Genetic characterization and plasmid profile of extended-spectrum β -lactamase and Quaternary ammonium compound E delta 1 gene producing multi-resistant *Shigella* species isolated from raw cow milk and milk products in Egypt

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Abstract

Background Multi-resistant *Shigella* species recovered from raw cow milk and milk products has predominated all over the world especially extended spectrum β -lactamases (ESBLs) and Quaternary ammonium compound E delta 1 (*qacE* Δ 1) genes. This study was conducted 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. The genotypic analysis was determined for the presence of β -lactamase encoding genes (*bla*TEM, *bla*CTX-M, *bla*OXA-1 and *bla*SHV), *tetA*(A) and *qacE* Δ . Results Twenty-one (7%) of *Shigella* isolates (*S. dysenteriae*, *S. flexneri* and *S. sonnei*) were recovered with *S. dysenteriae* as the most predominant types. Antibiotic sensitivity tests showed 71.4% of multidrug-resistant *Shigella* isolates. High resistance rates were observed to tetracyclines (100%), ampicillin, amoxicillin-clavulanate (90.5%, each) and cefaclor (66.7%), whilst no resistance was detected against imipenem, sulfamethoxazole/trimethoprim, and azithromycin. Disinfectant susceptibility test of *Shigella* isolates revealed resistance to phenolic compound (vanillic), while 85.7% of *Shigella* isolates were benzalkonium chloride resistant. Uniplex PCR analysis exhibit presence of β -lactamase encoding genes (*bla*TEM in all isolates and *bla*CTX-M in 28.6% of isolates), *tetA*(A) in all isolates and 85.7% isolates positive for *qacE* Δ 1, while all isolates were negative for *bla*OXA-1 and *bla*SHV. All *Shigella* extended spectrum β -lactamase (ESBL) producers (6, 100%) were positive for *bla*TEM, *bla*CTX-M, and *qacE* Δ 1 genes. Furthermore, plasmid profiling revealed seven distinct plasmid patterns (P1-P7) ranging from 1.26 to 33.61 kb among all *Shigella* strains; *S. dysenteriae* displayed the greatest variance. The co-transfer of β -lactamase genes (*bla*TEM and *bla*CTX-M) and *qacE* Δ 1 genes was observed by conjugation. Conclusions *S. dysenteriae* was the most common identified types in the examined sources. Also, the findings imply the emergence of multi-resistant *Shigella* species either multi-resistant to antibiotics (particularly ESBL producer strains) or disinfectants in Egypt. Thus, the resistance of *Shigella* species should regularly be monitored and appropriate measures should be taken to manage this problem.

Background

Shigella species infection is known as the main cause of bacillary dysentery disease which associated with high morbidity and mortality especially in developing countries, such as Egypt [1]. *Shigella* genus is classified into four species: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. Endemic shigellosis in developing countries is mainly occurred by *S. flexneri* but *S. sonnei* is commonly detected in industrialized countries [2]. Dairy products especially raw milk and unpasteurized cheese remain important vehicles for the transmission of *Shigella* to humans [3,4].

Antibiotic therapy for *Shigella* infection can decrease the extent and seriousness of the disease [5]. The consequence of the misuse of antibiotics administration, the multi-resistant (MR) *Shigella* has predominated all over the world, particularly extended spectrum β -lactamases (ESBLs) which is one of the greatest extended resistance mechanisms [6,7]. There were many ESBL variants known in a variety of pathogens, *bla*CTX-M, *bla*TEM, *SHV* and *bla*OXA-1 that were proved to be the most successful in terms of antibiotic resistance and for epidemiological niches [7]. Among *bla*TEMESBLs types, the *bla*TEM-1 is the most commonly plasmid-mediated β -lactamase of ampicillin resistant Gram-negative bacteria [8]. The selection of antibiotic treatment for shigellosis becomes more difficult as a result of the generation of *Shigella* spp. resistance, especially the resistance which was developed to the third-generation cephalosporins which is a common public health problem, mainly in developing countries [9, 10]. The evolution of multidrug-resistant (MDR) strains is commonly due to the existence of mobile genetic elements such as; plasmids, integrons, and transposons which assist the *Shigella* spp. to obtain and transfer the exogenous genes [11]. Antibiotic-resistance plasmids often include genes conveying resistance to numerous different antibiotics [12].

Disinfectants including phenolic compound (vanillic) 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 membrane-impairing permeability action [13]. A particular concern is that repeated use of disinfectants may cause the persistence of bacteria with reduced susceptibility not only to the antiseptics but possibly to antibiotics [14]. One of the major mechanisms underlying such resistance is the acquisition of resistance genes called *qacE* and *qacE* Δ 1 that confer resistance to QACs [15]. The *qacE* Δ 1, a mutant version of *qacE*, is widely distributed throughout gram-negative bacteria mainly in *Enterobacteriaceae* [16].

Multiple global studies have reported the molecular basis of antibiotic resistance in clinical *Shigella* isolates of human origin[17,18]; but, inadequate data are available on the genotypic and phenotypic characteristics of *Shigella* spp. isolated from raw cow milk and milk products in Egypt. Therefore, this study was planned to isolate and characterize *Shigella* from raw milk and milk products in Egypt. This work was also proposed to examine the prevalence of multi-resistant *Shigella* particularly ESBL producing strains, screen for the presence of β -lactamase, *tetA(A)* and *qacE Δ 1* genes and its plasmid profile.

Results

The occurrence of Shigella species in raw cow milk and milk products

A total of twenty-one (7%) organisms were isolated and identified to be *Shigella*. *S. dysenteriae* was the most frequently identified, comprising 12 isolates (57.1%), followed by *S. flexneri* with 6 isolates (28.6%) and *S. sonnei* with 3 isolates (14.3%). The occurrences of the isolate in the samples are presented in Table 2. The occurrence of *Shigella* spp. was higher in kareish cheese (13%) than in raw cow's milk (8%). In addition, the absence of *Shigella* spp. in all examined yoghurt samples was detected.

Antibiotic and disinfectant susceptibility profiles

The antibiotic resistance of 21 isolates of *Shigella* is revealed in Table 3. The highest resistance percentages occurred against tetracyclines (100%), ampicillin, amoxicillin-clavulanate (90.5%, each) and cefaclor (66.7%). The highest frequency of intermediate resistance was displayed against cefaclor (19%). All isolates were sensitive to imipenem, sulfamethoxazole/trimethoprim, and azithromycin. The majority of the isolates were sensitive to cefepime, streptomycin (90.5% each), chloramphenicol (85.7%), ciprofloxacin (80.9%), ceftazidime (66.7%) and cefotaxime (61.9%). Only one isolate from *S. dysenteriae* and *S. flexneri* yielded resistance to ciprofloxacin and chloramphenicol (9.5%). Moreover, the only one isolate to display streptomycin resistance was *S. dysenteriae* (4.8%) but *S. flexneri* (4.8%) revealed intermediate resistance to streptomycin. About 15 (71.4%) of *Shigella* isolates were resistant to three of the antimicrobial classes at a minimum. The multidrug antibiotic resistance index (MARI) of the isolates was 0.2-0.5. The highest MARI of 0.5 was recorded in five (23.8%) isolates, followed by 0.4 by 4 (19%) isolates, 0.3 by 6 (28.6%) isolates and 0.2 by 6 (28.6%) isolates. The *Shigella* strains showed eight multi-resistance (MR) phenotypes (Table 4). The predominant MR phenotypes for *Shigella* isolates were TE, AM, AMC, CEC and TE, AM, AMC in 28.6 % and 19 % of the isolates, respectively. Among all MR phenotypes of *Shigella* isolates, 23.8 % of them were resistant to six different antibiotics, 19 % to five antibiotics, 28.6 % to 4 antibiotics, 19% to 3 antibiotics and 9.5% to 2 antibiotics. The double disk synergy test confirmed that all ceftazidime and cefotaxime resistant strains (28.6%, 3 *S. flexneri* and 3 *S. sonnei*) were extended-spectrum β -lactamase (ESBL) producers.

Concerning the disinfectant resistance (Table 3), there was growth in all plates containing vanillic acid disc that referred to the phenolic compound tolerance of all isolates, while 18 (85.7%) of *Shigella* isolates were BKC tolerance.

Characterization of antibiotic and disinfectant resistance genes

Uniplex PCR assay results exhibited that all *Shigella* spp. isolates were positive for *bla*TEM and only 6 (28.6%) isolates were positive for *bla*CTX-M, while all *Shigella* isolates were negative for *bla*OXA-1 and *bla*SHV (Table 5) (Figures. S1, S2, S3, S4). Out of 21 *Shigella* isolates, 6 (28.6%, 3 *S. flexneri* and 3 *S. sonnei*) isolates were phenotypically considered as ESBLs producers based on cefotaxime and ceftazidime resistance, alone and in combination with clavulanate by double disk synergy test. The genotypic methods demonstrated the existence of the extended-spectrum β -lactamase-encoding genes in *Shigella* isolates which were responsible for ESBL production. All *Shigella* ESBL producers (6, 100%) were positive for *bla*TEM and *bla*CTX-M genes, whilst none of the isolates harbored the *bla*OXA-1 and *bla*SHV genes. The tetracycline resistance gene, *tetA(A)*, was identified in all *Shigella* isolates. Furthermore, 18 (85.7%) of *Shigella* isolates possessed quaternary ammonium compound resistance gene (*qacE Δ 1*) (Figure. S5). Association of the occurrence of *qacE Δ 1* with the resistance to antibiotics is presented in Table 5 which illustrates that all MR strains harbor *qacE Δ 1* gene. In addition, all *qacE Δ 1* gene positive strains were *bla*TEM and *tetA(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 (Table 5). All plasmid patterns (P1-P7) were distributed in a similar percentage (3, 14.3%) among *Shigella* spp. *S. dysenteriae* strains involved from two to four plasmids of approximate sizes 1.26, 2.23, 2.36, 2.67, 4.07, 4.66, 7.12, 8.89, 27.51, 30.80 and/ or 33.61 kb. *S. flexneri* contained from one to three plasmids of approximate sizes 2.23, 4.40, 18.59 and/ or 23.28 kb, while *S. sonnei* harbored two, 1.41 and 30.80 kb plasmid. 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 *qacE Δ 1* genes was observed by conjugation.

Discussion

Although milk and milk products have been represented as an important vehicle for the foodborne disease transmission to humans, in developing countries; limited publications were documented shigellosis outbreaks in related to the consumption of milk and milk products. The current study showed a high prevalence of *Shigella* spp. (7%) with the predominance of *S. dysenteriae* in comparison to those recorded by *Ahmed and Shimamoto* [19] who reported that *Shigella* spp. were detected in 0.5% of raw cows milk samples and 0.9% of Kareish cheese samples with *S. flexneri* as the most predominant isolates. *Tambekar and Bhutda* [20] detected 8.7% of *S. flexneri* in milk product (pedha) samples in India. The high rate of *Shigella* prevalence in this study might be regarded to poor hygienic measures during milking, processing, preparation, handling, and storage of milk and milk products.

Antibiotic resistance of *Shigella* spp. from raw milk and milk products samples in this study was compared with previous reports from Egypt to observe the trend in antibiotic resistance keeping in mind that the respective study samples collection. Overall, this study had displayed the entity of warning levels of *Shigella* spp. resistant to the prevalently used antibiotics (tetracycline, ampicillin, amoxicillin-clavulanate, cefaclor) [21,22]. This resistance might be due to the fact of frequently and improperly using such antibiotics either in animal therapy or as a growth promoter in the veterinary context in Egypt. Also, the current study showed reduced susceptibility to cefotaxime, ceftazidime (third-generation cephalosporins) and ciprofloxacin, which were considered as preferable drugs for shigellosis treatment [10]. Thus, the appearance of such resistance would indicate a great challenge for the efficient treatment of shigellosis.

In this study, about 71.4% of *Shigella* isolates were multidrug-resistant to at least three of the antimicrobial classes with multidrug antibiotic resistance index (MARI) of 0.2-0.5. In addition, many isolates of *Shigella* spp. were shown to have MR phenotypes against tetracycline, ampicillin, amoxicillin-clavulanate, and cefaclor pattern. Nearly similarity, the high prevalence of MR *Shigella* isolates in dairy products (90.9%) was reported by *Ahmed and Shimamoto* [19] in Egypt. Therefore, some measures must be considered to confirm that currently available antibiotics remain effective. These measures may include great awareness among the public, healthcare professionals and the food-agriculture sector concerning the importance of the proper use of these medicines.

The presence of *Shigella* in the analyzed samples was an indicator of poor hygiene and sanitation during milking, post-milking and during milk processing. The effectiveness of the disinfection depended on the use of suitable disinfectant which was considered from the most critical points of hygienic measures in dairy cattle farms. The phenolic compounds and benzalkonium chloride (BKC) were widely used as farm disinfectant due to their antimicrobial activity [23]. BKC is a cationic, surface-active QAC commonly used as a farm disinfectant for cleaning and sanitizing of livestock buildings, equipment, milk utensils, and vehicle. The present study exhibited that all the tested strains were resistant to phenolic compound, while 85.7% of isolates were BKC resistance. Similarly, *Bouzada et al.* [24] found that Gram-negative rods involving Enterobacteriaceae were less susceptible to BKC. Moreover, this work displayed that most of the *Shigella* isolates (85.7%) had *qacE Δ 1* gene.

There are various β -lactamases, which hydrolyse the β -lactam ring and thereby inactivate the antibiotic, have been described, but TEM-, OXA-, SHV- and CTX-M-type β -lactamases are dominant in the Gram-negative bacteria [25]. 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 current study. The *bla*TEM, a narrow-spectrum β -lactamase gene, which conveys resistance to penicillins and first-generation cephalosporins, was identified in all isolates. Also, the extended spectrum β -lactamase-encoding genes *bla*CTX-

Mwas identified in 28.6% of the isolates. The high incidence of β -lactamase-encoding genes (*bla*TEM-1, *bla*CTX-M, in 2 isolates, *bla*OXA, in 4 isolates) had been detected previously in *Shigella* strains isolated from dairy products in Egypt [19]. The *bla*TEM gene was the dominant β -lactamase gene in *Shigella* spp. in this work, while *bla*CTX-M was the most common types of cefotaximases identified among *Shigella* isolates in the previous study [9].

Alarmingly, in this research, the prevalence of ESBL producing *Shigella* isolates, accounting for 28.6% of all *Shigella* isolates, was higher than detecting rates observed in other countries such as England [26]. This discrepancy between such finding and previous studies might be attributed to the misuse of antibiotics during the treatment of bacterial infections. In addition, the high tetracycline resistance in all *Shigella* isolates might be described by the potential distribution of the *tetA(A)* resistance gene [27].

For epidemiological investigations of various enteric pathogens, plasmid profiling could be an attractive tool. *Shigella* usually harbor various plasmids that might be from 2 to 10 plasmids in one strain. These plasmids were required for antibiotic resistance and also for invading of bacteria into the epithelial cells of the intestine [28]. Seven Plasmid Patterns, with relative plasmid sizes ranging from 1.26 to 33.61 kb, were detected in this study. It had been reported earlier that *Shigella* species had numbers of plasmids with a heterogeneous combination ranging from 1.0 to 120 MDa in Egypt [29]. Also, some of the plasmid sizes documented among the *Shigella* isolates were previously reported in Egypt and other countries [30,31,32]. All MR strains particularly ESBL producing strains carried plasmids with pattern P7 as the predominant pattern in this study. The ESBL encoding gene (*bla*CTX-M) had been conveyed to plasmid more than other Class A β -lactamases [33]. The conjugation experiment results assisted the plasmid location of *bla*TEM, *bla*CTX-M, and *qacE Δ 1* genes, since transconjugants of MR phenotypes of *Shigella* isolates were done on MacConkey agar. Of the 12 *S. dysenteriae* isolates in this work, 75% contained large plasmids, which were associated with antibiotic resistance. Over the last half-century, the extraordinary ability of different isolates in acquiring plasmid-encoded resistance to the disinfectants and antibiotics such as ESBLs, that could quickly be transmitted to several other strains, had demonstrated [34,35].

Antibiotics and disinfectants had been commonly used in dairy farms in Egypt. However, the susceptibility of *Shigella* to disinfectant and its contribution to multidrug resistance phenotype and genotype by co-selection of plasmid had never been reported. Plasmid-mediated multidrug resistance should be considered for dealing with infectious diseases. Therefore, this work was planned to explore the link between *qacE Δ 1* gene, plasmid, and antibiotic resistance. The results of this study showed that all *qacE Δ 1* gene positive strains were multi-resistant and plasmid harbored. Recently, it was demonstrated that the *qacC* gene conveys resistance to a number of β -lactam antibiotics [36]. While it was found the ability of *qac* genes to directly acquire resistance to antibiotics. This indicated the close relationship between the resistance to antibiotics and antiseptics [37]. An approaching interdependence between resistance to antiseptics and antibiotics might be principally elucidated by the fact that genetic determinants of resistance to these agents were mainly associated. The plasmids frequently conveyed *qac* genes with a number of other antibiotic resistance genes [38,39]. The plasmid analysis and conjugation experiments showed that the isolates harbored variable detectable plasmids and the antibiotic and disinfectant resistance genes could be co-transferred. This indicated that the resistance was plasmid-mediated, so there was a high risk for the spread of antibiotic and disinfectant resistance genes among the bacteria.

Limitations

The limitations of this study should be mentioned. Although this work explored for the first time the relationship of resistance to antibiotics and disinfectants with plasmids in multi-resistant *Shigella* spp. in Egypt, it focused on raw cow milk and milk product samples collected from only one province of Egypt and didn't elucidate such relationship in other provinces. Therefore, additional studies are warranted to explore such relationship in other provinces of Egypt.

Conclusion

This study demonstrated the prevalence of multi-resistant *Shigella* species either multi-resistant to antibiotics (particularly ESBL producer strains) or disinfectants in raw cow milk and milk products in Egypt that causes a possible hazard to public health and difficulty in controlling of outbreaks. Thus, the resistance of *Shigella* species should regularly be monitored and appropriate

measures should be taken to avoid the emergence and spreading of multi-resistant strains. Also, strict hygienic measures must be applied to prevent infection through consumption of dairy products.

Methods

Sample collection and microbial analysis

A total of 300 dairy product samples (100 raw cow milk, 100 Karish cheese, and 100 yoghurts) were collected randomly from different dairy farms, shops, and supermarkets in Dakahlia Governorate, Egypt throughout 2018. After collecting samples, they were stored at 4 °C until examination (within 3-4 h), then homogenization of (25 ml or gm) from each has been done in 225 ml of 0.1% buffered peptone water (Oxoid, England) by shaking for 5 min in a sterile stomacher bags and incubating for 24 h 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 onto Selenite F broth, then subcultured to *Salmonella-Shigella* (S-S) agar, MacConkey agar and xylose-lysine-deoxycholate agar (XLD) (Oxoid, UK) and then incubated at 37 °C for 24 h. The presumptive *Shigella* isolates (colorless, non lactose fermenting on SS agar, white and translucent on MacConkey and pink to red colonies on XLD) were biochemically confirmed by triple sugar iron agar (TSI), lysine iron agar (LIA), methyl red (MR), Voges-Proskauer (VP) broth, indole test, urea agar (UA), Simmon's citrate agar (SCA) and motility test. Serotypes of isolates were determined by slide agglutination assays, using a commercially available kits as described by the manufacturer (Difco Laboratories). All bacterial isolates were stored in tryptic soy broth (TSB) containing 25% glycerol at -80 °C for further analysis.

Antibiotic resistance evaluation

The antibiotic susceptibility testing was performed using the disk diffusion method according to the standards and interpretative rules described by the guideline of Clinical and Laboratory Standards Institute [40] on Mueller-Hinton agar (Difco). *Shigella* spp. were tested for susceptibility to commercially available antibiotic disks (Oxoid, England), including: tetracycline (TE), 30 µg; ampicillin (AM), 10 µg; amoxicillin-clavulanate (AMC), 30µg; cefaclor (CEC), 30µg; cefotaxime (CTX), 30µg; ceftazidime (CAZ), 30µg; cefepime (FEP), 30µg; ciprofloxacin (CIP), 5 µg; chloramphenicol (C), 30 µg; streptomycin (S), 10 µg; azithromycin (AZM), 15µg; trimethoprim-sulfamethoxazole (SXT), 25 µg and imipenem (IPM), 10µg. The selection of antibiotics was depended on the frequency and availability of using 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 (MDR). According to the diameter of the inhibition zones, isolates were categorized as sensitive, intermediate, or resistant. The isolates were screened for ESBL production by using the double disk synergy test following recommendation and interpretations of *CLSI* standards [40]. *Escherichia coli* ATCC 25922 was used as quality reference strains. Multiple antibiotic resistance indexes (MARI) was calculated using the formula: a/b where 'a' represents the number of antibiotics to which a particular isolate was resistant and 'b' the total number of antibiotics tested.

Disinfectant susceptibility testing

To test the effectiveness of BKC (2%) [41] and phenolic compound (vanillic 5%) (Chemica Company) against *Shigella* isolates, disk diffusion assay was carried out according to the *CLSI* [42]. Briefly, 24-48 h incubated *Shigella* broth cultures of each strain were diluted in saline (NaCl 0.9%) to contain approximately 1×10^6 CFU /ml by McFarland turbidity standards, the diluted cultures were poured on the surface of nutrient agar plates. The plates were allowed to dry after which five assay disks, 6mm, saturated with test substances which were applied to the surface of each plate then incubated aerobically at 37 °C for 24 h. The sensitivity pattern was scored as tolerance (-) and sensitive (+).

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), tetracycline genes and quaternary ammonium compound resistance genes (*qacE Δ 1*), uniplex PCR assays were applied with primers provided by Metabion (Germany) (Table 1). These resistance genes were mostly encoded in Gram-negative bacteria. *Shigella* isolates chromosomal DNA was extracted by the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) considering modulations from the manufacturer's

recommendations. The PCR amplification reaction was carried out in an applied biosystem 2720 thermal cycler using specific profiles (Table 1). The PCR products were subjected to electrophoresis in 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer using gradients of 5 V/cm. After staining with ethidium bromide the gel was visualized under UV light.

Plasmid profile analysis and Conjugative transfer

Plasmid Midi kits (Qiagen, Germany, GmbH) were used for plasmid DNA isolation from clinical isolates. Gel electrophoresis of the PCR products was applied to 0.8% agarose gel (Applichem, Germany, GmbH). The fragment sizes were determined by a gene ruler 1Kb plus DNA ladder (Fermentas, Thermo Scientific, Germany). In a trial to prove the association of these plasmids with ESBL antibiotic resistance, conjugation experiments were carried out as described previously [46] using the azide-resistant E. coli J53 as the recipient strain. Transconjugants were detected by plating mating mixtures on MacConkey agar supplemented with 150 mg/L sodium azide and 2 mg/L cefotaxime. Co-transfer of resistance determinants was determined through amplification of the relevant genes in the transconjugants.

Abbreviations

S:Shigella; ESBLs:extended spectrum β -lactamases; QacE Δ 1:Quaternary ammonium compound E delta 1 gene; MR:multi-resistant; MDR: multidrug-resistant; QACs:quaternary ammonium compounds; BKC:benzalkonium chloride; MARI:multidrug antibiotic resistance index; AM:ampicillin; AMC:amoxicillin-clavulanate; CEC:cefaclor ; CTX:cefotaxime; CAZ:ceftazidime; FEP:cefepime; CIP:ciprofloxacin; C:chloramphenicol; S:streptomycin; AZM:azithromycin; SXT:trimethoprim-sulfamethoxazole; IPM:Imipenem (IPM), PCR:polymerase chain reaction; PP:Plasmid profiling; S-S:Salmonella-Shigella agar; XLD:xylose-lysine-deoxycholate agar; TSI:triple sugar iron agar, LIA:lysine iron agar; MR:methyl red, VP:Voges-Proskauer broth, UA:urea agar, SCA:Simmon’s citrate agar, TSB:tryptic soy broth.

Tables

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		
<i>bla</i> _{TEM}	ATCAGCAATAAACCCAGC	516	94°C	94°C	54°C	72°C	72°C	Colom <i>et al.</i> [43]
	CCCCGAAGAACGTTTTTC		5 min.	30 sec.	45 sec.	45 sec.	10 min.	
<i>bla</i> _{OXA-1}	ATATCTCTACTGTTGCATCTCC	619	94°C	94°C	54°C	72°C	72°C	
	AAACCCTTCAAACCATCC		5 min.	30 sec.	45 sec.	45 sec.	10 min.	
<i>bla</i> _{SHV}	AGGATTGACTGCCTTTTTG	392	94°C	94°C	54°C	72°C	72°C	
	ATTTGCTGATTTGCTCG		5 min.	30 sec.	45 sec.	45 sec.	10 min.	
<i>bla</i> _{CTX-M}	ATG TGC AGY ACC AGT AAR	593	94°C	94°C	60°C	72°C	72°C	Archambault <i>et al.</i> [44]
	GTK ATG GC		5 min.	30 sec.	45 sec.	45 sec.	10 min.	
	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG							
<i>tetA(A)</i>	GGTTCACCTCGAACGACGTCA	576	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 45 sec.	72°C 10 min.	Randall et al. [45]
<i>qacEA1</i>	TAA GCC CTA CAC AAA TTG GGA GAT AT	362	94°C 5 min.	94°C 30 sec.	58°C 40 sec.	72°C 40 sec.	72°C 10 min.	Chuanchuen <i>et al.</i> [16]
	GCC TCC GCA GCG ACT TCC ACG							

Table 2. The occurrence of *Shigella* species in dairy products (n=300).

Isolated bacteria	No of positive samples (%)			Total (n=300)
	Raw cow's milk (n=100)	Kareish cheese (n=100)	Yogurt (n=100)	
<i>Shigella dysenteriae</i>	5(5%)	7(7%)	0	12(57.1%)
<i>Shigella flexneri</i>	2(2%)	4(4%)	0	6(28.6%)
<i>Shigella sonnei</i>	1(1%)	2(2%)	0	3(14.3%)
Total	8 (8%)	13(13%)	0	21/300(7%)

Table 3. Antibiotic and disinfectant susceptibility profiles of *Shigella* species (n=21).

Antimicrobial class	Antimicrobial	<i>Shigella dysenteriae</i> (n=12)			<i>Shigella flexneri</i> (n=6)			<i>Shigella sonnei</i> (n=3)			Total (n=21) (%)		
		R	I	S	R	I	S	R	I	S	R	I	S
Tetracyclines	TE	12	0	0	6	0	0	3	0	0	21(100%)	0	0
B-lactam	AM	11	1	0	5	1	0	3	0	0	19(90.5%)	2(9.5%)	0
	AMC	12	0	0	4	1	1	3	0	0	19(90.5%)	1(4.8%)	1(4.8%)
Cephalosporines	CEC	8	2	2	4	1	1	2	1	0	14(66.7%)	4(19%)	3(14.3%)
	CTX	0	2	10	3	0	3	3	0	0	6(28.6%)	2(9.5%)	13(61.9%)
	CAZ	0	1	11	3	0	3	3	0	0	6(28.6%)	1(4.8%)	14(66.7%)
	FEP	0	2	10	0	0	6	0	0	3	0	2(9.5%)	19(90.5%)
Fluoroquinolones	CIP	1	2	9	1	0	5	0	0	3	2(9.5%)	2(9.5%)	17(80.9%)
Phenicol	C	1	1	10	1	0	5	0	0	3	2(9.5%)	1(4.8%)	18(85.7%)
Aminoglycosides	S	1	0	11	0	1	5	0	0	3	1(4.8%)	1(4.8%)	19(90.5%)
Macrolides	AZM	0	0	12	0	0	6	0	0	3	0	0	21(100%)
Sulfonamides	SXT	0	0	12	0	0	6	0	0	3	0	0	21(100%)
Carbapenems	IPM	0	0	12	0	0	6	0	0	3	0	0	21(100%)
QAC	BKC	9	-	3	6	-	0	3	-	0	18(85.7%)	-	3(14.3%)
Phenolic compound	V	12	-	0	6	-	0	3	-	0	21 (100%)	-	0

Resistance (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), trimethoprim sulfamethazole (SXT), imipenem (IPM), quaternary ammonium compound (QAC), benzalkonium chloride (BKC), vanillic (V),

Table 4. The antimicrobial resistance phenotypes of *isolated Shigella* strains (n=21).

Pattern	resistance phenotypes	No. of isolates	No. of resistant antibiotics	Ratio (%)	MARI
A	TE,AM,AMC,CEC,CIP,C	2	6	9.5	0.5
B	TE,AM,AMC,CEC,CTX,CAZ	3	6	14.3	0.5
C	TE,AM,AMC,CEC,S	1	5	4.8	0.4
D	TE,AM,CEC,CTX,CAZ	2	5	9.5	0.4
E	TE,AM,AMC,CTX,CAZ	1	5	4.8	0.4
F	TE,AM,AMC,CEC	6	4	28.6	0.3
G	TE,AM,AMC	4	3	19	0.2
H	TE,AMC	2	2	9.5	0.2

Multiple antibiotic resistance indexes (MARI) was calculated using the formula: a/b where 'a' represents the number of antibiotics to which a particular isolate was resistant and 'b' the total number of antibiotics tested.

Table 5. Characteristic features of *Shigella* species under study (n=21).

Strain ID	Species	Resistance Phenotype	Resistance genotype						Plasmid patterns	Plasmid (Kb)	Source
			ESBL				Tet(A)	Qac			
			blaTEM	blaCTX-M	blaOXA-1	blaSHV					
SD1	S. dysenteriae	TE,AM,AMC,CEC,S, BKC,P	+	-	-	-	+	+	P1	8.89, 4.07, 2.23, 1.26	Raw milk
SD2	S. dysenteriae	TE,AM,AMC,CEC, BKC,P	+	-	-	-	+	+	P1	8.89, 4.07, 2.23, 1.26	Raw milk
SD3	S. dysenteriae	TE,AMC,P	+	-	-	-	+	-	P1	8.89, 4.07, 2.23, 1.26	Raw milk
SD4	S. dysenteriae	TE,AM,AMC,CEC, BKC,P	+	-	-	-	+	+	P3	30.80, 7.12	Raw milk
SD5	S. dysenteriae	TE,AM,AMC,CEC, BKC,P	+	-	-	-	+	+	P3	30.80, 7.12	Raw milk
SD6	S. dysenteriae	TE,AM,AMC,CEC, BKC,P	+	-	-	-	+	+	P2	27.51, 4.66, 2.36	Kareish cheese
SD7	S. dysenteriae	TE,AM,AMC,CEC,CIP,C, BKC,P	+	-	-	-	+	+	P3	30.80, 7.12	Kareish cheese
SD8	S. dysenteriae	TE,AM,AMC,CEC, BKC,P	+	-	-	-	+	+	P4	33.617,2.677	Kareish cheese
SD9	S. dysenteriae	TE,AM,AMC, CEC, BKC,P	+	-	-	-	+	+	P2	27.51, 4.66, 2.36	Kareish cheese
SD10	S. dysenteriae	TE,AM,AMC,P	+	-	-	-	+	-	P2	27.51, 4.66, 2.36	Kareish cheese
SD11	S. dysenteriae	TE,AM,AMC,P	+	-	-	-	+	-	P4	33.617,2.677	Kareish cheese
SD12	S. dysenteriae	TE,AM,AMC, BKC,P	+	-	-	-	+	+	P4	33.617,2.677	Kareish cheese
SF1	S. flexneri	TE,AM,AMC,CEC,CIP,C, BKC,P	+	-	-	-	+	+	P5	18.59	Raw milk
SF2	S. flexneri	TE,AM,AMC,CEC,CTX, CAZ,BKC,P	+	+	-	-	+	+	P6	23.28, 4.40, 2.23	Raw milk
SF3	S. flexneri	TE,AM,CEC,CTX, CAZ,BKC,P	+	+	-	-	+	+	P5	18.59	Kareish cheese
SF4	S. flexneri	TE,AM,CEC,CTX, CAZ,BKC,P	+	+	-	-	+	+	P5	18.59	Kareish cheese

SF5	<i>S. flexneri</i>	TE,AM,AMC, BKC,P	+	-	-	-	+	+	P6	23.28, 4.40, 2.23	Kareish cheese
SF6	<i>S. flexneri</i>	TE,AMC, BKC,P	+	-	-	-	+	+	P6	23.28, 4.40, 2.23	Kareish cheese
SS1	<i>S. sonnei</i>	TE,AM,AMC,CEC,CTX, CAZ,BKC,P	+	+	-	-	+	+	P7	30.80, 1.41	Raw milk
SS2	<i>S. sonnei</i>	TE,AM,AMC,CEC,CTX, CAZ,BKC,P	+	+	-	-	+	+	P7	30.80, 1.41	Kareish cheese
SS3	<i>S. sonnei</i>	TE,AM,AMC,CTX, CAZ,BKC,P	+	+	-	-	+	+	P7	30.80, 1.41	Kareish cheese

P; plasmid

Supplemental Figure Legends:

1. Figure S1. Representative gel showing an amplification of blaTEM gene (516 bp) in *Shigella* isolates.
2. Figure S2. Representative gel showing an amplification of blaCTX-M gene (593 bp) in *Shigella* isolates.
3. Figure S3. Representative gel showing an amplification of tetA(A) gene (576 bp) in *Shigella* isolates.
5. Figure S4. Representative gel showing an amplification of qacEΔ1 gene (362 bp) in *Shigella* isolates.

Supplementary Files

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