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Bacteriology

Characterization of multi-resistant *Shigella* species isolated from raw cow milk and milk products

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ABSTRACT. This study was organized to investigate the prevalence, antibiotic and disinfectant resistance phenotypes and genotypes as well as plasmid profiles of *Shigella* species isolated from raw cow milk and milk products in Egypt. Genotypic analysis was performed to determine the presence of *β*-lactamase encoding genes (bla_{TEM}, bla_{CTX-M}, bla_{OXA-1} and bla_{SHV}), *tet*(A) and *qacEΔ*. Forty-two (7%) *Shigella* isolates (*S. dysenteriae, S. flexneri, and S. sonnei*) were recovered, with *S. dysenteriae* as the predominant type. Antibiotic sensitivity tests showed that 71.4% of *Shigella* isolates were resistant to three or more antibiotic classes (multidrug-resistant). High resistance rates were observed against tetracyclines (100%), ampicillin, amoxicillin-clavulanate (90.5%, each) and cefaclor (66.7%), while no resistance was detected against imipenem, sulfamethoxazole/trimethoprim, and azithromycin. Disinfectant susceptibility test of *Shigella* isolates revealed resistance to phenolic compound (vanillic acid), while 85.7% of the *Shigella* isolates were resistant to benzalkonium chloride. Uniplex PCR analysis declared the existence of *β*-lactamase encoding genes (bla_{TEM} in all isolates and bla_{CTX-M} in 28.6% of isolates) and, *tet*(A) in all isolates and 85.7% of the isolates were positive for *qacEΔ1,* while all isolates were negative for bla_{OXA-1} and bla_{SHV}. All *Shigella* extended spectrum β-lactamases (ESBL) producers (12, 100%) were positive for the bla_{TEM}, bla_{CTX-M}, and *qacEΔ1* genes. Furthermore, plasmid profiling revealed seven distinct plasmid patterns (P1–P7), ranging from 1.26 to 33.61 kb, among all the *Shigella* strains; *S. dysenteriae* exhibited the greatest variance. The co-transfer of β-lactamase genes (bla_{TEM} and bla_{CTX-M}) and *qacEΔ1* genes was observed by conjugation from all ESBL producers to a recipient strain. These findings indicate the emergence of *Shigella* species in Egypt that exhibited multi-resistance to either antibiotics (particularly ESBL producer strains) or disinfectants. Thus, the resistance of *Shigella* species should regularly be monitored and appropriate measures should be taken to manage this problem.

KEYWORDS: antibiotic resistance, β-lactamase encoding gene, dairy product, *qacEΔ1* gene, *Shigella* species

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Shigella is a pathogen mainly found in water and faeces causing contamination of the animal, human, environment and milk [12]. Infections caused by *Shigella* species are the main causes of bacillary dysentery, which is affiliated with high morbidity and mortality, especially in developing countries, such as Egypt [29]. Shigellosis is a universal public health concern; preceding researches have focused on the human gastrointestinal pathogens but have neglected animal groups. *Shigella* spp. infect and generate analogous clinical symptoms in cows, chickens, pigs, monkeys and other animals [33]. Consumption of calves to contaminated milk with *Shigella* leads to severe diarrhea [52]. Members of the *Shigella* genus are classified into four species: *S.*

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dysenteriae, S. flexneri, S. boydii, and *S. sonnei*. Endemic shigellosis in developing countries is caused mainly by *S. flexneri,* while *S. sonnei* is frequently detected in industrialized countries [48]. Dairy products especially raw milk and unpasteurized cheese as karish cheese, which is one of the most popular soft cheese in Egypt, remain important vehicles for the transmission of *Shigella* to the rural and urban population [5, 21, 39].

Antibiotic therapy for *Shigella* infection can decrease the extent and severity of the disease [26]. As a consequence of the misuse of antibiotic administration, multidrug-resistant (MDR)*Shigella* species are developed in China, particularly those with extended spectrum β-lactamases (ESBLs) [50, 51]. Many ESBL variants are known to be present in a variety of pathogens, including $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and $bla_{\text{OXA-1}}$, which have been proven to be the most successful in terms of antibiotic resistance and epidemiological niches [51]. Among bla_{TEM} types, $bla_{\text{TEM-1}}$ is a class A broad-spectrum beta-lactamase and the most commonly plasmid-mediated β-lactamase of ampicillin (AM) resistant gram-negative bacteria [36]. Also, the resistance to tetracyclines in Gram-negative bacteria is frequently due to the possession of *tet*(A) gene [18]. The selection of antibiotic treatment for shigellosis is made more difficult by the generation of resistance in *Shigella* spp., especially the resistance to third-generation cephalosporins, which is a common public health problem, mainly in developing countries [7, 19]. The evolution of multidrug-resistant (MDR) strains is common due to the existence of mobile genetic elements that assist *Shigella* spp. in the acquisition and transfer of exogenous genes [40]. Antibiotic resistance plasmids often include genes conveying resistance to numerous different antibiotics [17].

Disinfectants, including phenolic compounds (vanillic acid) and quaternary ammonium compounds (QACs), such as benzalkonium chloride (BKC), are commonly used in dairy cattle farms. The capability of phenolic compounds to inhibit the growth of any microorganism depends on their interaction with proteins and/or on their ability to impair membrane permeability [41]. A particular concern is that repeated use of disinfectants may cause the persistence of bacteria with reduced susceptibility not only to antiseptics but also, possibly, to antibiotics [38]. One of the major mechanisms underlying such resistance is the acquisition of the resistance genes *qacE* and *qacEΔ1*, which confer resistance to QACs [27]. *qacE*Δ*1*, a mutant version of *qacE,* is widely distributed throughout gram-negative bacteria, mainly in *Enterobacteriaceae* [13].

Multiple global studies have reported the molecular basis of antibiotic resistance in clinical *Shigella* isolates of human origin [1, 46] but, inadequate data are available on the genotypic and phenotypic characteristics of *Shigella* spp. isolated from raw cow milk and milk products, especially *Shigella* has a main public health concern for those population who still consumed unpasteurized raw milk in Egypt. Therefore, this study was planned to isolate and characterize *Shigella* spp. from raw cow milk collected on farms and milk products in Egypt. This work was also proposed to examine the prevalence of MDR *Shigella* particularly ESBL producing strains, screen the isolates for the presence of β-lactamase, *tet*(A) and quaternary ammonium compound E delta 1 (*qacE*Δ*1*) genes, and examine their plasmid profiles.

MATERIALS AND METHODS

Sample collection and microbial analysis

A total of 600 dairy product samples (200 raw cow milk from different dairy farms, 200 Kareish cheese, and 200 yoghurt samples from different shops, and supermarkets) were collected randomly from Dakahlia and Damiate Governorates, in northen Egypt, throughout 2018. This investigation was carried out according to the Animal Ethics Board Committee of Mansoura University, Egypt. Raw cow milk samples were collected from the udder of cows. After collection, the samples were stored at 4°C until bacteriological examination (within 3–4 hr). Then, homogenization of 25 ml or g of each sample was performed in 225 ml of 0.1% buffered peptone water (Oxoid, Hampshire, UK) by shaking for 5 min in sterile Stomacher bags and incubating for 24 hr at 44°C and 42°C for *Shigella sonnei* and other *Shigella* species, respectively, for recovery. A loop from the enriched cultures was directly inoculated into Selenite F broth and then subcultured onto *Salmonella-Shigella* (S-S) agar, MacConkey agar and xylose-lysine-deoxycholate (XLD) agar (Oxoid), followed by incubation at 37°C for 24 hr. The presumptive *Shigella* isolates (colourless and non-lactose fermenting on S-S agar, white and translucent on MacConkey agar, and pink to red colonies on XLD agar) were biochemically confirmed with triple sugar iron (TSI) agar, lysine iron agar (LIA), methyl red, Voges-Proskauer (VP) broth, the indole test, urea agar (UA), Simmon's citrate agar (SCA) and a motility test. Serotypes of the isolates were determined by slide agglutination assays, using a commercially available kits as described by the manufacturer (Difco Laboratories, Detroit, MI, USA). All bacterial isolates were kept at −80°C in tryptic soy broth (TSB) (Oxoid) having 25% glycerol for further analysis.

Antibiotic resistance evaluation

Antibiotic susceptibility testing was done using the disk diffusion method in accordence to the standards and interpretative rules described by the guidelines of the Clinical and Laboratory Standards Institute [15] on Mueller-Hinton agar (Difco). *Shigella* spp. were tested for susceptibility to commercially available antibiotic discs (Oxoid), including discs of ampicillin (AM), 10 µg; amoxicillin-clavulanate (AMC), 30 µg; cefaclor (CEC), 30 µg; cefotaxime (CTX), 30 µg; ceftazidime (CAZ), 30 µg; cefepime (FEP), 30 µg; tetracyclines (TE), 30 µg; ciprofloxacin (CIP), 5 µg; chloramphenicol (C), 30 µg; streptomycin (S), 10 µg; azithromycin (AZM), 15 µg; sulfamethoxazole/trimethoprim (SXT), 25 µg; imipenem (IPM), 10 µg. Antibiotic selection was based on the frequency and availability of these antibiotics in the study area, both in veterinary and human medicine. Resistance to at least one antibiotic belonging to at least three antibiotic classes was interpreted as multidrug resistance. According to the diameter of the inhibition zone, isolates were calssified as susceptible, intermediate, or resistant. The isolates were investigated for ESBL production by the double-disc synergy test following the recommendation and interpretations of *CLSI* guidelines [14]. *Escherichia*

coli ATCC 25922 was used as a quality control strain. Multiple-antibiotic resistance (MAR) index values were calculated using the formula a/b, where 'a' represents the number of antibiotics to which a particular isolate was resistant, and 'b' represents the total number of antibiotics tested [28].

Disinfectant susceptibility testing

To test the effectiveness of BKC (2%) [20] and a phenolic compound (vanillin, 5%) (Chemica Co., Mansoura, Egypt) against *Shigella* isolates, agar well diffusion assay was carried out according to Njagi *et al*. [35]. Briefly, *Shigella* broth cultures of each strain incubated for 24–48 hr were diluted in saline (NaCl 0.9%) to an approximately cell density of 1×10^6 CFU/ml based on McFarland turbidity standards. The bacteria suspensions were dispersed in the same approach as for the antibiotic susceptibility tests. After spreading out, wells were cut by a sterile 6 mm diameter agar well-puncher. Each disinfectant (50 µl) was distributed distinctly, with a micropipette, into various wells, by one petri-dish per disinfectant. The petri-dishes were then incubated in a vertical position at 37°C. Afterward 16 hr of incubation, every inhibition zone was measured and recorded in millimetres (mm). The results were interpreted as follows: a radius of 0–5 mm from the edge of the well to the inhibition front was considered as no inhibition; a radius of 6–9 mm was taken as a moderate inhibition; a radius of 10–14 mm was considered a strong inhibition, and beyond 15 mm was taken as a very strong inhibition [30].

Screening for antibiotic and disinfectant resistance genes

For amplification of β-lactamase-encoding *genes* (bla_{TEM}, bla_{CTX-M}, bla_{OXA-1}, and bla_{SHV}), TE resistance gene and QAC resistance genes (*qacEΔ1*), uniplex PCR assays were carried out with primers provided by Metabion (Martinsried, Germany) (Table 1). These resistance genes are most commonly found in gram-negative bacteria [18]. Genomic DNA from the *Shigella* isolates was extracted by the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. The PCR amplification reaction was performed in an Applied Biosystems 2720 thermal cycler using specific profiles (Table 1). The PCR products were subjected to electrophoresis on a 1.5% agarose gel (Applichem, Darmstadt, Germany) in 1 × TBE buffer using a gradient of 5 V/cm. After staining with ethidium bromide, the gel was visualized under UV light. *Escherichia coli* ATCC 25922 was used as a negative control in the PCR assay.

Plasmid profile analysis and Conjugative transfer

The Plasmid Midi kit (Qiagen) was applied for plasmid DNA isolation from all *Shigella* isolates (n=42) according to the manufacturer's recommendations. The extracted plasmid DNA was separated by electrophoresis on a 0.8% agarose gel (Applichem, Germany, GmbH) in 1 × TBE buffer at room temperature using gradients of 5 V/cm. For gel analysis, 20 µl of the plasmid was mixed with a loading dye and loaded in each gel slot. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through its computer software. A GeneRuler 1Kb plus DNA ladder (Fermentas, Thermo Scientific, Karlsruhe, Germany) were used to determine the molecular masses of the unknown plasmid DNA and to analyze the plasmid profiles.

In a trial to prove the association of these plasmids with ESBL-based antibiotic resistance, conjugation experiments were performed using the azide-resistant *E. coli* J53 as the recipient and all *Shigella* ESBL producers (n=12) as the donors, as described previously [1]. Transconjugants were demonstrated by the double-disk synergy test following the *CLSI* guidelines [14].

Table 1. PCR conditions employed for the detection of resistance associated genes of *Shigella*

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
bla_{TEM}	ATCAGCAATAAACCAGC CCCCGAAGAACGTTTTC	516	94°C 5 min	94°C 30 sec	54°C 45 sec	72°C 45 sec	72°C 10 min	[16]
bla_{OXA-1}	ATATCTCTACTGTTGCATCTCC AAACCCTTCAAACCATCC	619	94°C 5 min	94°C 30 sec	54°C 45 sec	72°C 45 sec	72°C 10 min	
bla_{SHV}	AGGATTGACTGCCTTTTTG ATTTGCTGATTTCGCTCG	392	94°C 5 min	94°C 30 sec	54°C 45 sec	72°C 45 sec	72°C 10 min	
bla_{CTX-M}	ATGTGCAGYACCAGT AARGTKATGGC TGGGTRAARTARGTS ACCAGAAYCAGCGG	593	94°C 5 min	94°C 30 sec	60°C 45 sec	72°C 45 sec	72°C 10 min	[6]
tetA(A)	GGTTCACTCGAACGACGTCA CTGTCCGACAAGTTGCATGA	576	94°C 5 min	94°C 30 sec	50°C 40 sec	72°C 45 sec	72°C 10 min	[37]
qacEΔ1	TAAGCCCTACAC AAATTGGGAGATAT GCCTCCGCAGCGACT TCCACG	362	94°C 5 min	94°C 30 sec	58°C 40 sec	72°C 40 sec	72°C 10 min	[12]

Plasmid DNA was extracted from *E.coli* J53 transconjugants and co-transfer of resistance determinants was determined through amplification of the relevant genes (bla_{TEM}, bla_{CTX-M} and *qacEΔ1*) in the transconjugants by PCR as previously described.

RESULTS

Occurrence of Shigella species in raw cow milk and milk products

A total of fourty-two (7%) organisms were isolated and identified as *Shigella* spp. *S. dysentriae* was the most frequently identified, comprising 24 isolates (57.1%), followed by *S. flexneri* with 12 isolates (28.6%) and *S. sonnei* with 6 isolates (14.3%). A single *Shigella* species was identified from each sample. The occurrence of the isolates in the samples is presented in Table 2. The occurrence of *Shigella* spp. was higher in Kareish cheese (13%) obtained from markets than in raw cow milk (8%) obtained from dairy farms. In addition, the absence of *Shigella* spp. was observed in all examined yoghurt samples. These are indicative of high contamination level at market point sampled kareish cheese.

Antibiotic and disinfectant susceptibility profiles

Phenotypic antibiotic resistance of 42 isolates of *Shigella* is presented in Table 3. The highest resistance percentages occurred against tetracyclines (TEs) (100%), ampicillin (AM), amoxicillin-clavulanate (AMC) (90.5%, each) and cefaclor (CEC) (66.7%). All isolates were sensitive to imipenem (IPM), sulfamethoxazole/trimethoprim (SXT), and azithromycin (AZM). A majority of the isolates were sensitive to cefepime (FEP), streptomycin (S) (90.5% each), chloramphenicol (C) (85.7%), ciprofloxacin (CIP) (80.9%), ceftazidime (CAZ) (66.7%) and cefotaxime (CTX) (61.9%). Only two isolate from *S. dysenteriae* and *S. flexneri* exhibited resistance to CIP and C (9.5%). Thirty (71.4%) *Shigella* isolates were resistant to at least three of the antimicrobial classes. The multiple-antibiotic resistance (MAR) index of the isolates ranged from 0.2–0.5. The highest MAR index of 0.5 was recorded in ten (23.8%) isolates, followed by 0.4 in 8 (19%) isolates. The *Shigella* strains showed eight multi-resistance phenotypes (Table 4). The

Table 2. Occurrence of *Shigella* species in dairy products (n=42/600)

Isolated bacteria	No of positive samples (%)			Total (n=600)
	At the dairy farms	At the market		
	Raw cow milk (n=200)	Kareish cheese (n=200)	Yoghurt (n=200)	
Shigella dysenteriae	10	14	0	24/42 (57.1%)
Shigella flexneri	4	8	0	12/42 (28.6%)
Shigella sonnei	2	4	0	6/42 (14.3%)
Total	16	26	0	42/600 (7%)

Table 3. Antibiotic and disinfectant susceptibility profiles of *Shigella* species (n=42)

Antimicrobial class	Antimicrobial	*Shigella dysenteriae* (n=24)			*Shigella flexneri* (n=12)			*Shigella sonnei* (n=6)			Total (n=42) (%)*		
		R	I	S	R	I	S	R	I	S	R	I	S
Tetracyclines	TE	24	0	0	12	0	0	6	0	0	42 (100)	0	0
β-Lactams	AM	22	2	0	10	2	0	6	0	0	38 (90.5)	4 (9.5)	0
	AMC	24	0	0	8	2	2	6	0	0	38 (90.5)	2 (4.8)	2 (4.8)
Cephalosporines	CEC	16	4	4	8	2	2	4	2	0	28 (66.7)	8 (19)	6 (14.3)
	CTX	0	4	20	6	0	6	6	0	0	12 (28.6)	4 (9.5)	26 (61.9)
	CAZ	0	2	22	6	0	6	6	0	0	12 (28.6)	2 (4.8)	28 (66.7)
	FEP	0	4	20	0	0	12	0	0	6	0	4 (9.5)	38 (90.5)
Fluoroquinolones	CIP	2	4	18	2	0	10	0	0	6	4 (9.5)	4 (9.5)	34 (80.9)
Phenicols	C	2	2	20	2	0	10	0	0	6	4 (9.5)	2 (4.8)	36 (85.7)
Aminoglycosides	S	2	0	22	0	2	10	0	0	6	2 (4.8)	2 (4.8)	38 (90.5)
Macrolides	AZM	0	0	24	0	0	12	0	0	6	0	0	42 (100)
Sulfonamides	SXT	0	0	24	0	0	12	0	0	6	0	0	42 (100)
Carbapenems	IPM	0	0	24	0	0	12	0	0	6	0	0	42 (100)
QACs	BKC	18	-	6	12	-	0	6	-	0	36 (85.7)	-	6 (14.3)
Phenolic compounds	V	24	-	0	12	-	0	6	-	0	42 (100)	-	0

Resistant (R), intermediate (I), sensitive (S), number (n), tetracycline (TE), ampicillin (AM), amoxacillin-clavulanate (AMC), cefaclor (CEC), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), ciprofloxacin (CIP), chloramphenicol (C), streptomycin (S), azithromycin (AZM), sulfamethoxazole/trimethoprim (SXT), imipenem (IPM), quaternary ammonium compound (QAC), benzalkonium chloride (BKC), vanillin (V). *The percentages were calculated across rows.

Table 4. Antimicrobial resistance phenotypes of isolated *Shigella* strains (n=42)

Pattern	Resistance phenotypes	No. of isolates	No. of resistant antibiotics	Ratio (%)	MAR index*
A	TE, AM, AMC, CEC, CIP, C	4	6	9.5	0.5
B	TE, AM, AMC, CEC, CTX, CAZ	6	6	14.3	0.5
C	TE, AM, AMC, CEC, S	2	5	4.8	0.4
D	TE, AM, CEC, CTX, CAZ	4	5	9.5	0.4
E	TE, AM, AMC, CTX, CAZ	2	5	4.8	0.4
F	TE, AM, AMC, CEC	12	4	28.6	0.3
G	TE, AM, AMC	8	3	19	0.2
H	TE, AMC	4	2	9.5	0.2

Tetracycline (TE), ampicillin (AM), amoxacillin-clavulanate (AMC), cefaclor (CEC), cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), ciprofloxacin (CIP), chloramphenicol (C), streptomycin (S), azithromycin (AZM), sulfamethoxazole/trimethoprim (SXT), imipenem (IPM). *Multiple-antibiotic resistance (MAR) index values were calculated using the formula a/b, where 'a' represents the number of antibiotics to which a particular isolate was resistant, and 'b' represents the total number of antibiotics tested.

predominant multi-resistance phenotypes for *Shigella* isolates were TE, AM, AMC, CEC and TE, AM, AMC in 28.6% and 19% of the isolates, respectively. The double-disc synergy test confirmed that all CAZ and CTX resistant strains (28.6%, 6 *S. flexneri* and 6 *S. sonnei*) were ESBL producers.

With regard to disinfectant resistance (Table 3), phenolic compound (vanillic acid) showed no efect on the growth of the *Shigella* isolates, no inhibition zone around the discs, as well as the effect of saline used as control. Although, it had larger zone of inhibition (19 mm) against *Escherichia coli* ATCC 25922 which was used as quality control strain. Thirty-six (85.7%) of the *Shigella* isolates exhibited BKC tolerance (no inhibition zone around the discs).

Characterization of antibiotic and disinfectant resistance genes

Uniplex PCR assay results revealed that all *Shigella* spp. isolates were positive for bla_{TEM}, and only 12 (28.6%) isolates were positive for bla_{CTX-M}, while all the *Shigella* isolates were negative for bla_{OXA-1} and bla_{SHV} (Supplementary Table 1). The genotype analysis demonstrated the existence of ESBL-encoding genes that were responsible for ESBL production in *Shigella* isolates. All *Shigella* ESBL producers (12, 100%) were positive for bla_{TEM} and bla_{CTX-M} genes, whereas none of the ESBL producers harbored the bla_{OXA-1} and bla_{SHV} genes. The TE resistance gene, *tet*(A), was identified in all *Shigella* isolates. Furthermore, 36 (85.7%) of the *Shigella* isolates possessed a QAC resistance gene (*qacEΔ1*). It seems that the presence of the *qacEΔ1* gene might coincide with an antimicrobial resistance profile (Supplementary Table 1), which shows that all MDR strains harbor the *qacEΔ1* gene with a MAR index of 0.2–0.5. In addition, all qacEΔ1 gene-positive strains were bla_{TEM} and *tet*(A) genes positive.

Plasmid profiling and conjugative transfer

Plasmid profiling (PP) revealed seven distinct plasmid patterns (P1–P7) ranging from 1.26 to 33.61 kb among the *Shigella* strains; *S. dysenteriae* yielded the greatest variance (Supplementary Table 1). All plasmid patterns (P1–P7) were distributed in a similar percentage (3, 14.3%) among *Shigella* spp. *S. dysenteriae* strains contained two to four plasmids with approximate sizes of 1.26, 2.23, 2.36, 2.67, 4.07, 4.66, 7.12, 8.89, 27.51, 30.80 or 33.61 kb. *S. flexneri* contained one to three plasmids with approximate sizes of 2.23, 4.40, 18.59 or 23.28 kb, while *S. sonnei* harbored two plasmids with sizes of 1.41 and 30.80 kb plasmids. All ESBL-producing strains harbored plasmids with pattern P7 (1.41 and 30.80 kb) as the predominant pattern.

The co-transfer of β-lactamase genes (bla_{TEM} and bla_{CTX-M}) and *qacEΔ1* genes was observed by conjugation. After the conjugative test using the plating mating method, the co-transfer of β-lactamase genes (bla_{TEM} and bla_{CTX-M}) and *qacEΔ1* genes was observed by conjugation from all ESBL producing strains (n=12) as donor strains to the azide-resistant *E. coli* J53 as the recipient strain. All obtained transconjugants successfully acquired these resistance (bla_{TEM}, bla_{CTX-M} and *qacEΔ1)* genes from donor strains.

DISCUSSION

Although milk and milk products represent as important vehicles for foodborne disease transmission to humans, in developing countries, limited publications have documented shigellosis outbreaks related to the consumption of milk and milk products [29, 45]. The present study showed a high prevalence of *Shigella* spp. (7%) with the predominance of *S. dysenteriae*. Although *S. flexneri* has been stated as the main cause of shigellosis in developing countries, other investigation revealed *S. dysenteriae* as the most common serogroup (A) of *Shigella* in Africa [8]. In comparison to the results of *Ahmed and Shimamoto* [2], who reported that *Shigella* spp. were detected in 1.4% of dairy product samples with *S. flexneri* as the predominant species. *Tambekar and Bhutda* [42] detected 8.7% *S. flexneri* in milk product (pedha) samples in India. In the current study, the level of contamination with *Shigella* spp. was higher in kareish cheese obtained from markets. The high rate of *Shigella* prevalence in this study might indicate poor hygienic measures used during milking, processing, preparation, and handling of milk and milk products [2, 24]; particularly

water and faeces are the major source of *Shigella* [31]. Thus, basic hygienic measures must be enforced in animal farms to minimize the risk of spread of *Shigella* to other animals and human using control of microbial pollutants, capability hygiene, cleanliness of cows, good animal health managing, in effect cleaning and disinfection measures of the milking mechanism, clean water and staff hygiene [43].

The risk of *Shigella* might be higher in the raw cow milk and cheese, as compared to yoghurt products because yoghurt has very effectively inhibitory effect; involving competing bacterial flora and low pH (4.5); on the growth of the most common enteric pathogens such as *Shigella* [34, 49]. Overall, it is important to observe all hygienic measures while dealing with milk and milk products. Also, pasteurization of milk intended for consumption or processing to human nutrition is necessary to reduce the number of foodborne pathogen, especially raw milk might be sold as it's and used for prepartion of karish cheese in Egypt.

The antibiotic resistance of *Shigella* spp. isolated from raw cow milk and milk product samples in the present work was compared with preceding studies from Egypt to notic the development in antibiotic resistance taking into account the sample collection method for each study [4]. Overall, this study revealed the presence of harmful level of *Shigella* spp. resistant to the prevalently used antibiotics (TE, AM, AMC, CEC) among human and livestocks [4, 47]. This resistance might be because of the frequent and improper use of such antibiotics either in animal therapy or as a growth promoter in the veterinary context in Egypt [3] and the horizontal transfer of resistance genes from animal food resources and environments to humans' normal flora and pathogens through food and drink chains [11]. Additionally, the current investigation showed reduced susceptibility to CTX, CAZ (third-generation cephalosporins) and CIP, which are considered preferable drugs for shigellosis treatment [7]. Thus, the appearance of such resistance would pose a great challenge for the efficient treatment of shigellosis.

Many isolates of *Shigella* spp. were shown to have multi-resistance phenotypes against TE, AM, AMC, and CEC. Similarly, a high prevalence of MDR *Shigella* isolates in dairy products (90.9%) was reported by *Ahmed and Shimamoto* [3] in Egypt. Therefore, some measures must be considered to confirm that the currently available antibiotics remain effective. These measures may include increasing the consciousness amongst the public, healthcare professionals and the food-agriculture sector concerning the necessity of the proper use of these medicines.

The presence of *Shigella* in the the examined samples was an indicator of poor hygiene and sanitation during milking, post milking and during milk processing. The effectiveness of disinfection depended on the use of a suitable disinfectant, which is considered the most critical aspect of hygienic measures used in dairy cattle farms. Phenolic compounds and BKC are widely used as farm disinfectants due to their antimicrobial activity [25, 32]. BKC is a cationic, surface-active QAC commonly used as a farm disinfectant for cleaning and sanitizing livestock buildings, equipment, milk utensils, and vehicles [22]. The current work demonstrated that all the studied strains were resistant to vanillic acid as one of phenolic compound, while 85.7% of the isolates were resistant to BKC. Similarly, *Bouzada et al.* [9] found that gram-negative rods of *Enterobacteriaceae* exhibited low susceptibility to BKC.

Various β-lactamases, which hydrolyse the β-lactam ring and thereby inactivate β-lactam antibiotics, have been stated. TEM-, OXA-, SHV- and CTX-M-type β-lactamases are dominant in gram-negative bacteria [10]. Thus, in this investigation, the presence of these β-lactamase-encoding genes in isolates was recognized by molecular methods, which provided data to support the present study. The bla_{TEM} gene, a broad-spectrum *β*-lactamase gene that confers resistance to penicillins and first-generation cephalosporins, was identified in all isolates. Additionally, the ESBL-encoding gene $bla_{\text{CTX-M}}$ was recognized in 28.6% of the isolates. The high incidence of β-lactamase-encoding genes ($bla_{\text{TEM-1}}$, $bla_{\text{CTX-M}}$, in 2 isolates; bla_{OXA}, in 4 isolates) had been detected previously in *Shigella* strains isolated from dairy products in Egypt [3]. The bla_{TEM} gene was the predominant β-lactamase gene in *Shigella* spp. in this work, while $bla_{\text{CTX-M}}$ was the furthermost type of cefotaximases identified among *Shigella* isolates in a previous study [19]. Alarmingly, in this research, the occurrence of ESBL-producing *Shigella* isolates, accounting for 28.6% of all *Shigella* isolates, was higher than the detection rates noticed in other countries, such as Turkey [44]. This discrepancy between these findings and previous studies might be attributed to many factors as the geographical location, and unsupervised use of antibiotics by the community and animal health professionals.

For epidemiological investigations of various enteric pathogens, PP could be an attractive tool. *Shigella* spp. usually harbor various plasmids, with 2 to 10 plasmids being harbored by one strain [31]. These plasmids are required for antibiotic resistance and for bacterial invasion of intestinal epithelial cells [23]. Seven plasmid patterns, with relative plasmid sizes ranging from 1.26 to 33.61 kb, were detected in this study. It had been reported previously that multiple plasmid patterns (n=23) were found in all *Shigella* species, varying from 3 in *S. dysenteriae* to 7 plasmid patterns in each of *S. flexneri* and *S. sonnei* [31]. Also, Ud-Din [46] found that *Shigella* spp. in Bangladesh harbor varying numbers of plasmids ranging in size from 1.0 to 120 MDa.

Limitation

It should be noted that there are some limitations to the present study. Although the current study showed various plasmid profile patterns among *Shigella* strains, it focused on the plasmid DNA analysis depending on plasmid molecular sizes using gel electrophoresis and did not elucidate the plasmid digestion with a restriction enzyme. The restriction enzyme digestion simplifies characterization of plasmids and warranted to be explored in further study.

The present study demonstrated the prevalence of MDR *Shigella* species that were either MDR to antibiotics (particularly ESBL-producing strains) or disinfectants in raw cow milk and milk products in Egypt, posing a possible hazard to animal and public health and causing difficulty in controlling outbreaks. Thus, the resistance of *Shigella* species should regularly be monitored, and appropriate measures should be taken to avoid the emergence and spread of MDR strains. Additionally, strict hygienic measures must be applied throughout dairy industry chain to prevent infection through the consumption of dairy products.

CONFLICT OF INTEREST. The authors have declared that they have no competing conflict of interest.

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