Jundishapur J Microbiol. 2024 April; 17(4): e145649. https://doi.org/10.5812/jjm-145649.

Published online: 2024 May 28. **Research Article**

Phenotypic and Genotypic Antibiotic Resistance Patterns of Clinical *Shigella* Isolates from Tehran, Iran

Saeede Tahmasbi [1], Akram Sadat Tabatabaee Bafroee [1,*], Babak Pakbin [2]

[1] Department of Biology, East Tehran Branch, Islamic Azad University, Tehran, Iran
[2] Werner Siemens Chair of Synthetic Biotechnology, Department of Chemistry, Technical University of Munich (TUM), Lichtenberg Str. 4, 85748 Garching bei München, Germany

[*] *Corresponding author:* Department of Biology, East Tehran Branch, Islamic Azad University, Tehran, Iran. Email: akram_tabatabaee@yahoo.com

Received 2024 February 6; **Revised** 2024 April 20; **Accepted** 2024 April 29.

Abstract

Background: The emergence of multidrug-resistant (MDR) *Shigella* is a health concern in both developed and developing countries. The indiscriminate use of antibiotics without considering resistance patterns has led to the emergence of resistant strains.

Objectives: This study aimed to investigate the phenotypic and genotypic patterns of antibiotic resistance and virulence gene distribution in *Shigella* isolates from patients in Tehran, Iran.

Methods: In this cross-sectional study, 60 *Shigella* isolates were collected from patients referred to Milad Hospital and Pasargad Laboratory in Tehran. The isolates were gathered over four months, from February 2023 to June 2023. After biochemical and genetic confirmation of the isolates, the disk diffusion method was employed to determine antibiotic resistance patterns. The distribution of beta-lactamase genes (*bla* $_{\text{CTX-M1}}$, *bla* $_{\text{CTX-M2}}$, *bla* $_{\text{CTX-M8}}$, *bla* $_{\text{SHV}}$, *bla* $_{\text{TEM}}$, and *bla* $_{\text{OXA}}$) and virulence genes (*ial, virF, invE, sigA*, and *pic*) was investigated in *Shigella* isolates using the multiplex PCR method. SPSS statistical software version 22 was used for data analysis.

Results: Thirty-six percent of isolates were found to be MDR. The highest rate of resistance was against tetracycline (90%) and ampicillin (80%). The lowest resistance rate was against azithromycin (1.9%) and amikacin (7.1%). The *bla* $_{\text{CTX-M1}}$ and *bla* $_{\text{CTX-M8}}$ genes were more prevalent, detected in 38.3% and 50% of the isolates, respectively. However, the *bla* $_{\text{SHV}}$ and *bla* $_{\text{CTX-M2}}$ genes were not detected in any of the isolates. Additionally, 32% of the MDR isolates harbored both the *bla* $_{\text{CTX-M1}}$ and *bla* $_{\text{CTX-M8}}$ genes simultaneously. The distribution of virulence genes *ial*, *virF*, *invE*, *sigA*, and *pic* in the studied isolates was 28.3%, 85%, 68.3%, 81.7%, and 15%, respectively.

Conclusions: The present study emphasizes the increasing trend of MDR *Shigella* isolates. Therefore, serious measures are needed to prevent the spread of resistant genes. Furthermore, the detection of virulence factors could help in controlling shigellosis, which is a significant global health issue.

Keywords: *Shigella*,Antibiotic Resistance, Cephalosporin, ESBL, Virulence Genes

1. Background

Shigellosis is a type of diarrhea known as bacillary dysentery caused by the Gram-negative rod-shaped bacterium *Shigella* spp. (1). This type of diarrhea can progress to a more severe form containing bloody mucus, which is associated with morbidity and mortality, especially in young children in developing countries (2). The infectious dose of *Shigella* spp. can be very low, as few as 10 organisms, facilitating person-to-person transmission (3). The *Shigella* genus consists of four subgroups based on biochemical and serological properties: (A) (*Shigella dysenteriae*), (B) (*S. flexneri*), (C) (*S. boydii*), and (D) (*S. sonnei*). Each subgroup can cause shigellosis, but *S. flexneri* and *S. sonnei* are the two major causes of the disease, especially in developing and industrialized countries (4). Antibiotics can help shorten the duration of illness and reduce the risk of spreading *Shigella* bacteria when treating patients with

shigellosis. Several different types of antibiotics are effective in treating this infection (5).

Fluoroquinolones, cephalosporins, and sulfonamides are the drugs typically used to treat acute diarrhea caused by *Shigella* spp., according to current medical guidelines (6). However, the overuse of antibiotics, along with the emergence of *Shigella* strains that are resistant to multiple drugs, has reduced the number of treatment options for *Shigella* infections due to various resistance mechanisms (7-9). There are different mechanisms by which *Shigella* spp. can develop drug resistance, such as drug efflux, decreased cellular permeability, drug modification or inactivation by producing enzymes, and drug target mutations (10, 11). The main reason gram-negative bacteria are resistant to beta-lactam antibiotics is the production of extended-spectrum beta-lactamase enzymes (ESBLs). These enzymes can break down penicillins and first-, second-, and third-generation cephalosporins (12).

The distribution of virulence genes among different types of *Shigella* spp. can cause varying symptoms and outcomes (13). The *Ipa* and *ial* genes are important in helping *Shigella* cells invade the intestinal epithelium (14). The *VirF* and *VirB* (*InvE*) genes are involved in regulating invasion-related genes (15). SPATE genes are a group of genes that can be divided into class 1 and class 2. SPATE class 1 genes include *sigA* and *sat*, and SPATE class 2 genes include *sepA* and *pic*. These genes work together to allow *Shigella* to survive and cause colonization (16).

Karimi Yazdi et al., in their study focused on pediatric patients under 14 years of age in Iran, found that *S. sonnei* was the dominant species isolated from children with shigellosis, confirming the findings of Abbasi et al.. These kinds of studies highlight the high prevalence of multidrug resistance among *Shigella* isolates in Iran and emphasize the importance of continuous monitoring of antibiotic resistance patterns in different regions of the country (17, 18).

2. Objectives

Understanding the antibiotic resistance pattern and the relationship between virulence genes and antimicrobial resistance in *Shigella* spp. is critical for developing new and effective treatments for shigellosis. In this study, after determining the antibiotic resistance pattern of *Shigella* clinical isolates to different antibiotic groups, the presence of antibiotic resistance genes (ARGs), including $bla_{\text{CTX-M1}}$, $bla_{\text{CTX-M2}}$, $bla_{\text{CTX-M8}}$, bla_{SHV}, bla_{TEM}, and bla_{OXA} in *Shigella* spp., was investigated. Finally, the distribution of five virulence genes (*ial, virF, invE, sigA*, and *pic*) in the studied *Shigella* isolates was evaluated.

3. Methods

3.1. Isolation and Identification of Shigella spp.

In this cross-sectional study, 60 *Shigella* isolates were collected from the microbiology laboratories of two hospitals (Imam Khomeini and Milad) in Tehran, Iran, from February 2023 to June 2023. These isolates had been previously obtained from stool samples of patients ranging in age from under one year to 32 years old. *Shigella* isolates were checked using standard biochemical tests (urease, carbohydrate fermentation test, H_2S, methyl red, motility, citrate utilization, and indole) and molecular tests (amplifying the *ipa*H gene) (6). All isolates were then maintained in Brain Heart Infusion (BHI) Broth containing 20% glycerol at -70°C.

3.2. Antimicrobial Susceptibility Testing

The antibiotic susceptibility of *Shigella* isolates was assessed using the Kirby-Bauer disk diffusion technique on Muller Hinton agar (MHA; Merck, Darmstadt, Germany) according to the Clinical and Laboratory Standards Institute (CLSI, 2021) guidelines (19). Ten different antibiotic disks (Padtanteb, Iran), representing eight classes, were used, including aminoglycosides (amikacin-30 µg, kanamycin-30 µg, and gentamicin-10 µg), carbapenems (imipenem-10 µg), macrolides (azithromycin-15 µg), tetracyclines (tetracycline-30 µg), penicillins (ampicillin-10 µg and amoxicillin-25 µg), fluoroquinolones (nalidixic acid-30 µg and norfloxacin-10 µg), cephalosporins (cefoxitin-5 µg and cefepime-30 µg), and chloramphenicol-30 µg. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 strains were used as reference strains. Isolates resistant to more than three classes of antibiotics were classified as multidrug-resistant (MDR) (20), and the following formula (21) was used to determine the multiple antibiotic resistance (MAR) index of isolates: MAR = (The number of antimicrobial agents to which a particular *Shigella* isolate shows resistance/the total number of antibiotics that were utilized in treating an isolate).

3.3. Molecular Detection of Beta-lactamase Resistance Genes and Virulence Genes Using Multiplex PCR

3.3.1. DNA Extraction

Genomic DNA was extracted from the bacterial samples using a commercial Genomic DNA extraction Kit (cat No: DM05050, Gene Transfer Pioneer, Pishgaman Co, Iran). The extraction procedure was performed according to the manufacturer's instructions. *DNA* concentration and *quality* were determined with a NanoDrop 2000 Spectrophotometer (Thermo Fisher, USA) and examined by gel electrophoresis.

3.3.2. Multiplex PCR Technique

The beta-lactamase genes *bla* $_{CTX-M1}$, *bla* $_{CTX-M2}$, *bla* $_{CTX-M8}$, *bla* $_{SHV}$, *bla* $_{TEM}$, and *bla* $_{OXA}$ were simultaneously investigated using specific primers and two sets of multiplex PCR techniques (Table 1). Furthermore, the *ipaH* gene encoding an E3 ubiquitin-ligase in the *Shigella* family was considered an internal positive control (Table 1). The validity of all primers was checked using the Blast databases available from NCBI, and they were synthesized by the oligonucleotide DNA synthesis service of Macrogen Inc., South Korea. Positive controls were sequencing confirmed and applied for the targeted beta-lactamase genes. A PCR reaction mixture was prepared by blending 2 µL of DNA template (concentration 50 ng/µL) with 10 µL of 2X master mix containing the standard buffer and 0.7 µL of each of the six primer pairs. This PCR mixture had a final volume of 20 µL.

Thermal cycling conditions for PCR were as follows: Initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s. For the set of *bla* $_{SHV}$, *bla* $_{TEM}$, and *bla* $_{OXA}$ genes, the annealing temperature was set at 55.5°C for 30 seconds. For the set of *bla* $_{CTX-M1}$,*bla* $_{CTX-M2}$, and *bla* $_{CTX-M8}$ genes, the annealing temperature was set at 57°C for 30 seconds. This was followed by a primer extension step at 72°C for 1 minute. After the last PCR cycle, an additional incubation step was included to complete the chain extension. This step included incubation at 72°C for 5 min. To analyze the PCR products, the multiplex PCR mixture was subjected to electrophoresis using 1.5% (w/v) agarose gel with a voltage of 110 V for 60 min. After the electrophoresis process, the DNA fragments in the gel were observed using a UV transilluminator (Protein Simple, Red imagen SA-1000). Two strains of *S. sonnei* ATCC 25931 and *Escherichia coli* ATCC 25922 were used as negative and positive controls, respectively.

To determine the distribution of virulence genes, including *ial*, *virF*, *invE*, *sigA*, and pic, a multiplex PCR assay and specific primers (Table 1) were applied. For this purpose, a new mixture was prepared. This 20 µL multiplex PCR mixture consisted of 2 µL of DNA template (concentration of 50 ng/µL), 10 µL of 2X master mix with the standard buffer, and 1 µL of each of the four primer pairs. The thermal cycling conditions were set as follows: Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s. The next steps included primer annealing and extension. For the annealing step, the temperature was set at 58°C for 30 s. This was followed by a primer extension step at 72°C for 1 min. After the cycling conditions mentioned earlier, an additional incubation step was included to complete the chain extension. This step consisted of incubation at 72°C for 7 min following the last PCR cycle. Then, multiplex PCR products of three genes including *virF*, *invE*, and *sigA* were purified for sequencing and sent to Niagenenoor Corporation, Tehran, Iran, along with the used primers. Online BLAST software was employed to examine the sequences in the NCBI database (https://www.ncbi.nlm.nih.gov/BLAST/).

3.4. Statistical Analysis

SPSS statistical software version 22 was used for data analysis. The chi-square test and Cramer's V test were employed to determine the significance and strength of the associations between antibiotic resistance genes and the antibiotic resistance observed in the studied bacterial samples. The P-value was set at less than 0.05.

4. Results

4.1. Isolation and Identification of Shigella spp.

Sixty bacterial isolates were confirmed as *Shigella* spp. after performing the morphological, biochemical and molecular tests.

4.2. Antibiotic Resistance Pattern

All *Shigella* isolates in this research were assessed separately for susceptibility to common clinically relevant antibiotics (Table 2 and Figure 1). Out of 60 isolates, 93% showed resistance to amoxicillin (56/60), 90% were resistant to tetracycline (54/60), and 80% were resistant to ampicillin (48/60). Resistance to nalidixic acid and chloramphenicol was observed in 33.3% (20/60) and 23.3% (14/60) of isolates, respectively. Twenty percent of isolates showed resistance to imipenem, cefepime,

Table 1. Sequences of Primers used for Detecting β-lactamase Genes, Virulence Genes, and *IpaH* Genes in Studied *Shigella* Isolates and Their PCR Product Length

Genes Group	Primer Sequence (5' - 3')	PCR Product Length (bp)	Reference
β-lactamase			
TEM	F: TCGGGAAATGTGCG	857	(22)
	R: GGGTTTGATACCGGCACCCGT		
OXA	F: GCAGCGCCAGTGCATCAAC	198	(23)
	R: CCGCATCAAATGCCATAAGTG		
SHV	F: GCCGGGTTATTCTTATTTGTCGC	768	(24)
	R: ATGCCGCCGCCAGTCA		
CTX-M1	F: CGTCACGCTGTTGTTAGGAA	593	(25)
	R: ACGGCTTTCTGCCTTAGGTT		
CTX-M2	F: TTAATGATGATCTCAGAGCATTC	901	(26)
	R: GATACCTCGCTCCATTTATTG		
CTX-M8	F: CGCTTTGCCATGTGCAGCACC	307	(26)
	R: GCTCAGTACGATCGAGCC		
Virulencefactors			
Ial	F: GCTATAGCAGTGACATGG	320	(27)
	R: ACGAGTTCGAAGCACTC		
Vir	F: AGCTCAGGCAATGAAACTTTGAC	618	(27)
	R: TGGGCTTGATATTCCGATAAGTC		
InvE	F: CGATAGATGGCGAGAAATTATATCCCG	766	(28)
	R: CGATCAAGAATCCCTAACAGAAGAATCA		
SigA	F: CCGACTTCTCACTTTCTCCCG	430	(27)
	R: CCATCCAGCTGCATAGTGTTTG		
Pic	F: ACTGGATCTTAAGGCTCAGGAT	572	(27)
	R: GACTTAATGTCACTGTTCAGCG		
Ipa			
IpaH	F: GCTGGAAAAACTCAGTGCCT	423	(29)
	R: CCAGTCCGTAAATTCATTCT		

and azithromycin (12/60). Regarding other tested antibiotics, 11.7% were resistant to amikacin (7/60), 3.3% were resistant to kanamycin and gentamicin (2/60), and 1.7% showed resistance to norfloxacin and cefoxitin (1/60). Additionally, among the sixty isolates, 36.66% were determined to be MDR. The most common MDR pattern was simultaneous resistance to chloramphenicol, ampicillin, tetracycline, and amoxicillin (Table 3).

4.3. Antimicrobial Resistance Genes Identified in *Shigella spp.*

All 60 *Shigella* isolates were tested for the presence of the *bla* $_{\text{CTX-M1}}$, *bla* $_{\text{CTX-M2}}$, *bla* $_{\text{CTX-M8}}$, *bla* $_{\text{SHV}}$, *bla* $_{\text{TEM}}$, and *bla* $_{\text{OXA}}$ genes. Figures 2 and 3 display the PCR gel electrophoresis results of these six resistance genes. All isolates were negative for *bla* $_{\text{CTX-M2}}$ and *bla* $_{\text{SHV}}$, but 30 (50%) isolates harbored *bla* $_{\text{CTX-M1}}$. The frequency of the *bla*CTX-M8 gene was 38.3% (23 isolates). Only two (3.33%) isolates contained the *bla* $_{\text{TEM}}$ gene, and five isolates (8.33%) carried *bla* $_{\text{OXA}}$. The *bla* $_{\text{CTX-M1}}$ gene had a significantly higher frequency than the *bla* $_{\text{CTX-M8}}$ gene. The results of multiplex PCR indicated the presence of 16 patterns in terms of carrying resistance genes in the 60 investigated *Shigella* isolates. Table 4 displays the occurrence of ARGs in Shigella isolates.

4.4. Distribution and Prevalence of Virulence Genes

During the present investigation, all 60 *Shigella* isolates were subjected to PCR to detect five virulence genes, including *ial*, *virF*, *invE*, *sigA*, and *pic*. Figure 4 displays the PCR gel electrophoresis results of these five virulence genes. The distribution of virulence genes *ial*, *virF*, *invE*, *sigA*, and *pic* in the studied isolates was 28.3% (17/60), 85% (51/60), 68.3% (41/60), 81.7% (49/60), and 15% (9/60), respectively. Table 3 also shows the multidrug

Table 2. Drug Resistance Rate of *Shigella* Isolates

Antibiotics	*Shigella* Isolates Total (n = 60) [a]	No. of Isolates	Valid Percent
Norfloxacin	1.7	1	1.7
Gentamicin	3.3	2	3.3
Kanamycin	3.3	2	3.3
Cefepime	20	12	20
Amoxicillin	93.3	56	93.3
Cefoxitin	1.7	1	1.7
Nalidixic acid	33.3	20	33.3
Tetracycline	90	54	90
Ampicillin	80	48	80
Azithromycin	20	12	20
Imipenem	20	12	20
Amikacin	11.7	7	11.7
Chloramphenicol	23.3	14	23.3

[a] Values are expressed as (%).

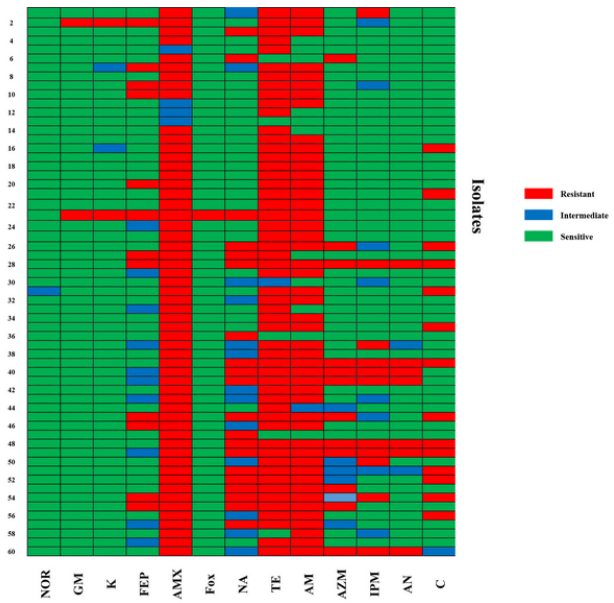

Figure 1. Antibiogram profiles of 60 *Shigella* isolates. NOR, norfloxacin; GM, gentamicin; K, kanamycin; FEP, cefepime; AMX, amoxicillin; FOX, cefoxitin; NA, nalidixic acid; TE, tetracycline; AM, ampicillin; AZM, azithromycin; IPM, imipenem; AN, amikacin; C, chloramphenicol

resistance profile of the *Shigella* isolates based on virulence genes.

4.5. Sequencing Results

Multiplex PCR products of three genes, including *virF*, *invE,* and *sigA*, were purified for sequencing along with the used primers. The sequences were evaluated against all nucleotide accessions available at the NCBI GenBank DNA Database using online BLAST software. The sequences of *virF*, *invE*, and *sigA* have been submitted to the GenBank database with accession numbers CP055137.1, CP055125.1, and CP055125.1, respectively.

Table 3. Multidrug Resistance Profile of the *Shigella* Isolates (n = 60) Based on the Distribution of *bla* Genes and Virulence Genes [a]

Pattern No.	Antibiotic Resistance Patterns	No. of Isolates	Overall MDR Isolates	Beta-lactamase Genes	Virulence Genes
1	C, AN, IPM, AZM, AM, TE, NA, AMX, FEP	1	22/60 (36.66%)	*bla* $_{CTX-M8}$, *bla* $_{OXA}$	*invE, virF, ial*
2	AM, TE, NA, FOX, AMX, FEP, K, GM	1		*bla* $_{CTX-M1}$, *bla* $_{CTX-M8}$	*invE, virF, sigA*
3	C, AN, IPM, AZM, AM, TE, NA, AMX	2		-ND	*invE, virF, sigA, ial*
4	C, IPM, TE, AM, NA, AMX, FEP	1		*bla* $_{CTX-M1}$, *bla* $_{CTX-M8}$	*virF, sigA*
5	C, AN, IPM, AM, TE, NA, AMX	1		-	*invE, virF, sigA, pic*
6	C, AZM, AM, TE, NA, AMX	2		*bla* $_{CTX-M1}$, *bla* $_{CTX-M8}$	*invE, virF, sigA*
7	AN, IPM, AZM, AM, TE, AMX	2		*bla* $_{CTX-M1}$	*invE, virF, sigA, pic*
8	AZM, TE, AM, NA, AMX	1		*bla* $_{CTX-M1}$, *bla* $_{CTX-M8}$	*virF, sigA*
9	AZM, TE, AM, AMX, FEP	1		*bla* $_{CTX-M8}$	*virF, pic*
10	C, AM, TE, NA, AMX	1		*bla* $_{CTX-M1}$, *bla* $_{CTX-M8}$	*virF, sigA*
11	TE, NA, AMP, FEP	1		-	*invE, virF, sigA, ial*
12	C, AM, TE, AMX	4		*bla* $_{CTX-M1}$	*invE, virF, sigA*
13	C, AM, TE, NA	1		*bla* $_{CTX-M1}$, *bla* $_{CTX-M8}$	*virF*
14	AM, TE, NA, AMX	1		*bla* $_{CTX-M1}$	*invE, virF, sigA, ial*
15	AM, TE, FEP, K	1		*bla* $_{CTX-M1}$	*invE, virF, sigA*
16	AZM, NA, AMX	1		*bla* $_{CTX-M8}$	*invE, virF, sigA*

Abbreviations: ND, no detected; NOR, norfloxacin; GM, gentamicin; K, kanamycin; FEP, cefepime; AMX, amoxicillin; FOX, cefoxitin; NA, nalidixic acid; TE, tetracycline; AM, ampicillin; AZM, azithromycin; IPM, imipenem; AN, amikacin; C, chloramphenicol.

[a] In this table, the antibiotic resistance patterns of 60 *Shigella* isolates are presented alongside their corresponding *bla* genes and virulence genes.

4.6. Investigating the Association Between Antibiotic Resistance Genes and the Phenotype of Antibiotic Resistance in Shigella Isolates

To investigate the association between the presence of ARGs and the phenotype of antibiotic resistance (IPM, AM, AMX, FOX, FEP), *Shigella* isolates were analyzed via chi-square and Cramer's V tests. Based on the results, significant and moderate associations were found between the imipenem and amoxicillin resistance phenotypes and the presence of the *bla* $_{CTX-M1}$ gene ($P < 0.05$), and also between the cefepime resistance phenotype and the presence of the *bla* $_{CTX-M8}$ gene ($P = 0.06$), but not with other antibiotics. The research showed that, apart from these genes, none of the other genes studied significantly correlated with antibiotic resistance. Table 5 shows the association between genes and the antibiotic resistance profile in *Shigella* isolates.

5. Discussion

Shigellosis is a significant health problem affecting people worldwide, resulting in approximately 700,000 deaths yearly due to severe diarrhea. Despite improved public health measures, reports of shigellosis persist (30). Antimicrobial resistance (AMR) is a looming crisis that poses a significant threat to public health, affecting humans, animals, and the environment (31). Overuse and misuse of antibiotics have contributed to AMR. Understanding regional drug resistance patterns and continuously monitoring the involved resistance genes can help elucidate mechanisms of drug resistance in *Shigella* spp. and aid in the development and implementation of preventive and control measures (32).

In a study by Beladi Ghannadi et al., out of 52 *Shigella* isolates, more than 67% were MDR. The highest rates of resistance were observed for cephalothin, tetracycline, amikacin, trimethoprim-sulfamethoxazole, and ampicillin, while the lowest resistance rate was observed for ciprofloxacin (33). In this study, 36% of the examined isolates (n = 60) were MDR, and the highest resistance was observed for tetracycline and beta-lactam antibiotics (ampicillin and amoxicillin). Furthermore, another study in 2019 aimed to identify the drug resistance and resistance mechanisms of *S. flexneri*. Out of 105 *S. flexneri* isolates collected, 34 (32.4%) were ESBL-producing isolates. All ESBL-producing isolates were sensitive to cefoxitin and imipenem and resistant to ciprofloxacin. ESBL-producing isolates were highly resistant to ampicillin, cefotaxime, tetracycline,

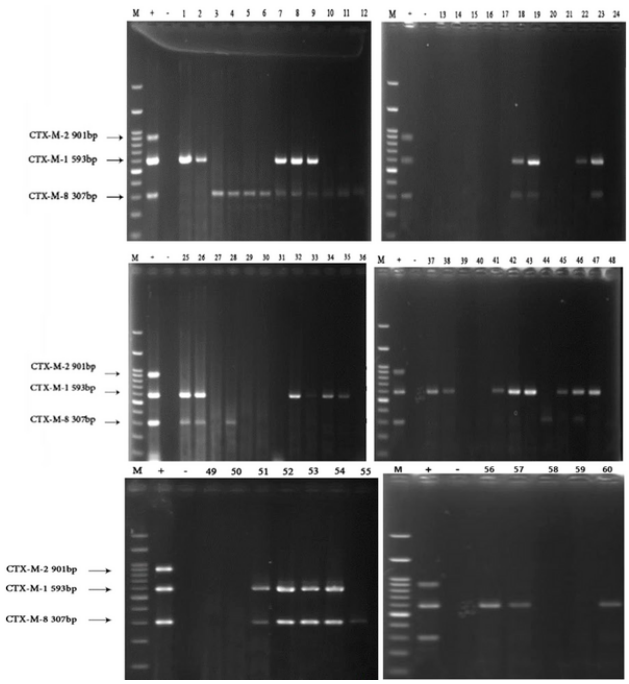

Figure 2. Multiplex PCR assay for detection of *bla* $_{CTX-M8}$, *bla* $_{CTX-M2}$, *bla* $_{CTX-M1}$ on 1.5% agarose gel. Lane 1, marker (100 bp); lane 2, positive control (*Escherichia coli* 25922 ATCC); lane 3, negative control (*Sh sonii* 25931 ATCC); lane 1 - 60, extracted DNA of *Shigella* isolates.

chloramphenicol, trimethoprim-sulfamethoxazole, ceftazidime, and cefepime (34). The resistance reported in the study by Bian et al. was higher than in the present study, especially for cephalosporins, which can be attributed to the spread of ESBL strains in the investigated isolates 34.

Our research and previous related studies have shown that *Shigella* isolates are resistant to amoxicillin, ampicillin, and tetracycline antibiotics, indicating that these antibiotics are no longer reliable choices for treatment. However, the observed differences in resistance to other antibiotics can be attributed to differences in the source of isolation, geographical region, and strain isolated (35-37).

Resistance to third-generation cephalosporins is a significant public health issue, especially in developing countries. This resistance is usually caused by the production of ESBL enzymes, which are often carried on plasmids (38, 39). Studies conducted in Iran found higher rates of ESBL-producing *Shigella* strains than have been reported in many other countries (40, 41). In this study, the presence of the six most common ESBL-producing genes (*bla* $_{CTX-M1}$, *bla* $_{CTX-M2}$, *bla* $_{CTX-M8}$, *bla* $_{SHV}$, *bla* $_{TEM}$, and *bla* $_{OXA}$) was investigated, and the most predominant ESBL gene was *bla* $_{CTX-M1}$.

The results generated by our study are consistent with those of the study by Beladi Ghannadi et al. In their study, the *bla* $_{TEM}$ gene was found to be the most prevalent among *Shigella* isolates, followed by the *bla* $_{CTX-M}$ gene. The *bla* $_{SHV}$ gene was not detected in any of the isolates, indicating it was the least abundant (33). The *bla* $_{SHV}$ appears to have a low detection rate among *Shigella* isolates in Iran (42). Additionally, Toy et al. reported the prevalence of *bla* $_{CTX-M1}$ more than other types of this gene (43). Some studies show the opposite of these results, such as a study by Hussain et al., which showed a higher prevalence of the *bla* $_{CTX-M2}$ gene compared to our study (44), and another study by Shahin et al. from Iran, which reported contrasting results for *Shigella* spp. isolated from food samples (45). These differences may be due to variations in the study year, geographical location, strains, and the number of samples (46).

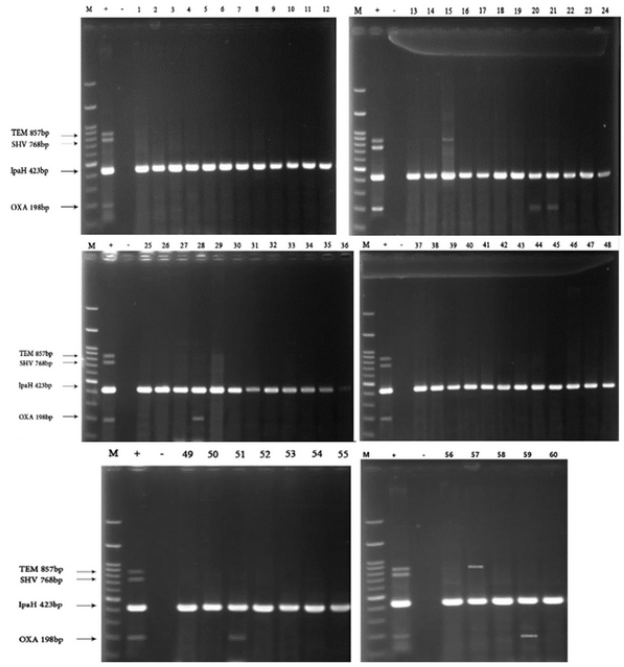

Figure 3. Multiplex PCR assay for detection of *bla* $_{OXA}$, *bla* $_{SHV}$, *bla* $_{TEM}$, *bla* $_{CTX-M1}$ and *ipaH* (as internal control) on 1.5% agarose gel. Lane 1, marker (100 bp); lane 2, positive control (*Escherichia coli* 25922 ATCC); lane 3, negative control (*Sh sonii* 25931 ATCC); lane 1 - 60, extracted DNA of Shigella isolates.

Table 4. Occurrence of ARGs in *Shigella* Isolates [a]

Antibiotic	*Sh* Isolates Total (n = 60) [b]	No. of Isolates	Valid Percent
bla $_{TEM}$	1.7	1	1.7
bla $_{SHV}$	1.7	1	1.7
bla $_{OXA}$	8.3	5	8.3
bla $_{CTX-M1}$	51.7	31	51.7
bla $_{CTX-M2}$	0.0	0	0.0
bla $_{CTX-M8}$	38.3	23	38.3

[a] In this table, the number of isolates and the percentage of isolates carrying each ARG are presented.

[b] Values are expressed as (%).

Generally, the results show that ESBL-producing *Shigella* isolates are increasing in prevalence and the beta-lactamase gene *bla* $_{CTX-M}$ is spreading rapidly. It seems that the clonal spread of strains carrying the *bla* $_{CTX-M}$ gene, the pattern of using cephalosporins, and the transfer of plasmids between different strains play an important role in the high regional prevalence of bla$_{CTX-M}$ genes in *Enterobacteriaceae* strains (47, 48).

The pathogenicity of *Shigella* spp. is connected to the presence of various virulence determinants, which enable the bacteria to invade and spread within the cells of the colonic epithelium (49). This study examined five virulence genes (*ial, virF, invE, sigA,* and *pic*) using a multiplex PCR assay. The distribution of virulence genes *ial, virF, invE, sigA,* and *pic* in the studied isolates was obtained. The high abundance of *virF, invE,* and *sigA* genes suggested that this classical regulatory pathway

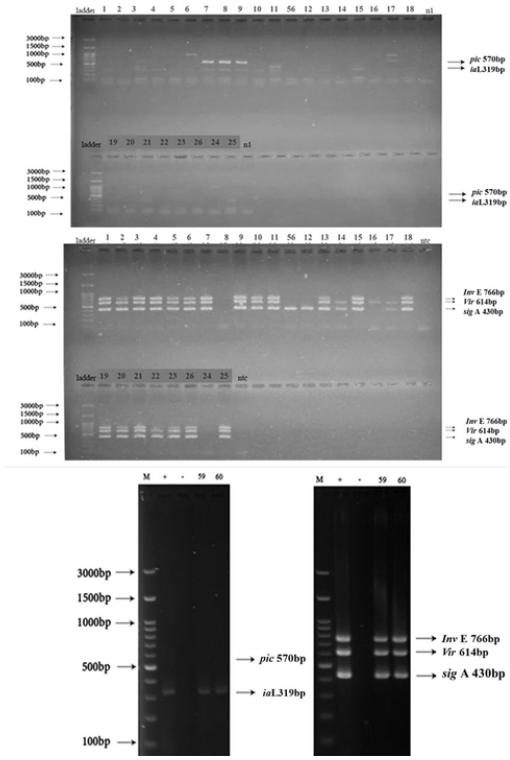

Figure 4. Multiplex PCR assay for detection of A, *pic* (570 bp) and *ial* (319 bp); B, *invE (766 bp), virF (614 bp)* and *sigA (430 bp)*; and C, *pic* (570 bp), *ial* (319 bp), *invE* (766 bp), *virF* (614 bp) and *sigA* (430 bp) on 1.5% agarose gel. First lane: marker (100 bp), Lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 56, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 26, 24, 25, 59, 60, extracted DNA of *Shigella* isolate.

of *Shigella* spp. virulence gene expression might play a key role in its pathogenesis.

Additionally, the current research showed that the simultaneous presence of more than two studied virulence genes in MDR isolates was prevalent, with the pattern of *invE, virF,* and *sigA* frequently observed among MDR isolates. Across various studies conducted in Iran, a total of 667 clinical isolates were identified as MDR and extended-ESBL producers (50). Research has shown that *Shigella* isolates collected from water sources, like those from human sources, exhibit high levels of antibiotic resistance. For example, a study by Shahin et al. reported all *Shigella* isolates obtained from water samples as MDR (42). Furthermore, some MDR isolates in this research did not carry any of the studied ARGs. This result indicates that resistance to these antibiotics in these isolates could be due to other mechanisms of resistance that need further research (51).

Some studies point to a possible association between increased antibiotic resistance in bacteria and increased virulence (13, 52, 53). A conceivable explanation is that bacteria repeatedly exposed to antibiotics may become resistant to multiple drugs, which could result in the emergence of highly virulent strains (54, 55). Therefore, this result (Table 3) indicates that isolates resistant to three or more antibiotics are also likely to be more virulent, providing evidence for the hypothesis that resistance and virulence may be related.

Additional findings from this study showed the presence of $bla_{\text{CTX-M}}$ in many (19/22) of the MDR isolates. Similarly, several virulence genes *(invE, virF, sigA, ial,* and *pic*) were also present in the MDR isolates. However, it is important to note that not all MDR isolates carried both the studied resistance and virulence genes. In some MDR patterns, beta-lactamase genes were not detected (marked as "-ND"), and a few patterns had MDR isolates without any beta-lactamase genes. Likewise, some patterns with MDR isolates did not have all the

Table 5. The Relationship Between the Genotypic Presence of Antibiotic Resistance Genes and the Phenotypic Expression of Resistance in *Shigella* Isolates, Along with the Chi-square Test Results, Cramer's Coefficient (V), and P-Value. Cramer's V Measures the Strength of This Association and the P-Value Indicates the Statistical Significance of the Association

Antibiotics	Chi-square	Cramer's Coefficient	P-Value
bla **$_{TEM}$**			
Imipenem	5.48	0.96	0.760
Ampicillin	2.45	0.65	0.881
Amoxicillin	0.73	0.35	0.788
Cefoxitin	0.17	0.17	0.896
Cefepime	5.08	2.91	0.079
bla **$_{OXA}$**			
Imipenem	1.12	0.43	0.946
Ampicillin	1.36	1.51	0.506
Amoxicillin	3.90	0.81	0.533
Cefoxitin	0.92	0.39	0.761
Cefepime	1.59	1.63	0.450
bla **$_{CTX-M1}$**			
Imipenem	2.94	2.22	0.229
Ampicillin	7.84	3.53	0.024
Amoxicillin	4.58	2.76	0.032
Cefoxitin	9.51	1.26	0.329
Cefepime	2.67	0.67	0.875
bla **$_{CTX-M8}$**			
Imipenem	1.45	1.56	0.483
Ampicillin	1.64	1.65	0.440
Amoxicillin	2.43	2.02	0.118
Cefoxitin	1.63	1.65	0.201
Cefepime	10.31	4.15	0.006

virulence genes listed. Further examination of additional genes within this group might likely lead to a modification of the results.

The current study is limited by the small sample size, which could affect the generalizability of the findings. Additionally, the study focused on Tehran, which may not be representative of the entire country. Information on patient clinical characteristics and treatment history that could lead to a clearer interpretation of antibiotic resistance patterns was also not available. Considering the high prevalence of multidrug resistance observed in this study, it is necessary to establish a continuous monitoring system to track the trend of antibiotic resistance in *Shigella* isolates in Iran.

5.1. Conclusions

In this study, high resistance to beta-lactams and tetracycline, as well as a high prevalence of *bla* $_{CTX-M}$ genes, were observed. Furthermore, high resistance to the first-line treatment of shigellosis, i.e., ampicillin, was also noted. With these limitations in treatment options and the emergence of cephalosporin-resistant strains, new strategies for treating shigellosis need to be developed, with a focus on continuous surveillance. This will help us develop more effective treatments and control strategies.

Footnotes

Authors' Contribution: A. T. and B. P. planned and designed the research. S. T. performed experiments. A. T. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interests Statement: Authors declared no conflict of interests.

Data Availability: The dataset presented in the study is available on request from the corresponding author during submission or after publication.

Ethical Approval: This study with research ethics code IR.IAU.ET.REC.1401.029 .The ethics committee of East

Tehran Branch, Islamic Azad University, Iran approved.

Funding/Support: No funding or financial support has been received for the current research.

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