Plasmid profile of extended-spectrum β-lactamase and quaternary ammonium compound E delta 1 gene held multi-resistant *Shigella* species isolated from raw cow milk and milk products in Egypt

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**Research article**

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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 (qacEΔ1) 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 (blaTEM, blaCTX-M, blaOXA-1 and blaSHV), tetA(A) and qacEΔ. 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-clavulananate (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 (blaTEM in all isolates and blaCTX-M in 28.6% of isolates), tetA(A) in all isolates and 85.7% isolates positive for qacEΔ1, while all isolates were negative for blaOXA-1 and blaSHV. All Shigella extended spectrum β-lactamase (ESBL) producers (6, 100%) were positive for blaTEM, blaCTX-M, and qacEΔ1 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 (blaTEM and blaCTX-M) and qacEΔ1 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

Infections caused by Shigella species are the main causes of bacillary dysentery, which is associated with high morbidity and mortality, especially in developing countries, such as Egypt [1]. Additionally, Shigella spp. infect and cause corresponding clinical symptoms in cows, chickens, pigs, monkeys and other animals which ignored in the previous studies [2]. Members of the Shigella genus are classified into four species: S. dysenteriae, S. flexneri, S. boydii, and S. sonnei. Endemic shigellosis in developing countries is caused mainly by S. flexneri, but S. sonnei is commonly detected in industrialized countries [3]. Dairy products especially raw milk and unpasteurized cheese remain important vehicles for the transmission of Shigella to humans [4,5]. Analyzing the epidemiological characteristics and resistance patterns of Shigella is essential for animal and human health [6].

There are many ESBL variants known in a variety of pathogens (blaCTX-M,blaTEM,SHV and blaOXA-1) that have been proved to be the most successful in terms of antibiotic resistance and for epidemiological niches.

Antibiotic therapy for Shigella infection can decrease the extent and severity of the disease [7]. As a consequence of the misuse of antibiotic administration, multi-resistant (MR) Shigella species defined as resistance to multiple antimicrobial drugs are predominant worldwide, particularly those with extended spectrum β-lactamases (ESBLs), which is one of the strongest extended resistance mechanisms [8,9]. Many ESBL variants are known to be present in a variety of pathogens, including blaCTX-M, blaTEM, SHV and blaOXA-1, which have been proven to be the
most successful in terms of antibiotic resistance and epidemiological niches [9]. Among blaTEM ESBL types, blaTEM-1 is the most commonly plasmid-mediated β-lactamase of ampicillin (AM) resistant gram-negative bacteria [10]. 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 [11,12]. The evolution of multidrug-resistant (MDR) strains, termed as resistance to at least one antibiotic belonging to at least three antibiotic classes, is common due to the existence of mobile genetic elements such as; plasmids, integrons, and transposons that assist Shigella spp. in the acquisition and transfer of exogenous genes [13]. Antibiotic resistance plasmids often include genes conveying resistance to numerous different antibiotics [14].

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 [15]. 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 [16]. One of the major mechanisms underlying such resistance is the acquisition of the resistance genes qacE and qacEΔ1, which confer resistance to QACs [17]. qacEΔ1, a mutant version of qacE, is widely distributed throughout gram-negative bacteria, mainly in Enterobacteriaceae [18].

Multiple global studies have reported the molecular basis of antibiotic resistance in clinical Shigella isolates of human origin [19,20] 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 spp. from raw cow milk and milk products in Egypt. This work was also proposed to examine the prevalence of MR Shigella particularly ESBL producing strains, screen the isolates for the presence of β-lactamase, tetA(A) and quaternary ammonium compound E delta 1 (qacEΔ1) genes, and examine their plasmid profiles.

Results

Occurrence of Shigella species in raw cow milk and milk products

A total of twenty-one (7%) organisms were isolated and identified as Shigella spp. S. dysentriae 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 occurrence of the isolates in the samples is presented in Table 1. 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 21 isolates of Shigella is presented in Table 2. 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),
chloramphenicol (C), ciprofloxacin (CIP), ceftazidime (CAZ) and cefotaxime (CTX). Only one isolate from *S. dysenteriae* and *S. flexneri* exhibited resistance to CIP and C (9.5%). Approximately 15 (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 five (23.8%) isolates, followed by 0.4 in 4 (19%) isolates. The *Shigella* strains showed eight multi-resistance phenotypes (Table 3). The 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%, 3 *S. exneri* and 3 *S. sonnei*) were ESBL producers.

With regard to disinfectant resistance (Table 2), phenolic compound (vanillic acid) showed no effect 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 (19mm) against *Escherichia coli* ATCC 25922 which was used as quality control strain. Approximately 18 (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 6 (28.6%) isolates were positive for *bla*CTX-M, while all the *Shigella* isolates were negative for *bla*OXA-1 and *bla*SHV (Table 4) (Figures S1, S2, S3, S4). Out of the 21 *Shigella* isolates, 6 (28.6%, 3 *S. flexneri* and 3 *S. sonnei*) were considered ESBL producers based on CTX and CAZ resistance phenotype, alone and in combination with clavulanate, by the double-disc synergy test. The genotype analysis demonstrated the existence of ESBL-encoding genes that were responsible for ESBL production in *Shigella* isolates. All *Shigella* ESBL producers (6, 100%) were positive for *bla*TEM and *bla*CTX-M genes, while none of the isolates harboured the *bla*OXA-1 and *bla*SHV genes. The TE resistance gene, *tetA(A)*, was identified in all *Shigella* isolates. Furthermore, 18 (85.7%) of the *Shigella* isolates possessed a QAC resistance gene (*qacEΔ1*) (Figure S5). The association of *qacEΔ1* with antibiotic resistance is presented in Table 4, which shows that all MR strains harbour the *qacEΔ1* gene. In addition, all *qacEΔ1* 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 4) (Figure S6). 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* harboured two plasmids with sizes of 1.41 and 30.80 kb plasmids. All ESBL-producing strains harboured plasmids with pattern P7 (1.41 and 30.80 kb) as the predominant pattern. According to spatial information, there were spatial relationships observed among *S. dysenteriae* strains (P1, P3) isolated from raw milk samples collected from Gamsa and Belqas districts, as showed in Table 4.
The co-transfer of β-lactamase genes (blaTEM and blaCTX-M) and qacEΔ1 genes was observed by conjugation. After the conjugative test using the plating mating method, the co-transfer of β-lactamase genes (blaTEM and blaCTX-M) and qacEΔ1 genes was observed by conjugation from all ESBL producing strains (n=6) as donor strains to the azide-resistant *E. coli* J53 as the recipient strain (Figures. S1, S2, S5). All obtained transconjugants successfully acquired these resistance (blaTEM, blaCTX-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. The present study showed a high prevalence of *Shigella* spp. (7%) with the predominance of *S. dysenteriae*, in comparison to the results of Ahmed and Shimamoto [21], 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 predominant species. Tambekar and Bhutda [22] 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, handling, and storage of milk and milk products. Therefore, basic hygienic measures must be enforced in animal farms to reduce the risk of spread of *Shigella* to other animals and human.

The risk of *Shigella* might be higher in the raw cows milk and cheese, as compared to yoghurt products because yoghurt has very effectively inhibitory effect on the growth of the most common enteric pathogens such as *Shigella* [23,24]. In addition, the production process of yoghurt is entirely industrial type, while in the case of karish cheese, this process is completely hand made. Therefore, results revealed whatever the type of the sample, the presence of *Shigella*, but the risk appears to decrease as we move from products obtained in informal to those are industrially manufactured [25]. Overall, it is important to observe all hygienic measures while dealing with milk and milk products.

The antibiotic resistance of *Shigella* spp. isolated from raw cow milk and milk product samples in this study was compared with previous reports from Egypt to observe the trend in antibiotic resistance taking into account the sample collection method for each study. 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 [26,27], though the presence of policies in Egypt regarding the use of antibiotics in both livestock and humans according to the World Health Organization. This resistance might be due to the frequent and improper use of such antibiotics either in animal therapy or as a growth promoter in the veterinary context in Egypt. Additionally, the current study showed reduced susceptibility to CTX, CAZ (third-generation cephalosporins) and CIP, which are considered preferable drugs for shigellosis treatment [12]. Thus, the appearance of such resistance would pose a great challenge for the efficient treatment of shigellosis.

In this study, approximately 71.4% of *Shigella* isolates were resistant to at least three of the antimicrobial classes with a MAR index of 0.2-0.5. In addition, many isolates of *Shigella* spp. were shown to have multi-resistance phenotypes against TE, AM, AMC, and CEC. Similarly, a high prevalence of MR *Shigella* isolates in dairy products (90.9%) was reported by Ahmed and Shimamoto [21] in Egypt. Therefore, some measures must be considered to confirm that the currently available antibiotics remain effective. These measures may include increasing the
awareness among the public, healthcare professionals and the food-agriculture sector regarding 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 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 [28]. BKC is a cationic, surface-active QAC commonly used as a farm disinfectant for cleaning and sanitizing livestock buildings, equipment, milk utensils, and vehicles. The present study demonstrated that all the tested strains were resistant to phenolic compounds, while 85.7% of the isolates were resistant to BKC. Similarly, Bouzada et al. [29] found that gram-negative rods of *Enterobacteriaceae* exhibited low susceptibility to BKC. Moreover, this work demonstrated that most of the *Shigella* isolates (85.7%) harboured the *qacEΔ1* gene.

Various β-lactamases, which hydrolyse the β-lactam ring and thereby inactivate β-lactam antibiotics, have been described, but TEM-, OXA-, SHV- and CTX-M-type β-lactamases are dominant in gram-negative bacteria [30]. 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 *blaTEM* gene, a narrow-spectrum β-lactamase gene that confers resistance to penicillins and first-generation cephalosporins, was identified in all isolates. Additionally, the ESBL-encoding gene *blaCTX-M* was identified in 28.6% of the isolates. The high incidence of β-lactamase-encoding genes (*blaTEM-1, blaCTX-M*, in 2 isolates; *blaOXA*, in 4 isolates) had been detected previously in *Shigella* strains isolated from dairy products in Egypt [21]. The *blaTEM* gene was the dominant β-lactamase gene in *Shigella* spp. in this work, while *blaCTX-M* was the most common type of cefotaximases identified among *Shigella* isolates in a previous study [11]. Alarmingly, in this research, the prevalence of ESBL-producing *Shigella* isolates, accounting for 28.6% of all *Shigella* isolates, was higher than the detection rates observed in other countries, such as England [31]. This discrepancy between these findings and previous studies might be attributed to the misuse of antibiotics during the treatment of bacterial infections. In addition, the high TE resistance in all *Shigella* isolates might be explained by the potential distribution of the *tetA(A)* resistance gene [32].

For epidemiological investigations of various enteric pathogens, PP could be an attractive tool. *Shigella* spp. usually harbour various plasmids, with 2 to 10 plasmids being harboured by one strain. These plasmids are required for antibiotic resistance and for bacterial invasion of intestinal epithelial cells [33]. 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 *Shigella* spp. in Egypt harbour varying numbers of plasmids ranging in size from 1.0 to 120 MDa [34]. All MR strains, particularly ESBL producing strains, carried plasmids with pattern P7 as the predominant pattern in this study. The ESBL-encoding gene (*blaCTX-M*) is present on plasmids with greater frequency than genes encoding other class A β-lactamases [35]. The results of the conjugation experiment aided the determination of plasmid locations of the *blaTEM, blaCTX-M*, and *qacEΔ1* genes because transconjugants of the MR *Shigella* isolates were grown on MacConkey agar. Over the last half-century, the extraordinary ability of different isolates to acquire plasmid-encoded resistance to disinfectants and antibiotics, such as ESBLs, which could quickly be transmitted to several other strains, has been demonstrated [36,37].

Antibiotics and disinfectants have been commonly used in dairy farms in Egypt. The most commonly used antimicrobials are the β-lactams, tetracyclines, aminoglycosides, lincosamides, macrolides and sulfonamides [38]. Antibiotics may be used indiscriminately for the treatment of bacterial diseases or they may be used to enhance
animal growth and feed efficiency. The ongoing hazard of antibiotic resistance is one of the biggest challenges to public health that is faced not only by the African people, but also by the human population worldwide [39].

However, the susceptibility of *Shigella* to disinfectants and its contribution to the multidrug resistance phenotype and genotype by plasmid co-selection had never been reported. Plasmid-mediated multidrug resistance should be considered when studying infectious diseases. Therefore, this work was planned to explore the link between the *qacEΔ1* gene, plasmids, and antibiotic resistance. The results of this study showed that all *qacEΔ1* gene-positive strains were MR strains and harboured plasmids. Recently, it was demonstrated that the *qacC* gene confers resistance to a number of β-lactam antibiotics [40]. The ability of *qac* genes to directly acquire resistance to antibiotics was found. This finding indicated a close relationship between resistance to antibiotics and antiseptics [41]. Plasmids frequently transfer *qac* genes with a number of other antibiotic resistance genes [42,43]. The plasmid analysis and conjugation experiments showed that the isolates harboured various detectable plasmids and that the antibiotic and disinfectant resistance genes could be co-transferred. This finding 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.

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 MR *Shigella* spp. in Egypt, it focused on raw cow milk and milk product samples which collected randomly from only one province of Egypt and did not elucidate such relationships in other provinces. Therefore, additional studies are warranted to explore such relationships in other provinces of Egypt.

**Conclusion**

This study demonstrated the prevalence of MR *Shigella* species that were either MR 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 MR strains. Additionally, strict hygienic measures must be applied throughout dairy industry chain to prevent infection through the consumption of dairy products.

**Methods**

**Sample collection and microbial analysis**

A total of 300 dairy product samples (100 raw cow milk from different dairy farms, 100 Kareish cheese, and 100 yoghurt samples from shops, and supermarkets) were collected randomly from three districts “Gamasa, Belqas and Dekernes” in Dakahlia Governorate, Egypt, throughout 2018. Raw cow milk samples were collected from the udder of cows (a composite sample from four quarters for each cow). After collection, the samples were stored at 4°C until examination (within 3-4 h). Then, homogenization of 25 ml or g of each sample was performed in 225 ml of 0.1% buffered peptone water (Oxoid, England) by shaking for 5 min in 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 into Selenite F broth and then subcultured onto Salmonella-Shigella (S-S) agar, MacConkey agar and xylose-lysine-deoxycholate (XLD) agar (Oxoid, UK), followed by incubation at 37 °C for 24 h. 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). All bacterial isolates were stored at ~80 °C in tryptic soy broth (TSB) containing 25% glycerol for further analysis.

**Antibiotic resistance evaluation**

Antibiotic susceptibility testing was performed using the disc diffusion method according to the standards and interpretative rules described by the guidelines of the Clinical and Laboratory Standards Institute [44] on Mueller-Hinton agar (Difco). *Shigella* spp. were tested for susceptibility to commercially available antibiotic discs (Oxoid, England), including discs of TE, 30 µg; AM, 10 µg; AMC, 30 µg; CEC, 30 µg; CTX, 30 µg; CAZ, 30 µg; FEP, 30 µg; CIP, 5 µg; C, 30 µg; S, 10 µg; AZM, 15 µg; SXT, 25 µg and 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 categorized as sensitive, intermediate, or resistant. The isolates were screened for ESBL production using the double-disc synergy test following the recommendation and interpretations of **CLSI** guidelines [44]. *Escherichia coli* ATCC 25922 was used as a quality control strain. 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 [45].

**Disinfectant susceptibility testing**

To test the effectiveness of BKC (2%) [46] and a phenolic compound (vanillin, 5%) (Chemica Company) against *Shigella* isolates, a disc diffusion assay was carried out according to the **CLSI** guidelines [47]. Briefly, *Shigella* broth cultures of each strain incubated for 24-48 h were diluted in saline (NaCl 0.9%) to an approximately cell density of 1x10^6 CFU/ml based on McFarland turbidity standards, and the diluted cultures were poured on the surfaces of nutrient agar plates. The plates were allowed to dry. Sterile 6 mm filter paper discs (Whatman. No. 3) were soaked in tested disinfectant for 30 min. After completely drying the discs at 55 ºC, they were plated on nutrient agar plates. The experiment was conducted in triplicate to minimize errors, after that, all plates were incubated aerobically at 37 ºC for 24h, [48]. *Escherichia coli* ATCC 25922 was used as quality control strain, and saline was selected as a control agent [49]. The sensitivity pattern was scored as tolerance (-) and sensitive (+).

**Screening for antibiotic and disinfectant resistance genes**

For amplification of β-lactamase-encoding genes (*blaTEM, blaCTX-M, blaOXA-1*, and *blaSHV*), TE resistance genes and QAC resistance genes (*qacEΔ1*), uniplex PCR assays were performed with primers provided by Metabion (Germany) (Table 5). These resistance genes were mostly encoded in gram-negative bacteria. Chromosomal DNA from the *Shigella* isolates was extracted by the QIAamp DNA Mini Kit (Qiagen, Germany, GmbH) with the modifications recommended by the manufacturer. The PCR amplification reaction was carried out
in an Applied Biosystems 2720 thermal cycler using specific profiles (Table 5). The PCR products were subjected to electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x 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, Germany, GmbH) was used for plasmid DNA isolation from all clinical isolates (n=21). Gel electrophoresis of the PCR products on an 0.8% agarose gel (Applichem, Germany, GmbH) was performed. The fragment sizes were determined by a GeneRuler 1Kb Plus DNA Ladder (Fermentas, Thermo Scientific, Germany) without using any restriction enzymes. In a trial to prove the association of these plasmids with ESBL-based antibiotic resistance, conjugation experiments were carried out using the azide-resistant *E. coli* J53 as the recipient and all Shigella ESBL producers (n=6) as the donors, as described previously [53]. Transconjugants were detected by plating mating mixtures on MacConkey agar supplemented with 150 mg/L sodium azide and 2 mg/L cefotaxime. Plasmid DNA was extracted from *E.coli* J53 transconjugants and co-transfer of resistance determinants was determined through amplification of the relevant genes (blaTEM, blaCTX-M and qacEΔ1) in the transconjugants by PCR as previously described.

**Abbreviations**

S.:Shigella; ESBLs: extended spectrum β-lactamases; qacEΔ1: quaternary ammonium compound E delta 1 gene; MR: multi-resistant; MDR: multidrug-resistant; QACs: quaternary ammonium compounds; BKC: benzalkonium chloride; MAR index: multiple-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: sulfamethoxazole/trimethoprim; IPM: imipenem, PCR: polymerase chain reaction; PP: plasmid profiling; S-S: *Salmonella-Shigella*; XLD:xylose-lysine-deoxycholate agar; TSI: triple sugar iron; LIA: lysine iron agar; VP: Voges-Proskauer; UA:urea agar; SCA: Simmon's citrate agar; TSB: tryptic soy broth.

**Declarations**

**Availability of data and materials**

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

This article does not contain any animal studies performed by any of the authors.

**Competing interests**

The authors have declared that they have no competing interests.

**Author contributions**
RME, RAE, MME, and MMA conceived and designed the experiments. RME, RAE, MME, and MMA performed the experiments. RME and MME analysed the data. RME, RAE, MME, and MMA contributed reagents/materials/analysis tools. RME, RAE, MME, and MMA wrote the paper. All authors reviewed the manuscript.

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Consent for publication

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Tables

Table 1. Occurrence of Shigella species in dairy products (n=300).

| Isolated bacteria | No of positive samples (%) | Total (n=300) |
|-------------------|---------------------------|--------------|
|                   | At the dairy farms | At the market |          |
| Raw cow milk (n=100) |            |             |          |
| Shigella dysenteriae | 5(5%)       | 7(7%)        | 12(57.1%) |
| Shigella flexneri   | 2(2%)       | 4(4%)        | 6(28.6%)  |
| Shigella sonnei     | 1(1%)       | 2(2%)        | 3(14.3%)  |
| Total              | 8 (8%)      | 13(13%)      | 21/300(7%)|
Table 2. Antibiotic and disinfectant susceptibility profiles of *Shigella* species (n=21).

| Antimicrobial class | Antimicrobial | *Shigella dysenteriae* (n=12) | *Shigella flexneri* (n=6) | *Shigella sonnei* (n=3) | Total (n=21) (%) |
|---------------------|--------------|-------------------------------|--------------------------|-----------------------|-----------------|
|                     |              | R    | I    | S    | R    | I    | S    | R    | I    | S    | R    | I    | S    |
| acyclines           | TE           | 12   | 0    | 0    | 6    | 0    | 0    | 3    | 0    | 0    | 21(100%) | 0    | 0    |
| actams              | 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%) |
| halosporines        | 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%) |
| norquinolones       | CIP          | 1    | 2    | 9    | 1    | 0    | 5    | 0    | 0    | 3    | 0    | 2(9.5%) | 2(9.5%) | 17(80.9%) |
| nolc                | C            | 1    | 1    | 10   | 1    | 0    | 5    | 0    | 0    | 3    | 2(9.5%) | 1(4.8%) | 18(85.7%) |
| noglycosides        | S            | 1    | 0    | 11   | 0    | 1    | 5    | 0    | 0    | 3    | 1(4.8%) | 1(4.8%) | 19(90.5%) |
| crrolides           | AZM          | 0    | 0    | 12   | 0    | 0    | 6    | 0    | 0    | 3    | 0    | 0    | 21(100%) |
| cronomides          | SXT          | 0    | 0    | 12   | 0    | 0    | 6    | 0    | 0    | 3    | 0    | 0    | 21(100%) |
| crpenem            | IPM          | 0    | 0    | 12   | 0    | 0    | 6    | 0    | 0    | 3    | 0    | 0    | 21(100%) |
| BKC                | 9            | -    | 3    | 6    | -    | 0    | 3    | -    | 0    | 18   | 1(4.8%) | 3(14.3%) |
| V                 | 12           | -    | 0    | 6    | -    | 0    | 3    | -    | 0    | 21(100%) | -    | 0    |

Resistant (R), intermediate (I), sensitive (S), number (n), tetracycline (TE), ampicillin (AM), amoxicillin-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).

Table 3. Antimicrobial resistance phenotypes of isolated *Shigella* strains (n=21).
| Pattern | Resistance phenotypes | No. of isolates | No. of resistant antibiotics | Ratio (%) | MAR index |
|---------|-----------------------|----------------|----------------------------|-----------|-----------|
| 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 (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.

Table 4. Characteristic features of *Shigella* species under study (n=21).
| Species | Resistance phenotype | Resistance genotype | ESBL | Tet(A) | Qac | Plasmids (kb) | Type & Source |
|---------|----------------------|---------------------|------|--------|-----|--------------|---------------|
| S. *nteriae* | TE, AM, AMC, CEC, S, BKC, P | ESBL: +, CTX-M: -, OXA-1: - | EM: +, SHV: + | P1 | 8.89, 4.07, 2.23, 1.26 | Raw cow milk from Gamasa farm |
| S. *nteriae* | TE, AM, AMC, CEC, BKC, P | ESBL: +, CTX-M: -, OXA-1: - | EM: +, SHV: + | P1 | 8.89, 4.07, 2.23, 1.26 | Raw cow milk from Gamasa farm |
| S. *nteriae* | TE, AMC, P | ESBL: +, CTX-M: -, OXA-1: - | EM: +, SHV: + | P1 | 8.89, 4.07, 2.23, 1.26 | Raw cow milk from Belqas farm |
| S. *nteriae* | TE, AM, AMC, CEC, BKC, P | ESBL: +, CTX-M: -, OXA-1: - | EM: +, SHV: + | P3 | 30.80, 7.12 | Raw cow milk from Belqas farm |
| S. *nteriae* | TE, AM, AMC, CEC, BKC, P | ESBL: +, CTX-M: -, OXA-1: - | EM: +, SHV: + | P3 | 30.80, 7.12 | Raw cow milk from Belqas farm |
| S. *nteriae* | TE, AM, AMC, CEC, BKC, P | ESBL: +, CTX-M: -, OXA-1: - | EM: +, SHV: + | P2 | 27.51, 4.66, 2.36 | Kareish cheese from Dekernes market |
| S. *nteriae* | TE, AM, AMC, CEC, CIP, BKC, P | ESBL: +, CTX-M: -, OXA-1: - | EM: +, SHV: + | P3 | 30.80, 7.12 | Kareish cheese from |
| S. enteriae | TE, AM, AMC, CEC, BKC, P | + | - | - | - | + | + | P4 | 33.617,2.677 | Gamasa market |
|------------|--------------------------|---|---|---|---|---|---|---|---|-------|
| S. enteriae | TE, AM, AMC, CEC, BKC, P | + | - | - | - | + | + | P2 | 27.51, 4.66, 2.36 | Kareish cheese from Dekernes market |
| S. enteriae | TE, AM, AMC, P | + | - | - | - | + | - | P2 | 27.51, 4.66, 2.36 | Kareish cheese from Dekernes market |
| S. enteriae | TE, AM, AMC, P | + | - | - | - | + | - | P4 | 33.617,2.677 | Kareish cheese from Dekernes market |
| S. enteriae | TE, AM, AMC, BKC, P | + | - | - | - | + | + | P4 | 33.617,2.677 | Kareish cheese from Dekernes market |
| S. exneri | TE, AM, AMC, CEC, CIP, C, BKC, P | + | - | - | - | + | + | P5 | 18.59 | Raw milk from Belqas farm |
| S. exneri | TE, AM, AMC, CEC, CTX, | + | + | - | - | + | + | P6 | 23.28, 4.40, | Raw milk |
| CAZ, BKC, P | | | | | | | 2.23 | from Gamasa farm |
|---|---|---|---|---|---|---|---|---|
| **exneri** | TE, AM, CEC, CTX, CAZ, BKC, P | + | + | - | - | + | + | P5 | 18.59 | Kareish cheese from Gamasa market |
| **exneri** | TE, AM, AMC, BKC, P | + | - | - | - | + | + | P6 | 23.28, 4.40, 2.23 | Kareish cheese from Gamasa market |
| **exneri** | TE, AMC, BKC, P | + | - | - | - | + | + | P6 | 23.28, 4.40, 2.23 | Kareish cheese from Gamasa market |
| **onrei** | TE, AM, AMC, CEC, CTX, CAZ, BKC, P | + | + | - | - | + | + | P7 | 30.80, 1.41 | Raw cow milk from Belqas farm |
| **onrei** | TE, AM, AMC, CEC, CTX, CAZ, BKC, P | + | + | - | - | + | + | P7 | 30.80, 1.41 | Kareish cheese from Belqas market |
Table 5. PCR conditions employed for the detection of resistance associated genes of *Shigella*

| Gene   | TE, AM, AMC, CTX, CAZ, BKC, P | + | + | - | + | + | P7 | 30.80, 1.41 | Kareish cheese from Belqas market |
|--------|-------------------------------|---|---|---|---|---|----|------------|---------------------------------|

P: plasmid
| Target gene | Primers sequences | Amplified segment (bp) | Primary denaturation | Amplification (35 cycles) | Final extension | Reference |
|-------------|-------------------|------------------------|----------------------|---------------------------|----------------|-----------|
| **blaTEM** | ATCAGCAATAAACAGC | 516 | 94 °C | 94 °C | 54 °C | 72 °C | 72 °C | Colom et al. [50] |
| | CCCCAGAAGACGTTC | | | | | | | |
| **blaOXA-1** | ATATCTCTACTGTTCATCTCC | 619 | 94 °C | 94 °C | 54 °C | 72 °C | 72 °C | |
| | AAACCTCTAAACCATCC | | | | | | | |
| **blaSHV** | AGGATTGACTGCTTTTTG | 392 | 94 °C | 94 °C | 54 °C | 72 °C | 72 °C | |
| | ATTTGCTGATTTCGCTCG | | | | | | | |
| **blaCTX-M** | ATGTCAGYACCGT | 593 | 94 °C | 94 °C | 60 °C | 72 °C | 72 °C | Archambault et al. [51] |
| | AARGTKATGCG | | | | | | | |
| | TGGGTRAARTGTS | | | | | | | |
| | ACCAGAAYCAGCGG | | | | | | | |
| **tet(A)** | GGTCACTCGAACGCTCA | 576 | 94 °C | 94 °C | 50 °C | 72 °C | 72 °C | Randall et al. [52] |
| | | | | | | | | |
| **qacEΔ1** | TAAGCCTACAC | 362 | 94 °C | 94 °C | 58 °C | 72 °C | 72 °C | Chuanchuen et al. [18] |
| | AAATTGGGAGATAT | | | | | | | |
| | GCCTCCGCAGCGACT | | | | | | | |

**Additional Files**

1. Figure S1. Representative gel showing amplification of the blaTEM gene (516 bp) from *Shigella* isolates.
2. Figure S2. Representative gel showing amplification of the blaCTX-M gene (593 bp) from *Shigella* isolates.
3. Figure S3. Representative gel showing amplification of the blaOXA gene (619 bp) from *Shigella* isolates.
4. Figure S4. Representative gel showing amplification of the tetA(A) gene (576 bp) from *Shigella* isolates.
5. Figure S5. Representative gel showing amplification of the qacEΔ1 gene (362 bp) from *Shigella* isolates.
6. Figure S6. Plasmid profiles of 7 representative *Shigella* isolates in 0.8% agarose gel.
Supplementary Files

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