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Prevalence and Distribution of Virulence Genes among Bacterial Isolates from Municipal Wastewater in Shahrekord County, Iran, during 2023–2024

Running title: Prevalence of virulence genes among bacterial isolates

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Abstract

Background and aims: Municipal wastewater may contain pathogenic and antimicrobial-resistant bacteria (ARB), posing public health risks. This study investigated bacterial frequency, antimicrobial resistance (AMR), extended-spectrum β-lactamase (ESBL) production, and resistance and virulence genes in wastewater from Shahrekord, Iran.

Methods: In this cross-sectional study, 384 wastewater samples were collected from June 2023 to May 2024. Bacterial identification, antimicrobial susceptibility, and ESBL production were assessed using standard methods and Clinical and Laboratory Standards Institute (CLSI) guidelines. β-lactamase genes (BLACTX-M, BLATEM, and BLASHV) and species-specific virulence genes were detected by polymerase chain reaction (PCR). Data were analyzed descriptively.

Results: Among the 384 wastewater samples, 195 bacterial isolates were recovered exclusively from influent samples, while no isolates meeting the predefined culture and identification criteria were recovered from the primary sedimentation, secondary biological treatment, or final effluent samples. The isolates included *Staphylococcus aureus* (45.6%, n=89), *Enterococcus faecalis* (18.5%, n=36), *Pseudomonas aeruginosa* (18.5%, n=36), and *Acinetobacter baumannii* (17.4%, n=34); *Salmonella* and *Shigella* were not detected. Phenotypic ESBL production occurred in 58.8% of *A. baumannii*, 83.3% of *P. aeruginosa*, 66.3% of *S. aureus*, and 25.0% of *E. faecalis*. BLACTX-M was the predominant β-lactamase gene in *A. baumannii* (29.4%) and *P. aeruginosa* (36.1%). The most frequent virulence genes were *exoS* in *P. aeruginosa* (80.5%), *efa* in *E. faecalis* (100%), and *fimH* in *A. baumannii* (82.3%); the highest frequency in *S. aureus* was 16.8%.

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Conclusion: Municipal wastewater contained clinically relevant bacteria with substantial ESBL production and diverse virulence determinants, emphasizing the need for continuous surveillance and effective control measures.

Keywords: Virulence genes, Wastewater treatment, *Pseudomonas aeruginosa*, Public health, AMR.

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Introduction

Water is a vital need for human life, but contamination of water with pathogenic and virulent bacteria is a considerable threat to health, especially in regions where water is scarce, like Iran. *Salmonella*, *Shigella*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii,* and *Enterococcus faecalis* are examples of microorganisms that can be found in municipal wastewater (1). Furthermore, virulence genes can also be present on mobile genetic elements, where they are associated with antibiotic resistance, thereby increasing bacterial pathogenicity and public health risk (2). In developing countries, contaminated water is responsible for killing about 1.7 million people each year, and 88% of diarrheal diseases are linked to poor water quality (3). Pathogenic bacteria may increase due to the low annual precipitation in Iran, where wastewater is reused, which is about 250 mm per year.

Some of the virulence factors in wastewater-associated pathogens are associated with severe infections. Typhoid fever is caused by *Salmonella typhi* and dysentery, especially in developing countries, by *Shigella dysenteriae*. *P. aeruginosa* and *A. baumannii* have the capacity to form biofilms and produce exotoxins and are linked to nosocomial pneumonia and sepsis. *E. faecalis* has genes coding for cytolysin and adhesin genes involved in endocarditis and UTIs (4). Conventional wastewater treatment reduces pathogens by only about 10–90%, leaving virulent bacteria in effluents used for irrigation (5).

ExoS and algD in *P. aeruginosa* are virulence factors involved in host-cell damage, immune evasion, and biofilm formation; ompA and blaOXA-51 in *A. baumannii* are involved in adhesion, biofilm formation, apoptosis, AMR, and species identification; and gelE and esp in *E. faecalis* are involved in tissue degradation, adhesion, colonization, and persistence (6). Wastewater surveillance (7) is important because of their environmental persistence.

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A large range of differences exists in previous studies that account for the regions. SpiC and misL virulence genes were found in 84-93% of *Salmonella* isolates from livestock systems in South Africa, and *Salmonella* was detected at a lower prevalence in treated wastewater in Spain (8). A few studies have been done about virulence genes in wastewater, but there is not enough evidence on the virulence of wastewater in Iran (9). Other studies have investigated the factors that affect the spread of antibiotic resistance genes in WWTPs (10) and water quality in Shahrekord, with some wells having suboptimal levels of total coliform and fluoride (11).

Resistance-associated genes (blaCTX-M, blaTEM, and blaSHV) can be detected by PCR, and phenotypic susceptibility testing can provide a comprehensive overview of bacterial pathogenicity and resistance across the wastewater treatment process (12). The use of antimicrobial agents in Iran has led to high resistance among waterborne bacteria (13), and increasing AMR adds complexity in the process of wastewater management, which is responsible for the deaths of around 700,000 people each year.

Hence, the present study aimed to investigate the prevalence and distribution of virulence genes of *S. dysenteriae*, *S. typhimurium*, *P. aeruginosa*, *A. baumannii*, and *E. faecalis* in municipal wastewater samples of Shahrekord city, which were collected in 2023–2024, and their prevalence and distribution were determined by PCR to support microbial risk management and water security.

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Materials and Methods

Study Design and Study Area

A cross-sectional study was conducted to evaluate the presence of pathogenic bacteria, AMR, and virulence-associated genes in municipal wastewater effluents from the Shahrekord Wastewater Treatment Plant (WWTP) of Chaharmahal and Bakhtiari Province, Iran. This study took place from June 2023 to May 2024. The WWTP of the municipality receives mostly domestic wastewater from Shahrekord city and is equipped with a conventional activated sludge treatment plant.

Sample Collection

During the study period, 384 wastewater samples were taken at various points of the wastewater treatment process in accordance with the guidelines recommended by the American Public Health Association (APHA) (14). Samples were collected aseptically in sterile 500 mL polyethylene containers and transported to the microbiology laboratory under refrigerated conditions (4°C). All samples were processed within the recommended holding time to maintain the viability of the bacteria.

The wastewater samples were taken from four treatment phases of the MWWTP: influent, primary sedimentation, secondary biological treatment, and final effluent. Samples were collected in aseptically filled sterile 500 mL polypropylene containers as per the Standard Methods for the Examination of Water and Wastewater (APHA). Samples were taken at regular intervals throughout the study period. Samples were collected and shipped directly to the microbiology lab in insulated ice boxes, where the temperature was kept close to 4°C, and processed immediately after collection within 4 hours. Of all the wastewater samples, 384 were collected, of which 96 samples were collected from each treatment stage. Bacterial isolates included in the final analysis

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were recovered exclusively from influent samples. No isolates meeting the predefined culture and identification criteria were recovered from the primary sedimentation, secondary biological treatment, or final effluent samples.

The number of samples was calculated based on the study design, sampling capacity of the municipal WWTP, and the set sampling time period in advance. As such, a formal sample size calculation for numbers was not performed.

All wastewater samples were then inoculated into selective and differential culture media to isolate the targeted bacterial pathogens in the samples after primary culture. There are several target bacterial species that may be present in a single sample because municipal wastewater contains a heterogeneous mix of microorganisms. Therefore, if more than one target pathogen was isolated from the same wastewater, each isolate was treated as a new bacterial isolate, biochemically and phenotypically identified, and then subjected to antimicrobial susceptibility testing and molecular characterization. The bacterial species included in this study (*S. aureus, P. aeruginosa, A. baumannii,* and *E. faecalis*) were the only species that were not duplicates and were included in the final analysis.

Isolation and Identification of Bacterial Isolates

Identification of Gram-negative bacterial isolates

Presumptive Gram-negative bacterial isolates were initially selected by morphological characteristics of the colonies grown on the selective and differential culture media and then confirmed by Gram staining (2, 15). The species was then identified by conventional biochemical tests suitable for each species of bacteria. The tests performed included oxidase, catalase, triple sugar iron (TSI) agar reaction, Simmons citrate utilization, urease production, indole production, methyl red (MR), Voges–Proskauer (VP) reaction, motility, lysine decarboxylase and oxidative–

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fermentative (O/F) glucose utilization tests. *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *P. aeruginosa* and *A. baumannii* were definitively identified by means of the combined use of cultural characteristics, microbiological examination and biochemical profiles, according to standard microbiological procedures. The culture media, biochemical confirmation tests, and incubation conditions used for bacterial isolation and identification are summarized in Table 1.

Identification of Gram-positive bacterial isolates

Colony morphology, Gram staining, and relevant biochemical properties were used to presumptively identify Gram-positive bacterial isolates. The confirmation of *S. aureus* isolates was done by catalase and coagulase tests and growth on Mannitol Salt Agar. Isolates of *E. faecalis* were detected by the ability to grow in 6.5% sodium chloride, catalase, pyrrolidonyl arylamidase (PYR), and bile esculin hydrolysis (2, 15). Conventional biochemical tests, in conjunction with colony morphology and microscopic characteristics, were used as per standard microbiological identification procedures to confirm the identification of Gram-positive isolates. The culture media, biochemical confirmation tests, and incubation conditions used for bacterial isolation and identification are summarized in Table 1.

Table 1. Culture media, biochemical characteristics, and incubation conditions used for bacterial isolation and identification.

Bacterial species	Selective/Differential culture medium	Biochemical confirmation tests	Incubation conditions
S. aureus	Mannitol Salt Agar (Merck, Darmstadt, Germany)	Gram staining, catalase test, coagulase test, mannitol fermentation	37°C for 24 h under aerobic conditions

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P. aeruginosa	Cetrimide Agar (Merck, Darmstadt, Germany)	Gram staining, oxidase test, catalase test, oxidative fermentative (O/F) glucose test, pigment production	37°C for 24-48 h under aerobic conditions
A. baumannii	MacConkey Agar (Merck, Darmstadt, Germany)	Gram staining, oxidase test (negative), catalase test, non-motility, oxidative–fermentative (O/F) glucose test	37°C for 24 h under aerobic conditions
E. faecalis	Bile Esculin Azide Agar (Merck, Darmstadt, Germany)	Gram staining, catalase test (negative), bile esculin hydrolysis, growth in 6.5% NaCl, PYR test	37°C for 24 h under aerobic conditions

Antimicrobial susceptibility testing

Antimicrobial susceptibility patterns of all the confirmed bacterial isolates were tested by the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (Merck, Darmstadt, Germany) following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (16). After incubation, the diameters of the inhibition zones were determined, and the results were considered susceptible, intermediate, or resistant based on the CLSI interpretive criteria. Quality control strains including *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *E. faecalis* ATCC 29212 were used as standard controls for antimicrobial susceptibility testing.

Phenotypic Detection of Extended-Spectrum β-Lactamase (ESBL)

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ESBL production was phenotypically confirmed by testing for production using CLSI guidelines for combined disk diffusion (17). Briefly, ceftazidime and cefotaxime disks alone and in combination with clavulanic acid were used. Any isolate that had an increment in inhibition zone diameter around the clavulanic acid-containing disks of ≥5 mm more than the cephalosporin disks alone was considered a producer of ESBL.

Confirmed ESBL-producing strains (e.g*., Klebsiella pneumoniae* ATCC 700603) and non-ESBL-producing strains (e.g., *E. coli* ATCC 25922) were used as positive and negative controls, respectively. Because conventional ESBL phenotypic criteria are primarily established for specific bacterial groups, we reported the findings as phenotypic ESBL screening results rather than definitive evidence of ESBL-mediated resistance in species for which standardized ESBL interpretive criteria are not established.

DNA Extraction

All confirmed bacterial isolates were isolated and used to extract genomic DNA by a boiling method as previously described (18). In brief, several fresh bacterial colonies were grown on nutrient agar and suspended in sterile distilled water and then incubated at 100°C for 10 min to lyse the bacterial cells. The suspension was immediately placed on ice and spun at 12,000 rpm for 10 min. The supernatant with genomic DNA was carefully transferred to sterile microtubes and stored at −20°C for PCR analysis. DNA was extracted using a Genomic DNA Extraction Kit (Sinaclon Co., Tehran, Iran) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

PCR was used for the molecular identification of the target bacterial isolates. Species-specific primers were designed from published data and purchased. All primers used, target genes, amplicon size, and annealing temp are summarized in Table 2.

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Table 2. Primer sequences and PCR amplification conditions used for molecular identification of bacterial isolates.

Target organism	Target gene	Primer sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference
Shigella spp.	Putative integrase	**F:** CCGTCATGCTGGATGAACGATGT **R:** GTTCAGGATTGCCCGAGACACA	159	60	(19)
Salmonella spp.	*invA*	**F:** GTATTGTTGATTAATGACATCCG **R:** ATATTACGCTACGGAAACACGTT	403	60	(19-21)
E. faecalis	*D-Ala-D-Ala ligase*	**F:** ATCAAGTACAGTTAGTCTTTATTAG **R:** ACGATTCAAAGCTAACTGAATCAGT	941	52.5	(22, 23)
A. baumannii	*sp4*	**F:** CACGCCGTAAGAGTGCATTA **R:** AACGGAGCTTGTCAGGGTTA	490	60	(24)
P. aeruginosa	*PA-SS*	**F:** GGGGGATCTTCGGACCTCA **R:** TCCTTAGAGTGCCCACCCG	956	58	(25)

Primer sequences and PCR cycling conditions used in this study were adopted from previously published studies. The corresponding references for each primer set are provided in Table 2.

The reaction mixture and cycling conditions from the original publication for each of the above were used for PCR amplification in a thermal cycler. Amplified products were then subjected to agarose gel electrophoresis and the presence of the predicted DNA fragments confirmed.

PCR amplification was performed with a final reaction volume of 25 µL, consisting of 12.5 µL of 2× PCR Master Mix, 1 µL of each forward and reverse primer (10 pmol/µL), and 2 µL of template DNA. PCR amplification was carried out using a final reaction volume of 25 µL, comprising 12.5

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µL of 2× PCR Master Mix, 1 µL of each forward and reverse primer (10 pmol/µL), and 2 µL of template DNA. The cycling conditions are indicated in Table 2, and the amplification was performed. The composition of the PCR reaction mixture is presented in Table 3.

Table 3. Composition of the PCR reaction mixture

Component	Final volume (µL)
2× PCR Master Mix	12.5
Forward primer (10 pmol/µL)	1.0
Reverse primer (10 pmol/µL)	1.0
Template DNA	2.0
Nuclease-free distilled water	8.5
Total volume	25

Table 4 represents the list of primers and PCR conditions used for amplification of virulence factors and antibiotic resistance genes in the *P. aeruginosa, A. baumannii, E. faecalis* and *S. aureus* isolates.

Table 4. Primer sequences of AMR and virulence genes investigated in this study**.**

Target organism	**Target gene**	**Gene category**	**Forward primer (5'→3')**	**Reverse primer (5'→3')**	**Amplicon size (bp)**	**Annealing temperature (°C)**	**References**
P. aeruginosa	BLACTX-M	β-lactamase	ATGGATATCATCGCGAGCCT	CGACTGCGACGACTATGCAG	350	55	(26)
P. aeruginosa	TEM	β-lactamase	CTTTAGCGGCGTTGCTGG	GACGATGAGCGATCGATCGA	280	54	(26)
P. aeruginosa	SHV	β-lactamase	TCGCTGAGCAATAACAGCGA	CGACGATCGACGACGATCGA	320	53	(26)

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A. baumannii	afa/draBC	Virulence	GATCAGAGAAGAAAGTTCAA	CGAGATACGACGATCGATAG	580	54	(27)
A. baumannii	cnf1	Virulence	AGGCTTTCCAAGCTCTCACA	TGACGATACGACGATCGACG	450	55	(27)
A. baumannii	cnf2	Virulence	TGGCTGCTTCACCGTGCT	CGAGACGATCGATACGATCG	397	56	(27)
A. baumannii	csgA	Virulence	GAAAGCCAACTTCCGCGAA	GACGATACGACGATCGACGA	410	54	(27)
A. baumannii	cvaC	Virulence	CGCCTCTCGTTTTCGACA	TGACGATCGACGATACGACG	524	55	(27)
A. baumannii	iutA	Virulence	ACCACGATCGTGTACGTCAT	CGACGATACGACGATCGACT	487	54	(27)
A. baumannii	fyuA	Virulence	GCCGATACTGCGACACAAAA	TGACGATCGATACGATCGAC	400	53	(27)
A. baumannii	fimH	Virulence	GGAGCCGTCACAGCCATGAG	CGACGATACGACGATCGACA	585	55	(27)
A. baumannii	kpsMT II	Virulence	CGGACGAAATGCGCAATAAT	TGACGATCGACGATACGACG	452	54	(27)
A. baumannii	PAI	Virulence	TGACGGCGTCAGCTGACAGT	CGACGATCGACGATACGACG	536	56	(27)
A. baumannii	ibeA	Virulence	CGCCACAGTCACAAACGAAA	TGACGATACGATCGACGATA	468	55	(27)
A. baumannii	papC	Virulence	AGTGGTGATGACAGTGCTAA	CGACGATCGACGATACACGAT	501	54	(27)
A. baumannii	papG II	Virulence	TGACGCCAAACAGCGCGCTA	TGACGATACGATACGATCGA	448	56	(27)
A. baumannii	papG III	Virulence	AGGCCAGAATTCCCGAAATA	CGACGATACGATACGATACG	461	55	(27)

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A. baumannii	sfa/focDE	Virulence	GAAGCCACGGTGATACGCAT	TGACGATACGACGATCGACG	620	54	(27)
A. baumannii	traT	Virulence	ATCAGAGTATTGTACGCTAA	CGACGATACGACGATCGACA	525	53	(27)
E. faecalis	efa	Virulence	TGTCGCAGTACACACATACG	TGACGATCGACGATACGACA	375	55	(28)
E. faecalis	ebpA	Virulence	TGGTGGCCTTGGATGAGCAG	CGACGATACGACGATCGACA	410	54	(28)
E. faecalis	ebpB	Virulence	CGAAACAAGATTCTCGCTCC	TGACGATACGACGATCGACG	484	56	(28)
E. faecalis	ebpC	Virulence	CGTCTGCGACGACGTGGTAT	CGACGATACGATCGACGATA	516	55	(28)
E. faecalis	esp	Virulence	AGCGGCACATACGAGACACC	TGACGATACGATACGATCGA	372	54	(28)
E. faecalis	cylL	Virulence	TGCCGACCTGACACATTCAG	CGACGATACGACGATCGACA	388	55	(28)
E. faecalis	cylS	Virulence	TCGCGATCGGTACGCCTGGT	TGACGATACGATACGATCGA	401	56	(28)
E. faecalis	fsrA	Virulence	TGCATTCGTATCCGTCCGAC	CGACGATACGATACGATCGA	467	54	(28)
E. faecalis	fsrB	Virulence	CGCGATACGCCGAGAAATAA	TGACGATCGACGATACGACA	498	55	(28)
E. faecalis	fsrC	Virulence	AGCGGCGACAGTACACGCAT	CGACGATACGATACGATCGA	502	54	(28)
S. aureus	coa	Virulence	TGGCTGGCACAAACGAACAA	TGACGATACGACGATCGACA	325	56	(29)
S. aureus	clfA	Virulence	GGTGCAGACATACGCGCTGA	CGACGATACGACGATCGACA	445	54	(29)

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S. aureus	X-region	Typing	AGGCTTAGACAGGATGTAAA	TGACGATCGATACGACGATA	435	55	(29)
S. aureus	IgG-binding region	Typing	TGACGATAGACGCGATAGAT	CGACGATACGACGATCGACA	427	54	(29)
S. aureus	TSST-1	Toxin	TGCAGAACGCAGACAGCGAA	TGACGATACGACGATCGACA	380	53	(29)
S. aureus	eta	Toxin	CGCGATTACGACGCGTACTA	CGACGATACGACGATCGACA	320	54	(29)
S. aureus	etb	Toxin	TGACGACGCGACGCGTCTAA	TGACGATACGACGATCGACA	410	55	(29)
S. aureus	agrI	Regulatory	TGGATCCTGACTACGCTGAA	CGACGATACGATACGATCGA	365	56	(29)
S. aureus	agrII	Regulatory	CGCGATACGGATGATGCTAA	TGACGATACGATACGATCGA	380	54	(29)
S. aureus	agrIII	Regulatory	TGACCGACGCACACACGAAT	CGACGATACGATACGATACG	390	55	(29)
P. aeruginosa	exoS	Type III secretion	GGAGCCAGTGAGCAAGATCC	TGACGATCGACGATACGACA	538	54	(30)
P. aeruginosa	exoU	Type III secretion	TGAGCGGCAAAGACGACGAA	CGACGATACGACGATCGACA	446	55	(30)
P. aeruginosa	exoY	Type III secretion	GCCGATACACGCGATAGCTA	TGACGATACGACGATCGACG	392	56	(30)
P. aeruginosa	exoT	Type III secretion	TGACGCACACGAGACGACTA	CGACGATACGACGATCGACA	484	54	(30)
P. aeruginosa	toxA	Exotoxin	CGACGGCGATATCAGACACC	TGACGATACGACGATCGACA	345	55	(30)
P. aeruginosa	algD	Alginate	TGAGCGATCGCGATTACGAT	CGACGATACGACGATCGACA	520	54	(30)

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P. aeruginosa	algU	Alginate regulation	GGATACGC GACGATCAC GAC	TGACGATAC GACGATCGA CA	480	53	(30)
P. aeruginosa	lasA	Protease	TGCGATCCA CGATTACGC TA	CGACGATAC GACGATCGA CA	455	55	(30)
P. aeruginosa	lasB	Elastase	CGCGACGAT CGCGATACT GA	TGACGATAC GATACGATC GA	386	56	(30)
P. aeruginosa	plcH	Hemolytic phospholipase	TGCAGACGC GACGATAC GCT	CGACGATAC GATACGATC GA	412	54	(30)
P. aeruginosa	plcN	Non-hemolytic phospholipase	CGACGATG ACGATCGCG ATC	TGACGATAC GATACGATA CG	468	55	(30)
P. aeruginosa	phzI	Quorum sensing	CGCGATCGC GATACGCG ATA	CGACGATAC GATACGATC GA	506	54	(30)
P. aeruginosa	phzII	Phenazine	TGACGATCG ACGATCGCG AT	TGACGATAC GATACGATC GA	542	53	(30)
P. aeruginosa	phzH	Phenazine	CGCGATAG ACGACGAT GACA	CGACGATAC GATACGATC GA	394	56	(30)
P. aeruginosa	phzM	Phenazine	TGACGATAG ACGATCGAC GA	TGACGATAC GATACGATA CG	421	54	(30)
P. aeruginosa	phzS	Phenazine	CGCGATACG CGACGATCG CA	CGACGATAC GATACGATA CG	459	55	(30)
P. aeruginosa	pvdA	Pyoverdine	TGCGATCGA CGATACGCG AT	TGACGATAC GATACGATC GA	475	56	(30)
P. aeruginosa	pilA	Pilus	GGACGATC GACGACGA TGAC	CGACGATAC GATACGATC GA	388	54	(30)
P. aeruginosa	pilB	Pilus biogenesis	TGCGATACG CGATACGAT CG	TGACGATAC GATACGATC GA	425	55	(30)
P. aeruginosa	apr	Alkaline protease	CGACGACG ATCGATACG CGA	CGACGATAC GATACGATC GA	486	54	(30)

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P. aeruginosa	nanI	Neuraminidase	TGCGATCGACGACGATACGA	TGACGATACGATACGATACG	392	53	(30)

Agarose Gel Electrophoresis

The PCR Products were run on a 2% agarose gel in 1× TAE buffer. About 5 µL of the amplified product was combined with loading dye and loaded in the wells. To determine the size of the amplified fragments, a DNA ladder was added to each run. Positive controls consisted of reference strains known to carry the target genes, including *P. aeruginosa* ATCC 27853 (for exoS, plcH, etc.), *A. baumannii* ATCC 19606 (for blaOXA-51 and fimH), *E. faecalis* ATCC 29212 (for efa, ebp genes), and *S. aureus* ATCC 25923 (for clfA, agr genes). Molecular-grade water was used as the negative control. The electrophoresis was carried out at 100 V, and the amplified fragments of the DNA were observed under UV light with a gel documentation system (18).

Statistical Analysis

Data obtained were analyzed using SPSS v16 statistical software. Results were summarized using descriptive statistics, including frequencies, percentages, means, and modes.

Results

During the study period, a total of 195 bacteria were isolated from the municipal wastewater samples. The isolates included *S. aureus* (n = 89), *E. faecalis* (n = 36), *P. aeruginosa* (n = 36), and *A. baumannii* (n = 34). The results of the distribution of bacterial isolates are shown in Table 5.

Table 5. Distribution of bacterial isolates recovered from municipal wastewater samples**.**

Bacterial species	Number of isolates (n)	Percentage (%)
S. aureus	89	45.6

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E. faecalis	36	18.5
P. aeruginosa	36	18.5
A. baumannii	34	17.4
Total	195	100

Phenotypic Detection of ESBL-Producing Isolates

Among the 195 bacterial isolates, 89 (45.6%) were positive, and 106 (54.4%) were negative in the phenotypic ESBL screening assay. *P. aeruginosa* (83.3%), *S. aureus* (66.3%), *A. baumannii* (58.8%), and *E. faecalis* (25.0%) showed the highest prevalence of ESBL production. The frequencies of ESBL-positive and ESBL-negative isolates by bacterial species are presented in Table 6.

Table 6. Results of phenotypic ESBL screening among bacterial isolates**.**

Bacterial species	**ESBL positive n (%)**	**ESBL negative n (%)**
A. baumannii	20 (58.8)	14 (41.2)
E. faecalis	9 (25.0)	27 (75.0)
S. aureus	59 (66.3)	30 (33.7)
P. aeruginosa	30 (83.3)	6 (16.7)
Total	89 (45.6)	106 (54.4)

Detection of β-Lactamase Genes

The presence of the β-lactamase-associated genes BLACTX-M, BLATEM, and BLASHV was investigated by PCR among the isolates that were positive in phenotypic ESBL screening. The most commonly isolated β-lactamase gene in *A. baumannii* isolates was BLACTX-M

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(29.4%), followed by BLASHV (23.5%) and BLATEM (5.8%). The distribution of these β-lactamase genes among ESBL-producing *A. baumannii* isolates is presented in Table 7.

Table 7. Frequency of β-lactamase genes among ESBL-producing *A. baumannii* isolates.

Gene	**Positive isolates (n)**	**Percentage (%)**
BLACTX-M	10	29.4
BLATEM	2	5.8
BLASHV	8	23.5

Within Pseudomonas aeruginosa, BLACTX-M was the most commonly detected β-lactamase gene (36.1%), followed by BLATEM (27.7%) and BLASHV (19.4%), as shown in Table 8.

Table 8. Frequency of β-lactamase genes among ESBL-producing *P. aeruginosa* isolates**.**

Gene	Positive isolates (n)	Percentage (%)
BLACTX-M	13	36.1
BLATEM	10	27.7
BLASHV	7	19.4

Distribution of Virulence Genes

The distribution of virulence genes was different for the various bacterial species studied. fimH, afa/draBC, and fyuA were the most common virulence genes detected in *A. baumannii* isolates (82.3%, 55.8%, and 55.8%, respectively). None of the isolates contained the csgA, iutA, PAI, or ibeA genes. Table 9 presents the distribution of these virulence genes.

Table 9. Frequency of virulence genes detected in *A. baumannii*.

Gene	**Positive isolates (n)**	**Percentage (%)**
afa/draBC	19	55.8
cnf1	10	29.4

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cnf2	12	35.2
csgA	0	0
cvaC	4	11.7
iutA	0	0
fyuA	19	55.8
fimH	28	82.3
kpsMT II	6	17.6
PAI	0	0
ibeA	0	0
papC	5	14.7
PapG II	6	17.6
PapG III	2	5.8
sfa/focDE	18	52.9
traT	2	5.8

In *E. faecalis*, the efa gene was detected in all isolates (100%), followed by ebpA (88.8%), ebpB (77.7%), ebpC (61.1%), esp (58.3%), fsrA (50.0%), fsrB (44.4%), cylS (38.8%), cylL (33.3%), and fsrC (27.7%), as shown in Table 10.

Table 10. Frequency of virulence genes detected in *E. faecalis.*

Gene	**Positive isolates (n)**	**Percentage (%)**
efa	36	100
ebpA	32	88.8
ebpB	28	77.7
ebpC	22	61.1
esp	21	58.3
cylL	12	33.3
cylS	14	38.8
fsrA	18	50.0

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fsrB	16	44.4
fsrC	10	27.7

Among *S. aureus* isolates, clfA was the most frequently detected virulence gene (16.8%), followed by agrIII (8.9%), etb (6.7%), agrII (5.6%), agrI (4.4%), eta (3.3%), and TSST-1 (2.4%). No coa, X-region or IgG-binding region genes were found. The corresponding frequencies for *S. aureus and P. aeruginosa* are presented in Table 11 and Table 12.

Among *P. aeruginosa* isolates, exoS was the predominant virulence gene (80.5%), followed by plcH (50.0%), plcN (47.2%), exoY (36.1%), pilA (30.5%), pvdA (27.7%), phzII (22.2%), pilB (22.2%), toxA (19.4%), algU (19.4%), exoT (19.4%), phzM (11.1%), lasB (11.1%), algD (8.3%), apr (8.3%), nanI (8.3%), exoU (5.5%), phzI (5.5%), lasA (5.5%), phzH (2.7%), and phzS (2.7%).

Table 11. Frequency of virulence genes detected in *S. aureus***.**

Gene	Positive isolates (n)	Percentage (%)
coa	0	0
clfA	15	16.8
X-region	0	0
IgG-binding region	0	0
TSST-1	2	2.4
eta	3	3.3
etb	6	6.7
agrI	4	4.4
agrII	5	5.6

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agrIII	8	8.9

Table 12. Frequency of virulence genes detected in *P. aeruginosa***.**

Gene	Positive isolates (n)	Percentage (%)
exoT	7	19.4
exoY	13	36.1
exoS	29	80.5
exoU	2	5.5
phzH	1	2.7
apr	3	8.3
phzII	8	22.2
phzM	4	11.1
phzI	2	5.5
phzS	1	2.7
lasA	2	5.5
lasB	4	11.1
pvdA	10	27.7
pilA	11	30.5
pilB	8	22.2
toxA	7	19.4
algD	3	8.3
algU	7	19.4
plcH	18	50.0

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plcN	17	47.2
nanI	3	8.3

Representative agarose gel electrophoresis of PCR amplification products of selected *P. aeruginosa* virulence genes is shown in **Figure 1**.

Figure 1: Representative agarose gel electrophoresis of PCR amplification products of selected *P. aeruginosa* virulence genes.

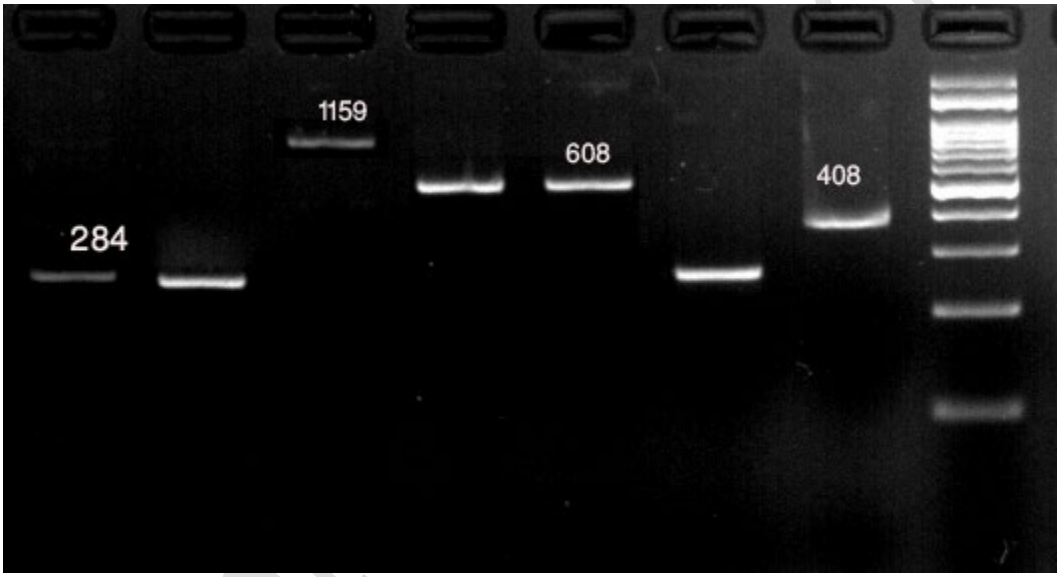

Figure 1. Representative agarose gel electrophoresis of PCR amplification products of selected *P. aeruginosa* virulence genes. PCR products were separated by agarose gel electrophoresis and visualized under UV illumination after staining. M indicates the DNA molecular weight marker. The expected amplicon sizes of the amplified products (284, 408, 608, and 1159 bp) are indicated above the corresponding bands. This figure represents successful amplification of representative virulence genes analyzed in the present study.

Discussion

As part of the present study, the antibiotic-resistant bacteria and virulence determinants in municipal wastewater were monitored. *S. aureus* (45.6%), *E. faecalis* (18.5%), *P. aeruginosa* (18.5%), and *A. baumannii* (17.4%) were the most common of the 195 isolates. 45.6% (89) of the isolates were capable of producing ESBL, highlighting wastewater as an important reservoir and dissemination route for AMR. ESBL production was highest in *P. aeruginosa* (83.3%), followed by *S. aureus* (66.3%), *A. baumannii* (58.8%) and *E. faecalis* (25.0%). The high rate in *P. aeruginosa* may be due to the ability to acquire and horizontally transfer resistance determinants and intrinsic resistance (31), and due to the environmental adaptability of this species.

The most common ESBL gene detected was BLACTX-M, which was found in 36.1% of *P. aeruginosa* and 29.4% of *A. baumannii*, which is in concert with the global predominance of BLACTX-M as an environmental ESBL (32). BLASHV and BLATEM were detected in *A. baumannii* (23.5% and 5.8%, respectively) and BLACTX-M and BLATEM in *P. aeruginosa* (36.1% and 27.7%, respectively). Several ESBL genes can be detected, suggesting plasmid- or chromosome-mediated acquisition and horizontal transfer and heightened resistance (33, 34).

In the case of *A. baumannii*, fimH was the most common virulence gene (82.3%), which has been associated with adhesion and biofilm formation (35). Other adhesion and iron-acquisition mechanisms, afa/draBC and fyuA, were found in 55.8% and 52.9%, respectively, of the isolates (36). In contrast, csgA, iutA, PAI, and ibeA were not present, which may indicate different environmental genetic backgrounds.

All *E. faecalis* isolates (100%) were found to carry efa, and ebpA, ebpB and ebpC were detected in 88.8%, 77.7% and 61.1% of the isolates, respectively (37). The presence of the esp gene was found in 58.3%, the cylS gene in 38.8%, and the cylL gene in 33.3%. Of these isolates, 50.0% and

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44.4% contained fsr quorum-sensing genes, indicating that there are multiple virulence mechanisms in place (38).

S. aureus had lower rates of virulence genes. clfA was the most common (16.8%), followed by agrIII at 2.4–8.9%, etb, agrII, agrI, eta, and TSST-1. The lack of coa, X-region, and IgG-binding region genes, and the rarity of agr locus occurrence, may suggest that the bacteria in the environment have different genetic traits from highly virulent clinical isolates.

P. aeruginosa was the most virulent with the highest number of virulence determinants. In 80.5% of the occurrences, exoS was detected, indicating the high pathogenicity potential of this bacterium (39). The plcH and plcN genes were found in 50.0% of samples and 47.2% of samples, respectively, and exoY was found in 36.1% and exoU in only 5.5% of samples. Phenazine-associated genes occurred at 22.2% (phzII), 11.1% (phzM), 5.5% (phzI), and 2.7% (phzH), while pvdA occurred in 27.7%. Pilus genes of type IV, pilA and pilB, were found in 30.5% and 22.2%, respectively. The algU and algD genes were present in 19.4% and 8.3%, respectively, suggesting alginate production ability and biofilm formation ability.

Overall, the results indicate the potential of WWTPs as reservoirs, amplification sites, and dissemination pathways for ARB and virulence determinants. The ESBL rate in influent (45.6%) is higher than those typically seen in community-acquired infections and is indicative of high selection pressure in wastewater (40). AMR genes may be present in combination with multiple virulence factors, which further enhances the potential public health threat via contaminated water, recreation and irrigation (41).

The high prevalence of BLACTX-M, BLASHV and BLATEM is consistent with other wastewater studies which found these three too often coexist on plasmids (42). Plasmid-mediated transfer can aid in transfer to other species of bacteria, and combinations of virulence determinants can enhance

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pathogenicity, as well as increase the potential for multidrug resistance (43). The wastewater resistome and mobilome need to be characterized using metagenomic and functional metagenomic approaches, and whole-genome sequencing could help to clarify the resistance plasmids, insertion sequences, and genomic islands and horizontal gene transfer mechanisms (44, 45).

Conventional treatment may be ineffective at removing the resistant bacteria and resistance genes, as *P. aeruginosa* and *A. baumannii* are prevalent and ESBL-producing (ESBL-PA and ESBL-AB, respectively) (41). Therefore, advanced oxidation, membrane filtration, and improved disinfection may minimize the release of advanced oxidation products into receiving waters (46). The high prevalence of efa in *E. faecalis* and the presence of adhesion- and biofilm-formation-related genes are also alarming, as they enable biofilm development that further enhances AMR and antimicrobial persistence (47). The coexistence of resistance and virulence factors can raise the threat for vulnerable groups (48).

A One Health approach identifies wastewater as a nexus of human, animal, and environmental sources of AMR, and wastewater can be an early warning system for emerging resistance (42). Culture-dependent and culture-independent approaches to systematic surveillance may help to detect resistance mechanisms early and provide public health evidence-based interventions. This diversity of ESBL combinations and virulence profiles indicates that genetic exchange within wastewater is still occurring. The occurrence of BLACTX-M-associated resistance in both *P. aeruginosa* and *A. baumannii* may be due to plasmid-mediated acquisition and horizontal transfer from environmental bacterial flora (49). Also, ESBL-producing bacteria in sewage sludge are an indication of adaptation to sewage and possibly environmental dissemination (32).

Conclusion

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The present study identified a high frequency of phenotypic ESBL-positive results among the recovered bacterial isolates from municipal wastewater and urban wastewater; a variety of virulence genes associated with ESBL-producing bacteria were recovered. Wastewater has emerged as a significant environmental source of AMR, especially for BLACTX-M, and also of bacterial pathogenicity, with multiple virulence determinants commonly found in the bacteria isolated from the wastewater samples of *P. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus*. The results highlight the critical need to improve wastewater treatment technologies, broaden routine environmental monitoring of AMRs, and develop comprehensive public health approaches to reduce the environmental spread of antimicrobial-resistant bacteria. Long-term longitudinal surveillance of AMR and functional studies of resistance plasmids and virulence determinants, combined with whole-genome sequencing, will generate more information on the ecology and evolution of AMR and help inform effective intervention strategies to reduce AMR dissemination.

Conflict of Interest Disclosure

The authors declare no conflict of interest.

Ethical Approval

Prior to conducting the study, all necessary approvals were obtained from the Ethics Committee of Islamic Azad University, Shahrekord Branch. The Ethics Committee of Islamic Azad University, Shahrekord Branch reviewed and approved the research protocol (IR.IAU.SHK.REC.1403.044).

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