific primers was not due to poor DNA quality or to failure of the PCR itself.

2.6. Statistical analysis

All data were included in the statistical package SPSS v.23.0 (SPSS Inc., Chicago, IL, USA), the difference in frequency of ESBL-PE and AmpC-PE isolates in livestock and poultry slaughterhouses wastewater was analyzed using the chi-squared test. A p-value of ≤ 0.05 was considered statistically significant.

3. RESULTS

3.1. Distribution of bacterial isolates

A total of 80 non-duplicate *Enterobacterales* isolates including 37 (46.2%) and 43 (54%) were collected from livestock and poultry slaughterhouse wastewater, respectively. The frequency of *Enterobacterales* in livestock and poultry slaughterhouse wastewater samples is shown in Table 2. Overall, *E. coli* ($n = 21/80$; 26.2%), *Citrobacter* spp. ($n = 18/80$; 22.5%), *Proteus mirabilis* (*P. mirabilis*) ($n = 13/80$; 16.2%), *Proteus vulgaris* (*P. vulgaris*) ($n = 8/80$; 10%), *K. pneumoniae* ($n = 7/80$; 8.7%), *Hafnia* spp. ($n = 5/80$; 6.2%), *Yersinia enterocolitica* (*Y. enterocolitica*) ($n = 5/80$; 6.2%), *Providencia* spp. ($n = 2/80$; 2.5%), and *Enterobacter* spp. ($n = 1/80$; 1.2%) were the most prevalent *Enterobacterales* species found in wastewater samples, respectively. Among *Enterobacterales* isolated from livestock slaughterhouse wastewater samples, *E. coli* ($n = 9/37$; 24.3%) and *Citrobacter* spp. ($n = 9/37$; 24.3%) were the most frequent organisms identified. On the other hand, *E. coli* ($n = 12/43$; 27.9%) was the most frequently isolated pathogen in poultry slaughterhouse wastewater samples. *Hafnia* spp. was not isolated from poultry wastewater samples.

3.2. Distribution of ESBL-PE and AmpC-PE isolates

The frequency of ESBL-PE and AmpC-PE isolates is shown in Table 3. The results of the DDST test showed that 18.7% ($n = 15/80$) of isolates were ESBL producers. Among ESBL-PE, 80% ($n = 12/15$) of isolates were isolated from poultry slaughterhouse wastewater, and 20% ($n = 3/15$) of isolates were obtained from livestock slaughterhouse wastewater ($p < 0.05$). ESBL production was observed in *E. coli* ($n = 9/21$; 42.8%) and *K. pneumoniae* ($n = 6/7$; 85.7%) isolates.

The results of the DPT test showed that 2.5% ($n = 2/80$) of isolates were AmpC β-lactamase producers. All AmpC-PE isolates were obtained from poultry slaughterhouse wastewater samples. Moreover, AmpC production was restricted to *E. coli* ($n = 2/21$; 9.5%) isolates. No isolate produced ESBL- and AmpC-β-lactamase simultaneously.

3.3. Antibiotic resistance

The antibiotic resistance profile of the isolates to commonly used antimicrobials is shown in Figure 1. In general, *Enterobacterales* isolates showed the highest rate of resistance to ampicillin ($n = 70/80$; 87.5%), cephalexin ($n = 49/80$; 61.2%, and TET ($n = 51/80$; 63.7%), respectively. While high susceptibility was observed against amikacin ($n = 78/80$; 97.5%) and gentamicin ($n = 77/80$; 96.2%) in *Enterobacterales* isolates.

Table 2 | The frequency of *Enterobacterales* spp in wastewater from livestock and poultry slaughterhouses

	Wastewater		
Bacteria	**Livestock slaughterhouse** ***N*** **= 37,** ***n*** **(%)**	**Poultry slaughterhouse** ***N*** **= 43,** ***n*** **(%)**	**Total** ***N*** **= 80,** ***n*** **(%)**
E. coli	9 (24.3)	12 (27.9)	21 (26.2)
Citrobacter spp.	9 (24.3)	9 (20.9)	18 (22.5)
P. mirabilis	6 (16.2)	7 (16.3)	13 (16.25)
P. vulgaris	3 (8.1)	5 (11.6)	8 (10)
K. pneumoniae	1 (2.7)	6 (13.9)	7 (8.7)
Y. enterolitica	3 (8.1)	2 (4.6)	5 (6.2)
Hafnia spp.	5 (13.5)	0 (0)	5 (6.2)
Providencia spp.	0 (0)	2 (4.6)	2 (2.5)
Enterobacter spp.	1 (2.7)	0 (0)	1 (1.2)

Table 3 | The frequency of ESBL and AmpC-producing *Enterobacterales in* wastewater from livestock and poultry slaughterhouses

Bacteria	Phenotypic resistance	Wastewater		Total *n* (%)*
		Poultry slaughterhouse	Livestock slaughterhouse	
E. coli, $N = 21$	ESBL, $N = 9$, *n* (%)	6 (66.6)	3 (33.3)	9 (42.8)
	AmpC, $N = 2$, *n* (%)	2 (100)	0 (0)	2 (9.5)
	Non ESBL/AmpC $N = 10$, *n* (%)	4 (40)	6 (60)	10 (47.6)
K. pneumoniae, $N = 7$	ESBL, $N = 6$, *n* (%)	6 (100)	0 (0)	6 (85.7)
	Non ESBL/AmpC, $N = 1$, *n* (%)	0 (0)	1 (100)	1 (14.3)
Citrobacter spp., $N = 18$	Non ESBL/AmpC, *n* (%)	9 (50)	9 (50)	18 (100)
P. mirabilis, $N = 13$	Non ESBL/AmpC, *n* (%)	7 (54)	6 (46)	13 (100)
P. vulgaris, $N = 8$	Non ESBL/AmpC, *n* (%)	5 (62.5)	3 (37.5)	8 (100)
Hafenia spp., $N = 5$	Non ESBL/AmpC, *n* (%)	0 (0)	5 (100)	5 (100)
Providencia spp., $N = 2$	Non ESBL/AmpC, *n* (%)	2 (100)	0 (0)	2 (100)
Y. enterolitica, $N = 5$	Non ESBL/AmpC, *n* (%)	2 (40)	3 (60)	5 (100)
Enterobacter spp., $N = 1$	Non ESBL/AmpC, *n* (%)	0 (0)	1 (100)	1 (100)
Total, $N = 80$	ESBL, $N = 15$, *n* (%)	12 (80)**	3 (20)	15 (18.7)
	AmpC, $N = 2$, *n* (%)	2 (100)**	0 (0)	2 (2.5)
	ESBL/AmpC $N = 0$, *n* (%)	0 (0)	0 (0)	0 (0)
	Non ESBL/AmpC, $N = 63$, *n* (%)	29 (46)	34 (54)	63 (78.7)

*The percentages were derived from the total number of each bacterium shown in the first column on the left side.
**Statistically significant ($p \leq 0.05$).

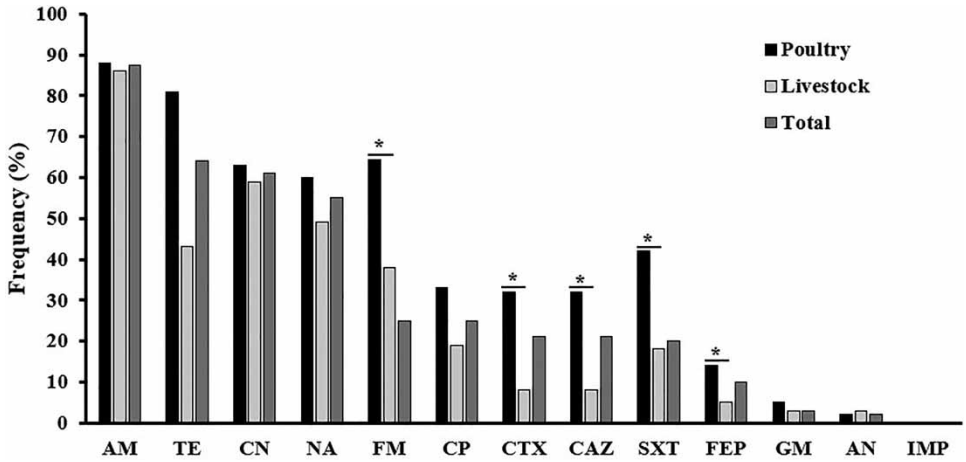

Figure 1 | The pattern of antibiotic resistance among *Enterobacterales* isolates in wastewater samples from livestock and poultry slaughterhouses. *Statistically significant ($p \leq 0.05$). AM, ampicillin; NA, nalidixic acid; CP, ciprofloxacin; AN, amikacin; CN, cephalexin; SXT, trimethoprim-sulfamethoxazole; TE, tetracycline; FM, nitrofurantoin; IMP, imipenem; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; GM, gentamicin.

In comparison, the resistance rate to CPM, CTX, CAZ, trimethoprim-sulfamethoxazole, and nitrofurantoin among isolates recovered from poultry slaughterhouse wastewater samples was significantly higher than the isolates recovered from livestock slaughterhouse wastewater samples ($p \leq 0.05$).

Results showed that 100% ESBL- and AmpC-PE isolates were MDR. At the same time, 47.5% ($n = 26/63$) of non-ESBL-/non-AmpC-PE isolates were MDR ($P \leq 0.05$).

The imipenem ($n = 12/12$; 100%), amikacin ($n = 11/12$; 92%), and gentamicin ($n = 10/12$; 83%) were the most effective antibiotics against ESBL-PE species isolated from poultry slaughterhouse wastewater samples. ESBL-PE isolates from livestock slaughterhouse wastewater samples had higher susceptibility rates to imipenem, TET, NA, and trimethoprim-sulfamethoxazole at 100% ($n = 3/3$), and to gentamicin and amikacin at 75% ($n = 2/3$) each. AmpC-PE isolates were more resistant than ESBL-PE isolates, but they were all susceptible to imipenem, CPM, gentamicin, and amikacin (Table 4).

Table 4 | The antibiotic resistance profile of the *Enterobacterales* isolates in wastewater samples from livestock and poultry slaughterhouses

Wastewater	Antibiotics		AM	CN	TE	NA	FM	CP	SXT	IPM	CAZ	CTX	FEP	GM	AN
Poultry slaughterhouse $N = 43$	ESBL, $N = 12$,, n (%)	S	0(0)	0(0)	3(25)	6(50)	2(17)	7(58)	4(33)	12(100)	0 (0)	0 (0)	6(50)	10(83)	11(92)
		I + R	12(100)	12(100)	9(75)	6(50)	10(83)	5(42)	8(67)	0 (0)	12(100)	12(100)	6(50)	2(17)	1(8)
	AmpC, $N = 2$, n (%)	S	0(0)	0(0)	0(0)	0(0)	0(0)	2(50)	0 (0)	2(100)	0 (0)	0 (0)	2(100)	2(100)	2(100)
		I + R	2(100)	2(100)	2(100)	2(100)	2(100)	1(50)	2(100)	0(0)	2(100)	2(100)	0(0)	0(0)	0(0)
Livestock slaughterhouse $N = 37$	Non ESBL/AmpC $N = 29$, n (%)	S	5(17)	16(55)	5(17)	11(38)	13(45)	21(72)	21(72)	29(100)	29(100)	29(100)	29(100)	29(100)	29(100)
		I + R	24(83)	13(45)	24(83)	18(62)	16(55)	8(28)	8(28)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	ESBL, $N = 3$, n (%)	S	0(0)	0(0)	3(100)	3(100)	0(0)	1(33)	3(100)	3(100)	0(0)	0(0)	1(33)	2(67)	3(67)
		I + R	3(100)	3(100)	0 (0)-	0 (0)	3(100)	2(67)	0 (0)	0 (0)	3(100)	3(100)	2(67)	1(33)	1(33)
	Non ESBL/AmpC $N = 34$, n (%)	S	5(15)	15(44)	18(53)	16(47)	23(68)	29(85)	27(79)	34(100)	34(100)	34(100)	34(100)	34(100)	34(100)
		I + R	29(85)	19(56)	16(47)	18(53)	11(32)	5(15)	7(21)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

S, susceptible; I, intermediate resistant; R, resistance.

3.4. Characterization of ESBL and AmpC β-lactamase encoding genes

A conventional PCR and multiplex PCR assay were used to investigate the frequency of encoding genes in ESBL-PE and AmpC-PE isolates, respectively. The results are shown in Table 5. Screening for ESBL-encoding genes revealed that 100% ($n = 9/9$) and 83% ($n = 5/6$) of ESBL-producing *E. coli* and *K. pneumoniae* isolates harbored $bla_{CTX\text{-}M\text{-}1}$ gene, respectively. All of these isolates were positive for the $bla_{CTX\text{-}M\text{-}15}$ subgroup. ESBL-encoding $bla_{OXA\text{-}1}$ gene was identified in 16.7% ($n = 1/6$) of ESBL-producing *K. pneumoniae* isolates. 44% ($n = 4/9$) and 50% ($n = 3/6$) of ESBL-producing *E. coli* and *K. pneumoniae* isolates contained the $bla_{TEM\text{-}1}$ gene, respectively. However, the $bla_{TEM\text{-}1}$ gene does not code for an ESBL enzyme. Other ESBL-encoding genes ($bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}25}$, and bla_{SHV}) were not identified in this study.

AmpC β-lactamases genes, bla_{FOX} and bla_{CIT}, were detected among 100% ($n = 2/2$) of AmpC-producing *E. coli* isolates. Other AmpC β-lactamases genes (bla_{MOX}, bla_{DHA}, bla_{ACC}, $bla_{EBC,}$ and bla_{CMY} genes were not detected in this study.

4. DISCUSSION

Over the last decades, antimicrobials have been frequently used in poultry and livestock, resulting in the emergence and development of ABR bacteria (Pormohammad *et al.* 2019). The wastewater of farm animals and poultry may act as a reservoir of ESBL-PE and AmpC-PE isolates and dissemination of these bacteria to environments leads to the colonization of humans (Gregova & Kmet 2020).

There are limited data about the frequency, antimicrobial resistance profile, and characterization of *Enterobacterales* in wastewater samples from livestock and poultry slaughterhouses in Iran. To our knowledge, the current study is the first comprehensive research on the occurrence and characterization of ESBL-PE and AmpC-PE in the wastewater of livestock and poultry slaughterhouses in Iran.

Overall, the results of the present study showed that *E. coli* and *Citrobacter* spp. were the most commonly isolated organisms in wastewater samples from both livestock and poultry slaughterhouses. This finding is in agreement with the findings of several studies conducted by Adelowo *et al.* (2020) from Germany, Ye *et al.* (2018) from China, Savin *et al.* (2020) from Germany, and Montso *et al.* (2019) from South Africa, which stated that the prevalence of *E. coli* isolates among wastewater samples is high. However, our findings are not consistent with those of a previously published study from Germany (Savin *et al.* 2021), which reported that the *Acinetobacter calcoaceticus–baumannii* complex is frequently isolated from wastewater from a poultry slaughterhouse.

In general, bacteria belonging to the *Enterobacterales* family can colonize the human and animal intestinal tract and are considered a part of normal microbiota in healthy humans and animals (Homeier-Bachmann *et al.* 2021). Consequently, the occurrence of these bacteria in livestock and poultry slaughterhouse waste is expected. Nevertheless, due to the extensive use of antibiotics in animal farming, various types of ARB are increasingly identified in livestock and poultry waste globally (He *et al.* 2020). The contamination of slaughterhouse wastewater with ABR bacteria represents a potential risk to human health (Gregova & Kmet 2020).

The phenotypic evaluation of the prevalence of β-lactamase-producing *Enterobacterales* revealed that 60 and 40% of ESBL-PE were *E. coli* and *K. pneumoniae* isolates, respectively. The frequency of ESBL-producing *E. coli* and *K. pneumoniae*

Table 5 | The frequency of ESBL and AmpC β-lactamases-encoding genes among ESBL and AmpC-producing *Enterobacterales* in wastewater from livestock and poultry slaughterhouses

	ESBL-encoding genes			
Bacteria	$bla_{CTX\text{-}M}$, ***n*** **(%)**	bla_{SHV}, ***n*** **(%)**	bla_{TEM}, ***n*** **(%)**	$bla_{OXA\text{-}1}$, ***n*** **(%)**
E. coli, $N = 9$	9 (100)	0 (0)	0 (0)	0 (0)
K. pneumonia, $N = 6$	5 (83.3)	0 (0)	0 (0)	1 (16.7)
Total, $N = 15$	14 (93.3)	0 (0)	0 (0)	1 (6.7)
	AmpC β-lactamases-encoding genes			
Organism	bla_{CIT}, ***n*** **(%)**		bla_{FOX}, ***n*** **(%)**	
E. coli, $N = 2$	2 (100)		2 (100)	

isolates was 42.8 and 85.7%, respectively. These results are consistent with those of studies by Montso *et al.* (2019) in South Africa, Ye *et al.* (2018) in China, and Lim *et al.* (2015) in Korea. These previously published studies found that the frequency of ESBL-producing *E. coli* in the wastewater samples was 58.2, 41.8, and 39.7%, respectively. However, our findings were in contrast with those of a previous study from Slovakia, which reported that the prevalence of ESBL-producing *E. coli* isolates was 20.4% (Gregova & Kmet 2020).

In our study, a significant proportion of ESBL-PE isolates were obtained from poultry slaughterhouse wastewater. This observation suggests a potential link to the intensified use of antibiotics within the poultry industry in our region. Consequently, to prevent the development and spreading of ARB, adopting a One Health approach using suitable treatment methods, antibiotic resistance surveillance programs, and robust wastewater treatment processes are crucial needs in poultry farms.

Our analyses regarding the prevalence of beta-lactamase encoding genes among phenotypically resistant organisms showed that the $bla_{CTX\text{-}M\text{-}15}$ subgroup was prevalent among ESBL-producing *K. pneumoniae* and *E. coli* isolates (83 and 100%, respectively). Obtained results are in agreement with those of published studies by Gregova *et al.* (2021) from Slovakia, Franz *et al.* (2015) from the Netherlands, and Kaesbohrer *et al.* (2019) from Germany, which reported that the prevalence of $bla_{CTX\text{-}M}$ gene among ESBL-producing *K. pneumoniae* and *E. coli* isolates was high. On the other hand, these findings are in contrast to other studies performed worldwide reporting high rates of ESBL-PE harbored other ESBL/AmpC-encoding genes such as bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{EBC}, bla_{FOX}, and bla_{CIT} (Khan *et al.* 2020; Savin *et al.* 2021). In the last decades, bla_{TEM} and bla_{SHV} genes were the most prevalent ESBL-encoding genes. However, in recent years, studies revealed that the $bla_{CTX\text{-}M}$ gene has spread worldwide and is the most prevalent ESBL-encoding gene. In most cases, the $bla_{CTX\text{-}M}$ gene is located on large plasmids. Organisms harboring the $bla_{CTX\text{-}M}$ gene have been related to widespread resistance to different classes of antibiotics such as quinolones, aminoglycosides, trimethoprim/sulfamethoxazole, and TET (Abbassi *et al.* 2008; Sghaier *et al.* 2019). Among different $bla_{CTX\text{-}M}$ subgroups, the CTX-M-15 subgroup has a high frequency among ESBL-producing *E. coli*. Globally, CTX-M-15 is one of the most prevalent ESBL genotypes in humans and is related to hospital-acquired infections or community-acquired urinary tract infections (Dolejska *et al.* 2011). It is presumed that organisms harboring the CTX-M-15 subgroup have different virulence factors and are more virulent than other bacteria (Hassen *et al.* 2020). The potential link between human and animal CTX-M-15 beta-lactamase underscores the importance of a holistic approach to antimicrobial stewardship, considering both human health and animal welfare.

In Iran, like many developed and developing countries, wastewater generated from livestock and poultry farms and their slaughterhouses, hospitals, and abattoirs do not get suitable treatment and resistant bacteria are present in effluents from wastewater treatment plants discharged to the nearby rivers and streams. The release of untreated wastewater from slaughterhouses and farms to the rivers that contain MDR pathogenic bacteria may lead to the environmental pool of resistant pathogenic bacteria and antimicrobial resistance genes. Moreover, untreated wastewater can act as a source of infection with ABR bacteria (Dolejska *et al.* 2011). On the other hand, it is presumed that *Enterobacterales* isolates may horizontally transmit the ABR genes to other *Enterobacterales* in the wastewater and contribute to the emergence and development of MDR bacteria (Kiros & Workineh 2019).

5. CONCLUSION

Our study showed the presence of ABR bacteria in the wastewater of poultry and livestock slaughterhouses in Ardabil, Iran. This contamination poses a significant risk of zoonotic transmission to humans through the consumption of contaminated food products and the spread of bacteria into the environment. Moreover, it is revealed that GNB isolated from wastewater carries different resistance genes and they show various resistance profiles to commonly prescribed antibiotics. Results revealed that among the *Enterobacterales* family members, the ESBL and AmpC β-lactamases are currently on the increase, especially among *E. coli* and *K. pneumoniae* isolates. Therefore, it can be concluded that sufficient sanitation infrastructure and ABR surveillance programs are critically required in livestock and poultry farms and slaughterhouses.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical clearance and approval for the study were obtained from the Institutional Ethics Committee of the Ardabil University of Medical Sciences (IR. ARUMS.1398.369).

CONSENT FOR PUBLICATION

All authors have read and agreed to the published version of the manuscript.

AUTHOR CONTRIBUTIONS

M. S. developed the methodology, investigated the data, rendered support in formal analysis, and supported in original draft preparation. H. P. D. conceptualized the whole article, reviewed, and edited the article. M. M. investigated the data and rendered support in formal analysis. K. H. investigated the work. N. H. investigated the work. T. A. supported in original draft preparation. M. A. supervised the work, rendered support in project administration, and revised the manuscript.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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