Iranian Journal of Microbiology

Volume 15 Number 4 (August 2023) 521-532

ORIGINAL ARTICLE

Characterization of beta-lactamase producing *Enterobacterales* isolated from an urban community wastewater treatment plant in Iran

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Received: March 2023**, Accepted:** July 2023

ABSTRACT

Background and Objectives: he occurrence and characteristics of Extended Spectrum- and AmpC-β-lactamase producing *Enterobacterales* (ESBL-PE and AmpC-PE) in an urban wastewater treatment plant (WWTP) were investigated.

Materials and Methods: A total of 30 wastewater samples were collected from all sections of WWTP. *Enterobacterales* were isolated and identified using standard microbiological tests. The antibiotic resistance profile was determined by the Kirby–Bauer disk diffusion method. Phenotypic screening for ESBL-PE and AmpC-PE isolates was performed by double-disk synergy and boronic acid disk potentiation tests, respectively. The isolates were examined for AmpC- and ESBL-encoding genes by PCR and sequencing methods.

Results: Among 146 *Enterobacterales* isolates, 8.9% (n=13) [ESBL-only; 5.48% (n=8) and ESBL + AmpC; 3.42% (n=5)] were ESBL-producers and 15.75% (n=23) [AmpC-only; 12.33% (n=18) and ESBL + AmpC; 3.42% (n=5)] AmpC-producers. *Hafnia* spp. with 33.33% (n=1/3) and *E. coli* with 20.58% (n=7/34) [ESBL-only; 17.64% (n=6/34) and ESBL + AmpC; 2.94% (n=1/34)] were the most common ESBL-producing bacteria. *Enterobacter* spp. with 37.50% (n=6/16) of isolates were the most common AmpC-producing organisms. ESBL- and/or AmpC-producing isolates were identified in all parts of the WWTP including 80% (n=8/10) of samples taken from effluent. Among ESBL-producing isolates, $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} ESBL-encoding genes were found in 61.5% (n=8), 15.3% (n=2), and 7.7% (n=1) of isolates, respectively. All CTX-M-type enzymes belonged to the CTX-M-1 group and CTX-M-15 subgroup. bla_{TEM} and bla_{SHV} type genes belonged to $bla_{\text{TEM-20}}$ and $bla_{\text{HSV-12}}$ subtypes, respectively. bla_{DHA} with 73.9% (n=17/23), and bla_{CIT} and bla_{FOX} with 30.4% (n=7/23) each, were the most common AmpC-encoding genes among AmpC-producing isolates. Overall, 75% of ESBL-producing and 55.5% of AmpC-producing isolates exhibited multi-drug resistance phenotypes. The organisms were most resistant against ampicillin (82.2%) nalidixic acid (43.8%) and cephalexin (41.1%).

Conclusion: ESBL- and AmpC-producing *Enterobacterales* spp. with diverse genetic resistance backgrounds in WWTP effluent poses a significant risk to public health.

Keywords: Extended spectrum beta-lactamase; AmpC beta-lactamase; *Enterobacterales*; Municipal sewage; Antibiotic resistance

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INTRODUCTION

The increasing use of antibiotics for the treatment of infectious diseases and as growth-promoting agents in animal husbandry leads to their uncontrolled release into the environment (1). The continuous exposure of the environmental microbial community to sub-inhibitory concentrations of antibiotics results in the selection of antibiotic-resistant bacteria (ARB) and dissemination of antibiotic-resistance genes (ARG), which substantially limits the therapeutic efficacy of antibiotics against pathogens (2, 3). Unfortunately, nowadays benefit of antibiotics in the control of infectious diseases is challenged by the emergence and spread of ARB (4). Aquatic ecosystems including wastewaters are one of the most important environments which provide favorable conditions for the development and dissemination of ARB (5). Wastewater treatment plants (WWTPs) could contribute to the spread of ARB into the environment as they accept and finally return wastewater to the natural environment (6).

Enterobacterales are one of the most important bacterial groups that are resistant to antibiotics (7). These are the largest group of Gram-negative bacilli in medicine. They are facultative anaerobes, non-spore-forming rods capable of fermenting sugars to various end products (8). The *Enterobacteriaceae* family with about 40 genera and 150 species is a large group of *Enterobacterales.* These genera are classified based on antigenic structure, biochemical and molecular characteristics. Despite the complexity of the family, less than 20 genera account for more than 95% of infections. These bacteria make up part of the normal intestinal flora of most animals as well as humans and cause various diseases in humans, including 30-35% of all septicemia, more than 70% of urinary tract infections, and many intestinal infections (9). Usually, opportunistic infections caused by *Enterobacterales* are associated with a high mortality rate (10).

β-lactam antibiotics including, cephalosporins, penicillins, and carbapenems are the most common antibiotics used by humans worldwide (11). These antibiotics enter wastewater with urine excretion and faeces from humans (7). The resistance to β-lactam antibiotics in *Enterobacterales* is mainly caused by β-lactam hydrolyzing enzymes such as extended-spectrum β-lactamase (ESBL) and AmpC β-lactamase (12, 13). ESBL-producing *Enterobacterales* (ESBL-PE) are increasing more and more throughout the world (14). *Escherichia coli* and *Klebsiella pneumoniae* are the main ESBL-producing organisms among *Enterobacterales* (15). ESBL enzymes cause resistance nearly against all β-lactam antibiotics excluding cephamycins and carbapenems (16). Carbapenems are used as first-line drugs in treating diseases caused by β-lactamase producing *Enterobacterales* (12). Among more than 300 different ESBL-type enzymes, TEM, SHV, CTX-M, and OXA groups are the most prevalent worldwide (17). The ESBL enzymes encoding genes ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{OXA}) are frequently located on mobile genetic elements (18).

AmpC β-lactamases are the second most prevalent β-lactam hydrolyzing enzymes (19). The AmpC-encoding genes are located either on chromosome or plasmid (20). About four decades ago several non-inducible AmpC β-lactamase genes were recognized on the chromosome of *Enterobacterales* species (21). However, to date, several plasmid-mediated AmpC β-lactamases have been identified including bla_{CMY}, bla_{FOX}, bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, and bla_{EBC} (22) which are structurally and functionally similar to their chromosomal origins (23). The main concern of AmpC β-lactamases is that, unlike ESBLs, they are capable of hydrolyzing cephamycins (e.g., cefotetan and cefoxitin) and are not inhibited by β-lactamase inhibitors (24). Reportedly, the co-existence of ESBL and AmpC enzymes in *Enterobacterales* is also on the rise worldwide (25, 26).

This study aimed to: (i) determine the prevalence of ESBL- and AmpC-producing *Enterobacterales* species (ESBL-PE and AmpC-PE) in a WWTP in Northwest Iran; (ii) study the genetic background responsible for ESBL and AmpC resistance and (iii) evaluate antibiotic resistance patterns of *Enterobacterales* isolates against commonly used antibiotics.

MATERIALS AND METHODS

Sample collection. In this study, samples were taken from the inlet, aeration lagoon, and effluent sections of Ardabil WWTP (Northwestern, Iran). The aerated lagoon is the biggest current WWTP in Ardabil (27). Thirty liquid wastewater samples were collected in 500 mL sterile bottles in 10 series over a period of 6 months (from March 2019 until August 2019). Collected samples were immediately transferred to the

microbiology laboratory in cold box containers and kept at 4°C. Microbiological analysis was performed in less than 2 h after sample collection.

Cultivation, isolation, and characterization of *Enterobacterales*. For enrichment of *Enterobacterales*, 5 mL samples were cultured into 5 mL double concentration *Enterobacteriaceae* enrichment broth (EE broth) (Merck, Darmstadt, Germany) for 24 h at 35 ± 2°C, then one loopful of each enrichment broth was streaked on a MacConkey agar medium (Pronadisa, Madrid, Spain). After 18-24 h incubation at 35 ± 2°C, raised colonies were examined based on colonial characteristics, and three colonies with the same appearance per colony morphology in each wastewater sample were selected for further analysis (28). Selected bacteria were isolated on Trypticase Soy Agar (Merck, Germany) and screened using oxidase test (impregnated filter paper disc, Padtan Teb, Iran), oxidative/fermentation glucose test [OF (Oxidation/Fermentation) medium] and nitrate reduction test (Nitrate Broth). Oxidase negative, nitrate reductase positive, and glucose fermenter isolates were characterized to the species level using a battery of biochemical differential tests with the help of standard manuals (29). The main tests used for this end include lactose fermentation, gas generation, and H2S production tests (Triple Sugar Iron (TSI) agar), indole production and motility tests (Sulfide Indole Motility (SIM) medium), urease test (Urease Agar), citrate utilization test (Simmons Citrate Agar), Voges-Proskauer and methyl red tests (MR/VP medium), lysine decarboxylase test [Lysine Iron Agar; (LIA)], arginine decarboxylase test (Arginine Decarboxylase Broth), ornithine decarboxylase test (Ornithine Decarboxylase Broth) and phenylalanine deaminase test (Phenylalanine Agar). All differential culture media used in this study were purchased from Himedia Laboratories Pvt. Ltd, India. The validity of the biochemical tests was ensured by using *E. 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 introduced by the enrichment process, repetitive isolates of each species and of each sample showing identical phenotypic and genotypic antimicrobial resistance were excluded from the study. The identified isolates were kept at -80°C in Trypticase soy broth (Merck, Germany) including 15% glycerol (Merck, Germany) for subsequent antimicrobial and molecular tastings.

Antimicrobial susceptibility testing. The isolates were tested for the antimicrobial susceptibility to 13 antibiotics: ampicillin (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), amikacin (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg) and cephalexin (30 µg) (Padtan teb, Tehran, Iran) using the disc diffusion Kirby-Bauer method on Mueller Hinton agar (MHA) (Pronadisa, Madrid, Spain) medium. Results were interpreted as sensitive (S), intermediate (I), and resistant (R) according to Clinical and Laboratory Standards Institute (CLSI; 2023) guidelines (30). For cephalexin interpretation was carried out according to European Committee on Antimicrobial Susceptibility Testing (EUCAST; 2023) guidelines (31). *E. coli* ATCC-25922 was used as a control strain for the antibiotic susceptibility tests. The isolates with resistance to three or more different classes of antibiotics were defined as multidrug resistant (MDR).

Phenotypic identification of ESBL and AmpC production. Initially, ESBL suspicious isolates were screened using cefotaxime (CTX, 30 µg) and ceftazidime (CAZ, 30 µg) discs according to CLSI (2023) guidelines. Then, the production of ESBL, AmpC, and ESBL/AmpC were examined as follows. In the confirmation stage, ESBL producer isolates were tested by the double-disc synergy test (DDST), as recommended by the CLSI 2023. DDST was performed by placing discs of cefotaxime (CTX, 30 µg), cefotaxime with clavulanic acid (CTX/CLA, Concentration ratio: 30/10 µg), ceftazidime (CAZ, 30 µg), and ceftazidime with clavulanic acid (CAZ/CLA, Concentration ratio: 30/10 µg) onto the plates of MHA medium inoculated with test bacterium. ESBL production was considered positive when, following incubation at 37°C for 24 h, the growth inhibitory zone, either around CTX/CLA or CAZ/CLA discs, increased by 5 mm or more compared with the diameter around the disc containing CTX or CAZ alone, respectively (30).

The isolates resistant to cefoxitin (FOX, 30 µg) and susceptible to cefepime (30 µg) were screened for AmpC type β-lactamases production. AmpC β-lactamases were detected using of cefoxitin disc alone and a combined disc of cefoxitin with boronic acid

(FOX/BRO, 30/300 µg) on plates of MHA medium inoculated with test bacterium. After incubation at 37°C for 24 h, the isolates that showed an increase of the growth inhibitory zone diameter by 5 mm or more around the FOX/BRO disc compared with the diameter around the disc containing FOX alone were considered AmpC-producer phenotypically (32).

The isolates that were suspected of producing ESBL but did not show an increase in growth inhibitory zone using DDST (as mentioned above) were examined for co-production of ESBL/AmpC (ESBL/AmpC-PE). The discs used for this test include CAZ 30 µg, CAZ/ BRO 30/400 µg, CAZ/CLA 30/10 µg, and CAZ/CLA /BRO 30/10/400 µg. The results were interpreted according to previous reports (33).

Characterization of ESBL and AmpC type genes. The isolates were grown on Trypticase Soy Agar (Merck, Germany) and then template DNA was extracted for PCR testing as follows: Initially, 3-5 colonies were picked up from MHA medium and dissolved in 400 µL of DNA extraction solution [including Tris base solution, Triton X-100 and Proteinase K (Sinaclon Co., Tehran, Iran)] in a 1.5 mL tube. The tube was incubated in a water bath for 3 h at 55°C; centrifuged for 10 min at 5000 rpm; incubated for 10 min at boiling water; again centrifuged for 10 min at 5000 rpm and supernatant containing genomic DNA was used for PCR testing.

The genes $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and bla_{OXA} encoding CTX-M, TEM, SHV, and OXA type β-lactamases respectively, were detected by PCR in ESBL and ESBL/AmpC producing isolates. PCR reactions were carried out in 25 µL in final volume using 0.3 µL of Taq DNA polymerase (1.5 U), 2.5 µL of 10X PCR buffer, 0.5 µL of dNTP mix (10 mM), 1.5 µL of $MgCl_2$ (50 mM), 1 µL of each primer (10 pmol), (Sinaclon Co., Tehran, Iran) and 1 µL of template DNA. Amplification was carried out using T100™ Thermal cycler (Bio-Rad, Germany) with specific temperatures and cycling conditions defined in Table 1.

The presence of AmpC encoding genes (bla_{FOX}, bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{CMY}, and bla_{EBC}) was detected by multiplex PCR in AmpC and ESBL/AmpC producing isolates as described by others (22). Primers and multiplex PCR conditions are shown in Table 1.

PCR products were separated by electrophoresis in 1.2% agarose gel (Sigma Co., Germany) in 0.5X TBE buffer (Sinaclon, Iran) stained with safe stain (Sinaclon Co., Iran) and visualized by UV transillumination (UVitec, England).

In this study, a representative PCR product for each β-lactamase encoding gene was randomly selected and sequenced to confirm its identity (Genomin Co., Iran). Sequences were aligned and analyzed using the BLAST program available at the National Center for Biotechnology Information. Confirmed isolates were used as a positive control in PCR testing. Additionally, the subtypes of the bla_{TEM} and bla_{SHV} genes were further determined by sequencing the PCR products.

Statistical analyses. SPSS software v.11.5 (SPSS Inc, Chicago, USA) was used for Statistical analyses. The association of distribution of β-lactamase-producing isolates within WWTP sections was calculated using Chi-square ($\chi 2$) test. A $P \leq 0.05$ was considered statistically significant.

Ethical approval. This study was approved by the regional Ethics Committee of Ardabil University of Medical Sciences [IR.ARUMS.REC.1398.057].

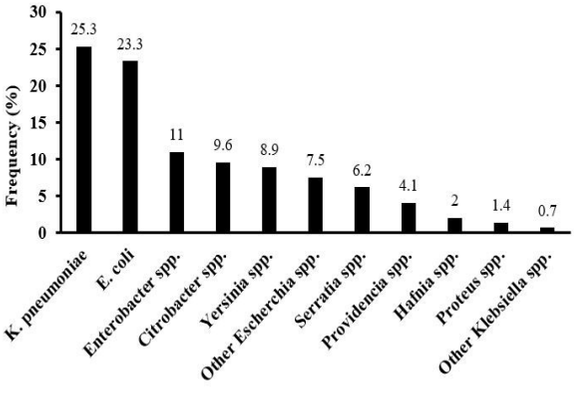
RESULTS

Distribution of *Enterobacterales* isolates from WWTP. The distribution pattern of *Enterobacterales* species in Ardabil WWTP is shown in Fig. 1. A total of 146 isolates were obtained from WWTP samples. Among them, 32.9% (n = 48), 35.6% (n = 52), and 31.5% (n = 46) of isolates were from inlet, lagoon, and effluent samples, respectively. *K. pneumoniae* and *E. coli* with 25% (n = 37) and 23% (n = 34) of isolates were the most common species identified. There was no significant difference in the distribution of bacterial species between the three sections of WWTP ($P > 0.05$).

Distribution pattern of ESBL-PE, AmpC-PE, and ESBL + AmpC-PE in WWTP. The frequency of distribution of ESBL-PE, AmpC-PE, and ESBL + AmpC-PE isolates in Ardabil WWTP is shown in Table 2. Among 146 *Enterobacterales* isolates, 8.9% (n = 13) [ESBL only; 5.48% (n = 8) and ESBL + AmpC 3.42% (n = 5)] were ESBL-producers and 15.75% (n = 23) [AmpC only; 12.33% (n=18) and ESBL + AmpC; 3.42% (n = 5)] AmpC-producers. ESBL-PE, AmpC-PE, and ESBL + AmpC-PE isolates were distributed among 3, 10, and 4 different *Enterobactera-*

Table 1. Specific primers and PCR conditions used for the amplification of ESBL and AmpC encoding genes.

Primers	Target gene	Primer sequence (5`→3`)	Size (bp)	PCR conditions	Ref
CTX-M-F	*bla*$_{CTX-M}$	SCSATGTGCAGYACCAGTAA	544	1 cycle of 5 min at 94°C; 34 cycle of 45 sec at 94°C, 40	
CTX-M-R		CCGCRATATGRTTGGTGGTG		sec at 51°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	
TEM-F	*bla*$_{TEM}$	TCGGGGAAATGTGCGCG	1076	1 cycle of 5 min at 94°C; 34 cycle of 1 min at 94°C, 1	
TEM-R		TGCTTAATCAGTGAGGCACC		min at 56°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	
SHV-F	*bla*$_{SHV}$	TTATCTCCCTGTTAGCCACC	1018	1 cycle of 5 min at 94°C; 34 cycle of 45 sec at 94°C, 40	
SHV-R		GATTTGCTGATTTCGCTCGG		sec at 51°C, 1 min at 72°C; 1 cycle of 10 min at 72°C	
OXA-F	*bla*$_{OXA}$	TATCAACTTCGCTATTTTTTTA	807	1 cycle of 5 min at 94°C; 30 cycle of 45 sec at 94°C, 45	
OXA-R		TTTAGTGTGTTTAGAATGGTGAC		sec at 55°C, 1 min at 72°C; 1 cycle of 5 min at 72°C	
CTX-M-1-F	*bla*$_{CTX-M-1}$	GGTTAAAAAATCACTGCGTC	864	1 cycle of 5 min at 94°C; 30 cycle of 45 sec at 94°C, 45	(34)
CTX-M-1-R		TTGGTGACGATTTTAGCCGC		sec at 55°C, 1 min at 72°C; 1 cycle of 5 min at 72°C	
CTX-M-15-F	*bla*$_{CTX-M-15}$	AGAATAAGGAATCCCATGGTT	884	1 cycle of 5 min at 94°C; 36 cycle of 30sec at 94°C, 40	(35)
CTX-M-15-R		ACCGTCGGTGACGATTTTAG		sec at 52°C, 50 sec at 72°C; 1 cycle of 5 min at 72°C	
MOX-F	*bla*$_{MOX}$	GCTGCTCAAGGAGCACAGGAT	520		
MOX-R		CACATTGACATAGGTGTGGTGC			
CIT-F	*bla*$_{CIT}$	TGGCCAGAACTGACAGGCAAA	462		
CIT-R		TTTCTCCTGAACGTGGCTGGC			
DHA-F	*bla*$_{DHA}$	AACTTTCACAGGTGTGCTGGGT	405	multiplex PCR for AmpC genes:	
DHA-R		CCGTACGCATACTGGCTTTGC			
ACC-F	*bla*$_{ACC}$	AACAGCCTCAGCAGCCGGTTA	346	1 cycle of 3 min at 94°C;	
ACC-R		TTCGCCGCAATCATCCCTAGC		25 cycle of 30 secs at 94°C, 30 secs at 64°C, 1 min at	(22)
EBC-F	*bla*$_{EBC}$	TCGGTAAAGCCGATGTTGCGG	302	72°C;	
EBC-R		CTTCCACTGCGGCTGCCAGTT		1 cycle of 7 min at 72°C	
FOX-F	*bla*$_{FOX}$	AACATGGGGTATCAGGGAGATG	190		
FOX-R		CAAAGCGCGTAACCGGATTGG			
CMY-F	*bla*$_{CMY}$	ATGATGAAAAAATCGTTATGCTGC	1030		
CMY-R		GCTTTTCAAGAATGCGCCAGG			



K. pneumoniae: Klebsiella pneumoniae, E. coli: Escherichia coli, Other *Escherichia* spp.: *Escherichia* species other than *E. coli,* Other *Klebsiella* spp.: *Klebsiella* species other than *K. pneumoniae*

Fig. 1. Distribution pattern of *Enterobacterales* isolates collected from Ardabil WWTP.

les species collected from wastewater, respectively. *Hafnia* spp. with 33.33% (n = 1/3) and *E. coli* with 20.58% (n = 7/34) [ESBL-only; 17.64% (n = 6/34) and ESBL + AmpC; 2.94% (n = 1/34)] were the most common ESBL-producing bacteria. *Enterobacter* spp. with 37.50% (n = 6/16) of isolates were the most common AmpC-producing organisms.

β-lactamase-producing isolates were present in all parts of Ardabil WWTP. These isolates were detected in 50 (n = 5/10), 70 (n = 7/10), and 80% (n = 8/10) of samples taken from the inlet, aeration lagoon, and effluent, respectively. The aerated lagoon with 4 (50%) and 8 (44.5%) isolates, was the most contaminated section with ESBL-PE and AmpC-PE isolates, respectively. ESBL + AmpC-PE isolates were mainly isolated from the inlet section with a frequency of 3 isolates (60%) ($P < 0.05$).

Antibiotic susceptibility testing. The results of antibi-

Table 2. Distribution of ESBL-PE, AmpC-PE and ESBL + AmpC-PE isolates in different parts of Ardabil WWTP.

Bacteria[a]	Phenotypic resistance	Total	WWTP sections		
		n (%)	Inlet	Aeration Lagoon	Effluent
			n (%)	n (%)	n (%)
E. coli	ESBL n (%)	6 (17.64)	2 (33.33)	3 (50)	1 (16.66)
N = 34	AmpC n (%)	2 (5.88)	-	-	2 (100)
	ESBL + AmpC n (%)	1 (2.94)	-	1 (100)	-
	Non ESBL/AmpC n (%)	25 (73.54)	5 (20)	12 (48)	8 (32)
K. pneumoniae	ESBL n (%)	1 (2.7)	-	1 (100)	-
N = 37	AmpC n (%)	4 (10.8)	1 (25)	3 (75)	-
	Non ESBL/AmpC n (%)	32 (86.5)	11 (34.37)	10 (31.25)	11 (34.37)
Yersinia spp.	AmpC n (%)	1 (7.7)	-	-	1 (100)
N = 13	ESBL + AmpC n (%)	1 (7.7)	1 (100)	-	-
	Non ESBL/AmpC n (%)	11 (84.6)	8 (72.72)	-	3 (27.27)
Citrobacter spp.	AmpC n (%)	2 (14.30)	-	2 (100)	-
N = 14	ESBL + AmpC n (%)	1 (7.10)	1 (100)	-	-
	Non ESBL/AmpC n (%)	12 (85.7)	4 (33.33)	5 (41.66)	3 (25)
Other *Escherchia spp.*[b]	AmpC n (%)	1 (9.09)	1 (100)	-	-
N = 11	ESBL + AmpC n (%)	2 (18.18)	1 (50)	-	1 (50)
	Non ESBL/AmpC n (%)	8 (72.73)	3 (37.5)	4 (50)	1 (12.5)
Serratia spp.	AmpC n(%)	1 (11.11)	1 (100)	-	-
N = 9	Non ESBL/AmpC n (%)	8 (88.89)	3 (37.5)	2 (25)	3 (37.5)
Enterobacter spp.	AmpC n (%)	6 (37.50)	1 (16.66)	3 (50)	2 (33.33)
N = 16	Non ESBL/AmpC n (%)	1 (20)	-	0 (0)	1 (100)
Hafnia spp.	ESBL n (%)	1 (33.33)	1 (100)	-	-
N = 3	AmpC n (%)	1 (33.33)	-	-	1 (100)
	Non ESBL/AmpC n (%)	1 (33.33)	-	-	1 (100)
Total	ESBL n (%)	8 (5.48)	3 (37.5)	4 (50)	1 (12.5)
Enterobacterales	AmpC n (%)	18 (12.33)	4 (22.22)	8 (44.5)	6 (33.33)
N = 146	ESBL + AmpC n (%)	5 (3.42)	3 (60)	1 (20)	1 (20)
	Non ESBL/AmpC n (%)	115 (78.76)	38 (33)	39 (34)	38 (33)

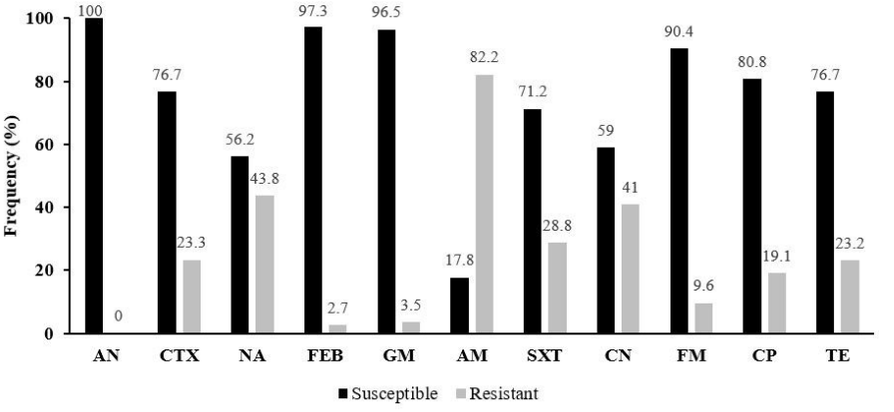
a. Non-ESBL-/non-AmpC-PE isolates were not listed in this table.

b. *Escherichia* species other than *E. coli*

otic susceptibility testing for *Enterobacterales* isolates are shown in Fig. 2. The highest resistance rate was observed for ampicillin (82.2%), nalidixic acid (43.8%) cephalexin (41.1%). Amikacin (100%), cefepime (973%), and gentamicin (96.5%) were the most active antibiotics against the isolates collected in this study.

Table 3 shows the profile of antibiotic resistance among β–lactamase-producing isolates. Collectively, 75% (n = 6/8), 55.5% (n = 10/18), and 20% (n = 1/5), of ESBL-PE, AmpC-PE, and ESBL + AmpC-PE showed MDR phenotype, respectively.

Distribution of ESBL and AmpC type enzymes encoding genes among *Enterobacterales*. Fig. 3 shows the results of genotyping in ESBL-PE (a) and AmpC-PE (b) isolates. Overall, $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} type genes were detected in 61.50% (n = 8/13), 15.30% (n = 2/13), and 7.70 % (n = 1/13) of ESBL-producing isolates (ESBL only and ESBL + AmpC-PE), respectively. bla_{OXA} type was not found in our isolates. All identified $bla_{\text{CTX-M}}$ genes belonged to $bla_{\text{CTX-M-1}}$ group and $bla_{\text{CTXM-15}}$ subgroup. bla_{TEM} genes detected belonged to $bla_{\text{TEM-20}}$ subtype and a single bla_{SHV} type gene detected in this study belonged to $bla_{\text{HSV-12}}$subtype. Of note, in 53.84% (n = 7/13) of ESBL-positive isolates $bla_{\text{TEM-1}}$ subtype was observed. However, these were not included in the results (Tables and Figs) as the $bla_{\text{TEM-1}}$ gene does not code an



AN: Amikacin; CAZ: Ceftazidime; CTX: Cefotaxime; NA: Nalidixic Acid; FEP: Cefepime; GM: Gentamicin; AM: Ampicillin; SXT: Trimethoprim-sulfamethoxazole; CN: Cephalexin; FM: Nitrofurantoin; CP: Ciprofloxacin; TE: Tetracycline.

Fig. 2. Antibiotic resistance (intermediate resistant + resistant) and susceptibility patterns of *Enterobacterales* isolated from Ardabil WWTP.

Table 3. Combined antibiotic resistance (intermediate + resistant) pattern of β-lactamase producing *Enterobacterales* species isolated from Ardabil" WWTP.

***Enterobacterales* spp. N = 31**	**Enzyme type**	**Phenotypic resistance pattern**	**Isolates n (%)**	**Antibiotic Classes (n)**	**Total Isolates[b] n (%)**	**MDR n (%)**	**Total MDR n (%)**
E. coli	ESBL	CAZ-CTX-NA-AM-CN-CP	1 (16. 66)	2	1 (16. 66)	5	
N = 9	N = 6	CTX-AM-SXT-CN-TE	1 (16. 66)	3	2 (33. 33)	(83. 33)	
		CAZ-CTX-FEP-CN-AM-SXT-TE	1 (16. 66)	3			
		CAZ-CTX-SXT-CN-TE-AM-NA	2 (33. 33)	4	3 (50)		
		CAZ-CTX-NA-AM-SXT-IMP-CN-CP-TE	1 (16. 66)	4			
	AmpC	CAZ-CTX-AM-SXT-CN	1 (50)	2	1 (50)	1 (50)	
	N = 2	CAZ-CTX-NA-CN-AM-IMP-SXT-CP-TE	1 (50)	4	1 (50)		
	ESBL + AmpC N = 1	CAZ-CTX-NA-FEP-GM-AM-CN	1 (100)	3	1 (100)	1 (100)	
K. pneumoniae	ESBL N = 1	CAZ-CTX-AM-NA-FEP-SXT-IMP-CN-FM-CP	1 (100)	4	1 (100)	1 (100)	
N = 5	AmpC N = 4	CTX-NA-CN-AM-IMP	1 (25)	2	2 (50)	2 (50)	ESBL
		CAZ-CTX-NA-AM-CN –CP	1 (25)	2	2 (50)		6/8 (75)
		CAZ-CTX-NA-AM-SXT-CN	1 (25)	3			
		CAZ-CTX-NA-AM-CN-FM-CP	1 (25)	3			
Yersinia spp.	AmpC N= 1	CAZ-CTX-NA-AM-SXT-CN-CP-TE	1 (100)	4	1 (100)	1 (100)	AmpC
N = 2	ESBL + AmpC N= 1	CAZ-CTX-NA-CN-AM-IMP-CP	1 (100)	2	1 (100)	-	10/18
Citrobacter spp. N = 3	AmpC N= 2	CAZ-AM-NA-CN	1 (50)	2	2 (100)	-	(55.5)
		CAZ-CTX-AM-SXT-CN	1 (50)	2			
	ESBL + AmpC N = 1	CAZ-CTX-AM-NA-IMP-CN-CP	1 (100)	2	1 (100)	0 (0)	
Other *Escherchia* spp.[a]	AmpC	CAZ-CTX-NA-AM-CN-IMP-CP	1 (100)	2	1 (100)	-	
N = 3	N= 1	CAZ-CTX-AM-CN	1 (50)	1	1 (50)	-	
	ESBL + AmpC N = 2	CAZ-CTX-NA-AM-CN-IMP-CP	1 (50)	2	1 (50)		
Serratia spp. N = 1	AmpC N = 1	CAZ-CTX-AM-NA-IMP-CN-CP	1 (100)	2	1 (100)	-	
Enterobacter spp N = 6	AmpC N = 6	CAZ-CTX-AM-NA-SXT-CN	6 (100)	3	6 (100)	6 (100)	
Hafnia spp.	ESBL N = 1	CAZ-CTX-AM-NA-FEP-CN-CP	1 (100)	2	1 (100)	0 (0)	ESBL+
N = 2	AmpC N = 1	CAZ-CTX-AM-NA-CN-IMP	1 (100)	2	1 (100)	0 (0)	AmpC
							1/5 (20)

a. *Escherichia* species other than *E. coli*

b. Total number of isolates resistant to same number of antibiotic classes.

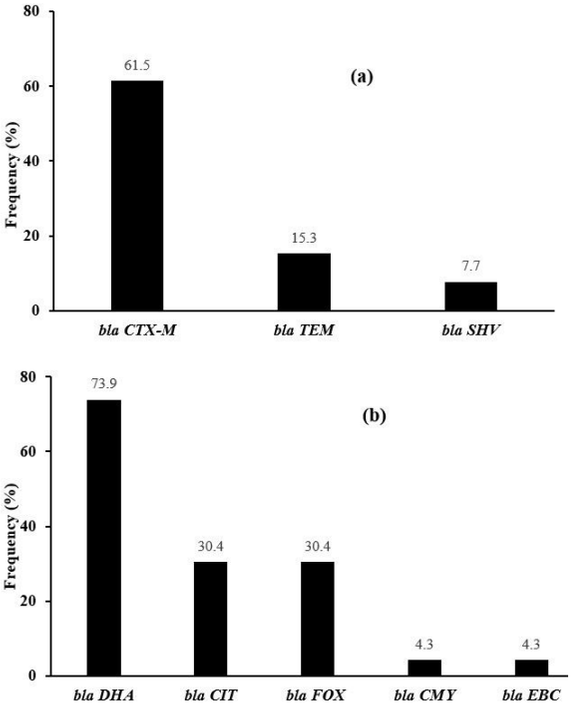


Fig. 3. Distribution of ESBL (a) and AmpC (b) enzymes encoding genes among *Enterobacterales* species from Ardabil WWTP.

ESBL phenotype enzyme. The bla_{DHA} type gene was detected in 73.9% (n =17 /23) of AmpC-producing isolates (AmpC only and ESBL + AmpC-PE isolates) followed by bla_{CIT} and bla_{FOX} (each one; 30.4%, n = 7/23), and bla_{CMY} and bla_{EBC} type genes (each one; 4.30%, n = 1/23). The bla_{MOX} and bla_{ACC} genes were not found in our isolates.

Table 4 shows the combination profiles of β-lactamase enzymes among AmpC-PE, ESBL-PE, and ESBL + AmpC-PE isolates. Multiple patterns of β-lactamase encoding genes were observed in ESBL-PE, AmpC-PE, and ESBL + AmpC-PE isolates. In AmpC-PE isolates, the profiles with bla_{DHA} alone and bla_{CIT} + bla_{FOX} were the most predominant ones identified. The bla_{CIT} + bla_{FOX} and bla_{CTX-M} alone were the main profiles found in ESBL-PE and ESBL + AmpC-PE isolates, respectively.

DISCUSSION

Municipal WWTPs contain various antibiotics and pharmaceutical compounds that are transferred to WWTPs through domestic and hospital wastewater due to human consumption (36). Therefore, wastewater can contribute to the development and dissemination of ARB including *Enterobacterales* species (37). As one of the main inhabitants of the human gut, *Enterobacterales* are often detected in municipal wastewater (38). β-lactams are the most widely used antibiotics for the treatment of infections caused by *Enterobacterales* (11). Bacteria in this family become resistant to β-lactam antibiotics mainly by producing β-lactamases including ESBL and AmpC-type enzymes (39).

In the present study, the heterogeneous diversity of *Enterobacterales* isolates was identified in municipal wastewater. *K. pneumoniae* (25%) and *E. coli* (23%) were the most frequent species isolated. In general, *E. coli* has been reported as the most common *Enterobacterales* spp. recovered from municipal wastewater. For instant, in a study conducted in Spain, *E. coli* included 72.9% of *Enterobacterales* isolates collected from urban wastewater, while the frequency of *K. pneumoniae* isolates was much lower compared to *E. coli* (5). However, consistent with our results in a study reported by Roederova et al. *K. pneumoniae* with 13.7% and *E. coli* with 9.8% composed the main *Enterobacterales* isolates collected from municipal and hospital WWTPs (40). In another study recently reported from South Africa, *K. pneumoniae* (24%) and *E. coli* (17%) were among the most common *Enterobacterales* isolated from municipal wastewater (41). The differences in the abundance of *Enterobacterales* spp. in different studies can be attributed to the origin of wastewater entering the treatment plant and also the effect of WWTP on the load of microorganisms (42).

The overall estimate of ESBL-PE isolates in municipal wastewater has been reported to be 18.83% (ranging from 11.15% to 27.88%) (37). In comparison, the prevalence of ESBL-producing isolates in our study (8.9%) was below the lower limit of the reported range. In this study, although, ESBL production was not restricted to a specific species, however, *E. coli* with 20.58% (ESBL-only; 17.64% and ESBL + AmpC; 2.94%) was the most common ESBL-producing bacterium. Studies by other researchers have also shown that the highest frequency of ESBL-producing isolates among *Enterobacterales* spp. is mainly related to *E. coli* (43). The prevalence of ESBL-producing isolates in municipal wastewater varies based on geographical location. The overall estimate of ESBL-producing *E. coli* in municipal wastewater was reported between 11.14-19.41% (pooled estimate;

15.05%) (37). The bacterial composition of municipal wastewater has been found to reflect human fecal flora, in a way that municipal wastewater samples can be used as a surrogate for studying human commensal *E. coli* in a local population (44). Our findings are somewhat akin to the profile obtained from fecal samples of healthy children in Ardabil, where 29.50% of *E. coli* isolates were ESBL producers (39). However, other factors such as local antibiotic use, sampling methods, and resistance detection techniques can affect the results.

In this study, out of the total 146 isolates, 23 (15.75%) isolates were AmpC β-lactamases-producers, among them *Enterobacter* spp. with 37.50% being the most common AmpC-producing species. In a similar study performed in Portugal 44.4% of *Enterobacterales* isolates were AmpC-producers (45), which was much higher than the present study. In the present study, 3.42% of *Enterobacterales* isolates were co-producers of ESBL and AmpC enzymes. Findings of the Amador et al study in Spain showed that 35.2% of *Enterobacterales* isolates collected from wastewater were jointly ESBL and AmpC enzyme-producers (44), which is substantially higher than our study.

In the present study, the $bla_{\text{CTX-M}}$ (61.5%) was the most prevalent ESBL-encoding gene in ESBL-positive isolates. This is in accordance with the global estimate of ESBL-encoding genes which identified $bla_{\text{CTX-M}}$ as the predominant ESBL-encoding gene in ESBL-producing isolates in wastewaters. Similar results with 67.4%, were reported on ESBL-PE isolates collected from wastewater in Spain (5). In our study, 12.5% of the ESBL-PE isolates simultaneously contained two different ESBL-encoding genes. Other studies also showed that ESBL-positive isolates commonly harbor multiple ESBL-encoding genes (5, 42).

In this study, bla_{DHA} (73.9%), bla_{CIT} and bla_{FOX} (30.4% each) were the most prevalent AmpC-encoding genes characterized in AmpC-positive isolates. In a study recently conducted by authors on commensal *Enterobacterales* isolates collected from healthy children in Ardabil bla_{DHA} and bla_{CIT} with 77.8% each one were the most common AmpC-encoding genes detected (39). Akin to our results, in a study performed on enteric gram-negative rods isolated from hospitalized patients and municipal wastewater in the Czech Republic bla_{DHA} (19.6%) followed by bla_{CIT} (15.7%) were the most common AmpC-encoding genes reported. However, in this study, the overall prevalence was lower compared to our results

Table 4. Combined profile of β-lactamase enzymes encoding genes in *Enterobacterales* species isolated from Ardabil WWTP.

Bacterium	AmpC-PE							ESBL + AmpC-PE							ESBL-PE			
	bla_{DHA} n (%)	$bla_{\text{DHA}}+bla_{\text{CIT}}$ n (%)	$bla_{\text{DHA}}+bla_{\text{EBC}}$ n (%)	$bla_{\text{DHA}}+bla_{\text{FOX}}$ n (%)	$bla_{\text{CIT}}+bla_{\text{FOX}}$ n (%)	$bla_{\text{CIT}}+bla_{\text{CMY}}+bla_{\text{FOX}}$ n (%)		bla_{DHA} n (%)	$bla_{\text{CTX-M}}+bla_{\text{DHA}}$ n (%)	$bla_{\text{CIT}}+bla_{\text{FOX}}$ n (%)	$bla_{\text{TEM}}+bla_{\text{CIT}}+$ n (%)		$bla_{\text{CTX-M}}$ n (%)	$bla_{\text{CTX-M}}+bla_{\text{TEM}}$ n (%)	bla_{SHV} n (%)
E. coli N = 2 n (%)	2 (100)	-	-	-	-	-	*E. coli* N = 1 n (%)	-	1 (100)	-	-	*E. coli* N = 6 n (%)	4 (66.6)	1 (16.7)	1 (16.7)
K. pneumonia N= 4 n (%)	2 (50)	1 (25)	-	1 (25)	-	-	Other *Escherichia* spp. [a,b] N = 2 n (%)	-	-	2 (100)	-	*K. pneumoniae* N = 1 n (%)	1 (100)	-	-
Hafnia spp N = 1 n (%)	-	-	1 (100)	-	-	-	*Yersinia* spp. N =1 n (%)	-	-	-	1 (100)	*Hafnia* spp. N = 1 n (%)	1 (100)	-	-
Serratia spp. N =1 n (%)	1 (100)	-	-	-	-	-	*Citrobacter* spp. [a] N = 1 n (%)	1 (100)	-	-	-	Total N = 8	6 (75)	1 (12.5)	1 (12.5)
Enterobacter spp. N = 6 n (%)	6 (100)	-	-	-	-	-	-								
Citrobacter spp. N = 2 n (%)	1 (50)	-	-	-	1 (50)	-									
Other *Escherichia* spp. N=1 n (%)	-	-	-	-	1 (100)	-									
Yersinia spp. N = 1 n (%)	-	-	-	-	-	1 (100)									
Total N = 18 n (%)	12 (67)	1 (5.5)	1 (5.5)	1 (5.5)	2 (11)	1 (5.5)	Total N = 5 n (%)	1 (20)	1 (20)	2 (40)	1 (20)				

a. Common ESBL encoding genes ($bla_{\text{CTX-M}}$, bla_{TEM} bla_{SHV} and $bla_{\text{}}$) were not detected.
b. *Escherichia* species other than *E. coli*

(40). Overall, 33.3% of our AmpC-PE isolates carried two or three different AmpC-encoding genes, simultaneously. A similar result was reported by Roederova et al. which indicated the concomitant occurrence of two or three different AmpC-encoding genes in 34.8% of AmpC-PE isolates (40). Accumulation of multiple resistance genes with similar functions in a bacterium could create a high-level resistance to a given antibiotic.

In *Enterobacterales* isolates collected in this study, a high rate of resistance was observed for ampicillin (82.2%), followed by nalidixic acid (43.8%) and cephalexin (41.1%). Antibiotic resistance patterns could significantly differ according to geographical area and wastewater sources. A similar study reported from Singapore showed a high prevalence of resistance to ampicillin (100%), cefazolin (100%), and ciprofloxacin (92%) in *Enterobacterales* isolated from wastewater (46). Our isolates exhibited the highest susceptibility to aminoglycoside antibiotics (amikacin 100% and gentamicin 96.5%) and cefepime (97.3%). Consistent with our study, Tokajian et al. reported amikacin as the most active antibiotic against *Enterobacterales* isolated from wastewater in Lebanon (47).

ESBL-encoding genes are usually carried on plasmids that contain several other resistance genes. Therefore, ESBL-PE isolates are MDR commonly. In the present study, 75% of the ESBL-PE isolates and 55.5% of AmpC-PE isolates exhibited MDR phenotypes. A similar finding has been reported previously indicating that 63.2% of ESBL-PE isolates collected from urban wastewater showed MDR phenotypes (48).

In this study, contamination with β-lactamase-producing isolates was found in all three parts of Ardabil WWTP. The presence of these organisms in the effluent indicates that the efficiency of WWTP has not been sufficient to eliminate resistant bacteria, a claim that has also been made by other researchers (5). Although wastewater treatment in WWTPs greatly reduces the burden of microorganisms, it does not necessarily eliminate all of them (42). The *Enterobacterales* species is one of the antibiotic-resistant bacteria that can be transmitted to other geographical environments through the effluent if it is not sufficiently destroyed in WWTPs (49).

CONCLUSION

This study indicated the occurrence of MDR *Enterobacterales* including ESBL- and AmpC-PE species in all sections (inlet, aeration lagoon, and effluent) of a WWTP in the city of Ardabil, Iran. The presence of clinically relevant MDR pathogenic *Enterobacterales* with high genetic resistance diversity in the effluent of a WWTP could be a risk to public health, because in many developing and even developed countries the effluent is used for irrigation in the agricultural sector. It is worth noting that, in addition to antibiotic-resistant bacteria, free circulating resistance genetic elements such as plasmids are also found in WWTPs effluents, which can contribute to the dissemination of antibiotic resistance by the horizontal transfer of resistance genes to the environmental bacteria. To prevent the release of resistant bacteria into the environment continuous monitoring of the presence of antibiotic-resistant bacteria and antibiotic-resistance genes in wastewater effluent and applying efficient wastewater disinfection methods are recommended.

ACKNOWLEDGEMENTS

This work was performed in partial fulfillment of the requirements for a Master's thesis in Environmental Health Engineering by KH at Ardabil University of Medical Sciences. The authors wish to thank the Vice-Chancellor of Research and Technology for financial support. This study was financially supported by Ardabil University of Medical Sciences, Iran (grant No. 13980301).

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