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Genotypic characterization and antimicrobial susceptibility profile of *Shigella* Species isolated from different sources at Kaliobia governorate

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ABSTRACT

The current investigation focused on 13 *Shigella* isolates. These isolates were derived from 260 randomly sampled specimens including beef, chicken meat, cow's milk, and diarrheic child stool (with 65 samples each). The samples were collected from diverse sources such as butcher and poultry shops, supermarkets, and hospitals in Kaliobia governorate, Egypt. The study's objectives encompassed the genetic identification of the isolates, analysis of their antimicrobial sensitivity profiles, and the determination of specific antimicrobial resistance genes. The results revealed pronounced resistance to amoxicillin, then ampicillin, tetracycline, nalidixic acid, streptomycin, and cefotaxime. Conversely, heightened sensitivity was observed towards meropenem, norfloxacin, gentamicin, and ciprofloxacin. Notably, all isolates exhibited resistance to a minimum of three distinct antimicrobials, categorizing them as multi-drug resistant (MDR).Genetically, all five studied *Shigella* isolates were *Shigella* species as all of them had *ipa*$_{H}$ gene of *Shigella* genus, the isolate No. 1, was *S. sonnei* strain as it carried the *wbg*$_{Z}$ gene of *S. sonnei* and the other four isolates were confirmed as *S. flexneri* strains as they had the *ipa*$_{H1}$ gene of *S. flexneri*. Moreover, β-lactam resistance gene, *bla*$_{TEM}$; quinolones resistance gene, *qnr*$_{A}$ and streptomycin resistance gene, *aad*$_{A1}$ were amplified in all five studied *Shigella* strains giving products of 516 bp.; 516 bp. and 484 bp., respectively. Therefore, this study concluded that, these isolates (*S. flexneri* and *S. sonnei*) are MDR to three or more antimicrobials of public health importance and there is correlation between antimicrobial resistance phenotypes and genotyping of *Shigella* isolates.

1. INTRODUCTION

Shigella species, categorized as Gram-negative, facultative anaerobic, non-spore-forming, rod-shaped, non-motile, and facultative intracellular pathogens within the *Enterobacteriaceae* family, holds substantial clinical significance (Pakbin *et al*., 2023). As one of the most prevailing causative agents of diarrheal illnesses globally, *Shigella* species have elicited considerable attention (Salleh et al., 2022). These pathogens induce Shigellosis, a gastrointestinal infection recognized as bacillary dysentery, characterized by the invasion and disruption of the epithelial lining of the terminal ileum, colon, and rectum. This leads to pronounced clinical manifestations encompassing acute watery diarrhea, dysentery with bloody stools, elevated temperature, and abdominal cramps (Kotloff *et al*., 2018). Within the *Shigella* genus, four major serological groups are recognized: *Shigella flexneri* (10 serotypes), *S. dysenteriae* (15 serotypes), *S. boydii* (20 serotypes), and *S. sonnei* (1 serotype). However, *S. flexneri* and *S. sonnei* account for over 90% of Shigellosis cases worldwide (Sabour et al., 2022). Given the potential for severe outcomes, antibiotic therapy plays a pivotal role in managing Shigellosis by curbing disease duration, mitigating transmission, and averting life-threatening complications (Pakbin et al., 2021a; Sabour et al., 2022).

Multidrug resistance (MDR) poses a substantial challenge in Shigellosis treatment. This resistance phenotype, often exhibited by *S. flexneri* and *S. sonnei*, involves resistance to two or more antibiotics from distinct classes. Resistance patterns frequently encompass sulfonamides, tetracyclines, streptomycin, ampicillin, cephalosporins, azithromycin, and quinolones, often attributed to point mutations and horizontal plasmid-mediated mechanisms (Thompson *et al*., 2015; Ranjbar and Farahani, 2019). Genetic factors such as *sul*$_{2}$, *tet*$_{A}$, *tet*$_{B}$, *str*$_{AB}$, and extended-spectrum beta-lactamases (ESBLs) like CTX-M, TEM, and SHV contribute to this resistance (Ud-Din *et al*., 2013; Shahsavan *et al*., 2017; Chung *et al*., 2021; Salleh *et al*., 2022). The proliferation of MDR *Shigella* strains accentuates the predicament of antimicrobial resistance (Ma *et al*., 2018; Pakbin *et al*., 2021a, b). While multiple studies have explored antibiotic resistance in MDR *Shigella* isolates from clinical and food sources, genetic profiling of such isolates remains limited (Zamanlou *et al*., 2018; Shahin *et al*., 2019; Pakbin *et al*., 2021a). Furthermore, updates on antimicrobial resistance within *Shigella* spp. are vital for informed therapeutic strategies, aimed at alleviating the morbidity and mortality linked to Shigellosis in Egypt. Thus, this study aimed to genetically identify and assess the antibiotic sensitivity profile, alongside antibiotic resistance genes, within previously isolated *Shigella* strains originating from diverse sources in Kaliobia Governorate, Egypt.

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2. MATERIAL AND METHODS

2.1. Ethical Approval

The protocol of this work was approved by Institutional Animals Care and Use Committee of faculty of veterinary medicine, Benha university (approved number BUFVTM)11-07-23)

2.2. Samples

Thirteen *Shigella* isolates were included in this study. These isolates were previously isolated and identified by the same authors from 260 random samples of beef; chicken meat; cow's milk and diarrheic child stool of patients (65 for each),that collected from different butcher and chicken shops; supermarkets and hospitals, respectively, at Kaliobia governorate Egypt . Each examined sample was taken alone in sterile plastic bags, kept in icebox and transferred under possible aseptic conditions with minimum delay to the laboratory for bacteriological examination , following ISO (2004); Gaurav *et al (* 2013) and Pakbin *et al* (2021a).

2.3. In-vitro antimicrobial sensitivity test

The antimicrobial susceptibility of the 13 *Shigella* isolates was determined using the Kirby–Bauer disk diffusion method on Mueller-Hinton agar (oxoid) plates, following the guidelines of CLSI (2019). Twelve standardized antimicrobial disks were used in the antibiograms, including: amoxicillin (AML/10μg), ampicillin (AM/10μg), azithromycin (AZM/15μg), cefotaxime (CTX/30μg), ciprofloxacin (CIP/5μg), co-trimoxazole (COT/25μg), gentamicin (GEN/10μg), meropenem (MEM/10μg), nalidixic acid (NA/30μg), norfloxacin (NOR/10μg), streptomycin (S/10μg), and tetracycline (TE/30μg).

2.4. Genotypic identification and detection of antibiotic resistance genes

Genotypic identification of *Shigella* isolates was performed by PCR amplification of the *ipa*$_{H}$ (invasion plasmid antigen H) gene of *Shigella* genus and species-specific genes (*ipa*$_{H1}$ and *wbg*$_{Z}$) for differentiation. A negative control (*E. coli* ATCC 25922) and positive controls (field *Shigella* strains) were included. Additionally, three antibiotic-resistance genes were detected using PCR: β-lactam gene *bla*$_{TEM}$ (Colom et al., 2003);, quinolone gene *qnr*$_{A}$ (Robicsek et al., 2006) , and streptomycin gene *aad*$_{A1}$(Randall et al. 2004) . Five random *Shigella* isolates were selected for this analysis, including isolates from diarrheic child stool, chicken meat, beef, and cow's milk. DNA extraction was performed using the QIAamp® DNA Mini Kit (Qiagen, Germany) following manufacturer's instructions. PCR was carried out using Emerald Amp GT PCR mastermix (Takara, Japan), and agarose gel electrophoresis (Sambrook et al., 1989) was performed for visualizing the amplicons. All primer sequences listed in this study are listed in table (1).

3. RESULTS

The results of in- vitro sensitivity tests for the studied Shigella isolates (Table, 2) showed that, they were highly resistant for amoxicillin (92.3%) followed by ampicillin (84.6%) then tetracycline (76.9%); Nalidixic acid (69.2%), streptomycin (69.2%) and cefotaxime (61.5%). Meanwhile, they were intermediate sensitive to Co- Trimoxazole (61.5%) and azithromycin (53.8%). Moreover, they were highly sensitive to meropenem (92.3%) followed by norfloxacin (84.6%) then gentamycin (76.9%) and ciprofloxacin (69.2%). In addition, Table,(2) showed that, all studied Shigella isolates were resistant to at least three different antimicrobials and considered multi-drug resistant (MDR).

Table 1: Primers sequences, amplicons sizes used in this study

Oligonucleotides	Description/Sequence (5'-3')	Amplified segment (bp.)	References
Shigella ipaH	F GCCGGTCAGCCACCCT CTGAGACTAC RGTTCCTTGACCGCCTTTCCGTACCGT	600 bp	Jiménez *et al*., 2010
S. sonnei wbg$_{Z}$	F ATGTTGCTAATACCAGTTGG R TAGAGAGAACTTCACACGGT	460bp	Radhika *et al*., 2014
S. flexneri ipa$_{H1}$	F TGAGAATTTGCCTCCACA R CTAGCCTTCCTTGTGCAA	595bp	
bla$_{TEM}$	F ATCAGCAATAAACCAGC R CCCCGAAGAACGTTTTC	516 bp.	Colom *et a*l., 2003
qnr$_{A}$	F ATTTCTCACGCCAGGATTTG R GATCGGCAAAGGTTAGGTCA	516 bp.	Robicsek *et al*., 2006
aad$_{A1}$	F TATCAGAGGTAGTTGGCGTCAT R GTTCCATAGCGTTAAGGTTTCATT	484bp	Randall *et al.* 2004

Table 2: *In-Vitro* antimicrobial sensitivity test following the guidelines of CLSI (2019). for the isolated *shigella* spp. n=13 from different sources *(ISO 2004, Gaurav et al 2013 and Pakbin et al. 2021a)*

Serial No.	Shigella Species	Isolate Sources	Antimicrobial agents											
			AML /10	AM/10	TE/30	NA/30/	S/10	CTX/30	COT/25	AZM/15	MEM/10	NOR/10	GEN/10	CIP/5
1	*S. sonnei*	DCS	R	R	R	R	R	S	IM	R	S	S	S	R
2	*S. flexneri*	DCS	R	R	R	R	R	R	S	IM	S	S	IM	R
3	*S. flexneri*	DCS	R	R	R	R	IM	R	IM	S	S	S	S	S
4	*S. flexneri*	DCS	R	R	IM	R	R	S	IM	IM	S	S	S	S
5	*S. flexneri*	DCS	R	R	S	S	R	IM	IM	R	S	S	S	S
6	*S. flexneri*	DCS	IM	S	R	IM	R	R	S	IM	S	S	S	S
7	*S. flexneri*	CM	R	R	R	R	R	R	IM	IM	IM	S	S	R
8	*S. flexneri*	CM	R	R	R	S	IM	R	IM	S	S	IM	R	S
9	*S. flexneri*	CM	R	R	R	R	R	IM	S	IM	S	S	S	S
10	*S. flexneri*	B	R	R	R	R	R	R	S	IM	S	S	S	S
11	*S. flexneri*	B	R	R	IM	IM	S	R	IM	R	S	S	S	S
12	*S. flexneri*	CoM	R	IM	R	R	IM	R	R	S	S	IM	R	S
13	*S. flexneri*	CoM	R	R	R	R	R	S	IM	IM	S	S	S	IM
		S %	0.0	7.7	7.7	15.4	7.7	23.1	30.8	23.1	92.3	84.6	76.9	69.2
		IM %	7.7	7.7	15.4	15.4	23.1	15.4	61.5	53.8	7.7	15.4	7.7	7.7
		R %	92.3	84.6	76.9	69.2	69.2	61.5	7.7	23.1	0.0	0.0	15.4	23.1
		AA	R	R	R	R	R	R	IS	IS	S	S	S	S

%: Percentage in relation to total number of the studied *Shigella* spp. (n=13),DCS: Diarrheic child stool,CM: Chicken meat,B: Beef , CoM: Cow's milk,S: Sensitive, IM: Intermediate R: Resistant,AA: Antibiogram activity

The results of genotypic identification of five studied *Shigella* isolates cleared that, all of them carrying the invasion plasmid antigen H (*ipa*$_{H}$) gene of *Shigella* genus and were amplified at 600 bp. (Fig., 1). So, all isolates were *Shigella* strains. Also, the putative epimerase/ dehydratase(*wbg*$_{Z}$) gene for *S. sonnei* was amplified at 460 bp. (Fig., 2) in isolate No. 1 only, considered *S. sonnei* strain and the invasion plasmid antigen H(*ipa*$_{H1}$)of *S. flexneri* was amplified at 595 bp. (Fig., 3) in the other four isolates, confirmed as *S. flexneri* strains . Moreover, the results of genotypic detection of antibiotic resistant genes, showed that, β-lactam, *bla*$_{TEM}$; quinolones, *qnr*$_{A}$ and streptomycin: *aad*$_{A1}$ were amplified At 516,516 and 484 bp respectively in all the five isolates of *shigella*.

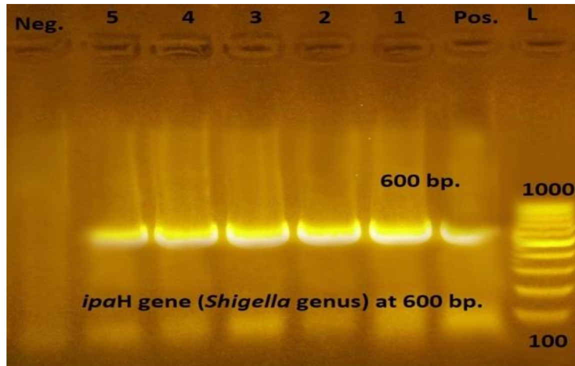

Fig. 1: Agarose gel electrophoresis of invasion plasmid antigen H (*ipa*$_{H}$) gene, Lane (L): 100-1000 bp. DNA Ladder, Lane (Pos.): Positive control (filed *Shigella* strain form RLQP,AHRI. at 600 bp.), Lane (Neg.): Negative control (*E. coli* ATCC 25922), Lanes 1-5: Positive *Shigella* strains at 600 bp. (1and 2 DCS; 3CM; 4B and 5 CoM), DCS: Diarrheic child stool, CM: Chicken meat,B: Beef, CoM: Cow's milk

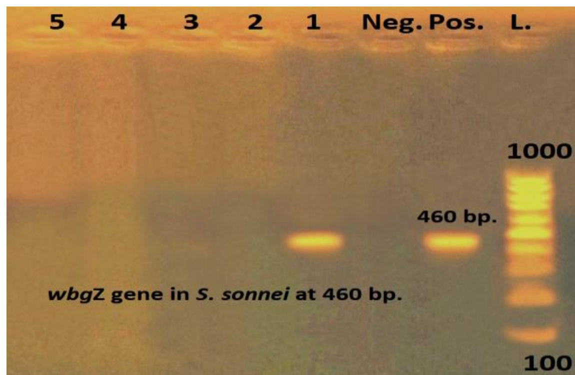

Fig.2: Agarose gel electrophoresis of putative epimerase/dehydratase (*wbg*$_{Z}$) gene, Lane (L): 100-1000 bp. DNA Ladder, Lane (Pos.): Positive control (*S. sonnei* ATCC25931 at 460 bp.), Lane (Neg.): Negative control (*E. coli* ATCC 25922), Lane 1: Positive *S. sonnei* strain at 460 bp. (1DCS), Lanes 2-5: Negative *S. sonnei* strains ((2 DCS; 3 CM; 4 B and 5 CoM))

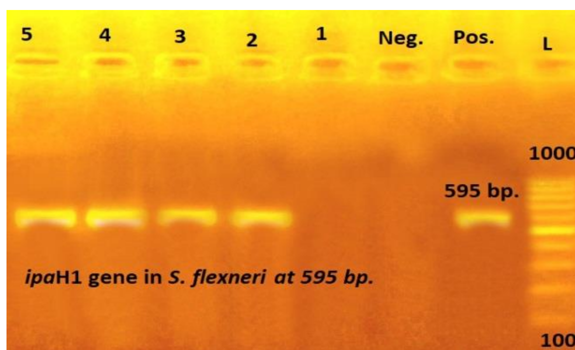

Fig. (3): Agarose gel electrophoresis of invasion plasmid antigen H (*ipa*$_{H1}$) gene, Lane (L): 100-1000 bp. DNA Ladder, Lane (Pos.): Positive control (*S. flexneri* ATCC 25875 at 595 bp.),Lane (Neg.): Negative control (*E. coli* ATCC 25922),Lane 1: Negative *S. flexneri* strain at 595 bp. (1DCS),Lanes 2-5: Positive *S. flexneri* strains at 595 bp. ((2 DCS; 3 CM; 4 B and 5 CoM))

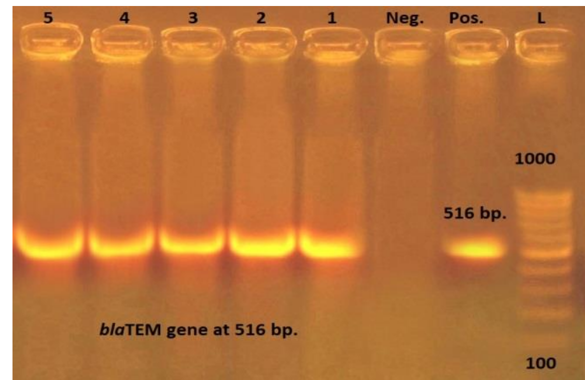

Fig. (4): Agarose gel electrophoresis of β-lactam resistant (*bla*$_{TEM}$) gene, Lane (L): 100-1000 bp. DNA Ladder,Lane(Pos.): Positive control (*Shigella*strain form RLQP, AHRI. positive for *bla*$_{TEM}$ at 516 bp.),Lane (Neg.): Negative control (*E. coli* ATCC 25922),Lanes 1-5: Positive*Shigella*strains for *bla*$_{TEM}$ at 516 bp. (1and 2 DCS;3CM;4B and 5 CoM)

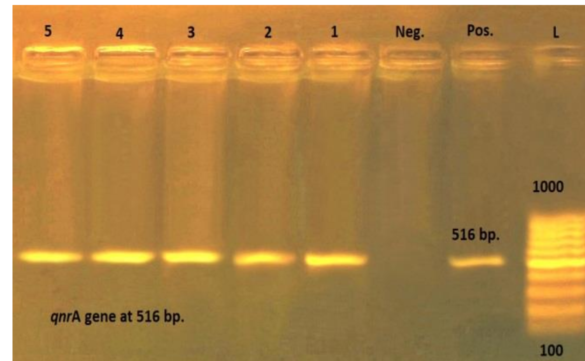

Fig. (5): Agarose gel electrophoresis of quinolones resistant (*qnr*$_{A}$) gene, Lane (L): 100-1000 bp. DNA Ladder ,Lane (Pos.): Positive control (*Shigella*strain form RLQP, AHRI. positive for *qnr*$_{A}$ at 516 bp.) ,Lane (Neg.): Negative control (*E. coli* ATCC 25922),Lanes 1-5: Positive*Shigella*strains for *qnr*$_{A}$ at 516 bp. (1and 2 DCS;3CM;4B and 5 CoM)

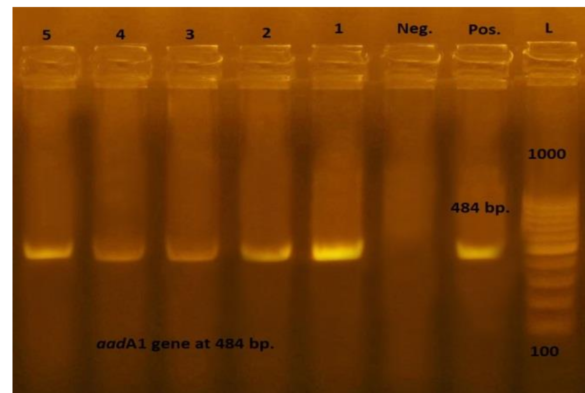

Fig. (6): Agarose gel electrophoresis of streptomycin resistant (*aad*$_{A1}$) gene, Lane (L): 100-1000 bp. DNA Ladder, Lane (Pos.): Positive control (*Shigella*strain form RLQP, AHRI. positive for*aad*$_{A1}$at 484 bp.), Lane (Neg.): Negative control (*E. coli* ATCC 25922),Lanes 1-5: Positive *Shigella* strains for *aad*$_{A1}$at 484 bp. (1and 2 DCS;3CM;4B and 5 CoM)

4. DISCUSSION

Shigellosis, particularly caused by multidrug-resistant (MDR) *Shigella* spp., has emerged as a significant public health challenge. The prevalence of this pathogen is on the rise, particularly in developing countries (Kotloff *et al.*, 2018). In developing countries, there is a lack of specific guidelines for employing antibiotic therapy for Shigellosis cases. As a result, antibiotics are often prescribed by physicians and veterinarians without proper cultures. To address this, the present study aimed to genetically identify and characterize antibiotic sensitivity profiles, as well as antibiotic resistance genes, in the previously isolated *Shigella* strains obtained from diarrheic child stool, chicken

meat, beef, and cow's milk samples in Kaliobia governorate, Egypt. The conventional methods utilized for isolating and identifying *Shigella* species are established on selective media and followed by an array of biochemical tests. However, these approaches are recognized for their limitations of being labor-intensive, costly, and time-consuming, as reported in prior research (Radhika *et al*., 2014; Sabour *et al*., 2022). In light of these challenges, our study opted for a more efficient and accurate identification strategy by confirming previously identified *Shigella* isolates using PCR amplification of specific genes. We employed PCR amplification of the *ipa*$_{H}$ (invasion plasmid antigen H) gene, which is characteristic of the *Shigella* genus. Additionally, we utilized species-specific genes, *ipa*$_{H1}$ for *S. flexneri* and *wbg*$_{Z}$ (putative epimerase/ dehydratase) for *S. sonnei*, to differentiate between the two species. The PCR results clearly indicated that all five studied Shigella isolates were indeed *Shigella* strains, with each carrying the *ipa*$_{H}$ gene amplified at 600 bp. Our findings corroborate those of prior studies by Jiménez *et al*. (2010), Younis *et al*. (2018), and Sabour *et al*. (2022). Furthermore, our study's genetic characterization allowed us to distinguish between *S. sonnei* and *S. flexneri* strains. The amplification of the *wbg*$_{Z}$ gene at 460 bp. in the *Shigella* isolate, obtained from diarrheic child stool, unequivocally identified it as an *S. sonnei* strain. This finding aligns with previous studies by Zou *et al*. (2001), Radhika *et al*. (2014), and Sabour *et al*. (2022), where the *wbg*$_{Z}$ gene was utilized for the molecular cloning and precise identification of *S. sonnei* strains. Additionally, the four remaining Shigella isolates were conclusively identified as *S. flexneri*, with the presence of the *ipa*$_{H1}$ gene amplified at 595 bp., consistent with studies by Farfan *et al.* (2010) and Radhika *et al.* (2014). The studied 13 Shigella isolates demonstrated pronounced resistance to several antibiotics, including amoxicillin, ampicillin, tetracycline, nalidixic acid, streptomycin, and cefotaxime. This pattern of resistance closely mirrors results reported in previous studies involving clinical and food samples, conducted by Ahmed and Shimamoto (2015), Shahin *et al*. (2019), Pakbin *et al.* (2021a,b), Elkenany *et al*. (2022), and Salleh *et al.* (2022). The unchecked use of antibiotics in both animal husbandry and medical applications has been identified as a driving force behind the surge in antimicrobial-resistant bacterial strains. This phenomenon has been notably observed by Pakbin *et al*. (2021a) , Sabour *et al*. (2022) and Salleh *et al.* (2022). Remarkably, our investigation also illuminated the emergence of multidrug-resistant (MDR) *Shigella* isolates. All studied *Shigella* strains exhibited resistance to at least three distinct antimicrobial agents, classifying them as MDR. This alignment with prior research findings by Ahmed and Shimamoto (2015), Karimi-Yazdi *et al*. (2020), Pakbin *et al*. (2021a,b), Rabins (2021), and Elkenany *et al*. (2022) reinforces the consistent and concerning presence of MDR *Shigella* species in foods of animal origin, such as meat, milk, and stool samples. The persistence of MDR *Shigella* strains constitutes a global threat to public health, warranting urgent attention and intervention. Interestingly, our study also revealed a nuanced sensitivity profile among the studied *Shigella* isolates. They demonstrated intermediate sensitivity to Co-Trimoxazole and azithromycin, while showing notable sensitivity to meropenem, norfloxacin, gentamycin, and ciprofloxacin. This sensitivity pattern suggests potential alternative treatment options when diagnosing Shigellosis. Our results closely mirror those of Obi and Ike (2018), Okoli *et al.* (2021), Pakbin *et al*. (2021a,b), and Rabins (2021), thus lending support to the potential use of these drugs as effective therapeutic choices. The increasing prevalence of multidrug resistance (MDR) among *Shigella* strains of both food and clinical origins represents an urgent global concern and a potential threat to public health (Shahin *et al*., 2019). Our current study corroborates this growing concern by demonstrating that all studied *Shigella* isolates exhibited the MDR phenotype. This observation was further genetically confirmed through the detection of three antibiotic resistance genes, namely *bla*$_{TEM}$, *qnr*$_{A}$, and *aad*$_{A1}$, in all five *Shigella* strains under investigation. The emergence of MDR *Shigella* strains is a multifaceted challenge that demands comprehensive investigation. Beta-lactamases, pivotal enzymes in conferring resistance among gram-negative bacteria like *Shigella* spp., *E. coli*, and *Salmonella*, play a significant role in rendering these pathogens impervious to beta-lactam antibiotics such as amoxicillin, ampicillin, penicillin, cefepime, and cefoxitin (Sabour *et al*., 2022). In our study, the presence of the β-lactam resistance gene, *bla*$_{TEM}$, was consistently amplified in all five *Shigella* isolates, producing products of 516 bp. (Fig., 4). These findings concur with previous studies by Ahmed and Shimamoto (2015), Ranjbar and Farahani (2019), Shahin *et al*. (2019), Hawkey *et al*. (2021), Elkenany *et al*. (2022), and Sabour *et al*. (2022). These researchers identified the *bla*$_{TEM}$ gene in *Shigella* spp. isolated from various sources, including meat, milk, and children with diarrhea. However, our study diverges from the findings of Pakbin *et al*. (2021b), who reported the absence of the *bla*$_{TEM}$ gene in *Shigella* isolates from meat and milk samples. Notably, investigations into quinolone resistance (*qnr*$_{A}$) and aminoglycoside adenyltransferase, specifically for streptomycin resistance (*aad*$_{A1}$), remain limited in the context of *Shigella* isolates sourced from food, animals, and humans (Ranjbar and Farahani, 2019). Our PCR results provide novel insights by detecting the *qnr*$_{A}$ gene in all five studied *Shigella* isolates, with amplicons of 516 bp. (Fig., 5). This aligns with findings by Bhattacharya *et al*. (2014), Taneja *et al*. (2014), Gu *et al*. (2017), and Ranjbar and Farahani (2019). Similarly, we detected the *aad*$_{A1}$gene in all five isolates, producing products of 484 bp. (Fig., 6), consistent with phenotypic resistance to streptomycin. These findings resonate with research conducted by McIver *et al*. (2002), Barman *et al*. (2010), Ranjbar and Farahani (2019), and Hawkey *et al*. (2021).

5. CONCLUSIONS

In conclusion, our study underscores the alarming increase in MDR *Shigella* strains and the critical role played by antibiotic resistance genes, namely *bla*$_{TEM}$, *qnr*$_{A}$, and *aad*$_{A1}$, in fueling this trend. These genetic determinants of resistance align with phenotypic resistance patterns observed in our study. Our findings emphasize the urgent need for robust surveillance, prudent antibiotic use, and targeted interventions to curtail the dissemination of MDR *Shigella* strains, safeguarding public health on a global scale. Moreover, our research contributes valuable genetic insights to the limited body of knowledge regarding quinolone and aminoglycoside resistance genes in *Shigella* isolates from diverse sources.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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