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ORIGINAL ARTICLE

Frequency and antibiotic resistance pattern of *Salmonella* spp. isolated from traditional dairies and raw milks collected in Yazd province, Iran

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ABSTRACT

Background and Objectives: Salmonellosis is among the most common food-born infections, caused by *Salmonella* spp. bacteria. Present study has investigated the frequency and antibiotic resistance pattern of *Salmonella* spp. isolated from traditional dairy products and raw milk supplied in Yazd, Iran.

Materials and Methods: In a cross-sectional study, 350 samples of raw milk and traditional dairy products were randomly collected from July to September 2018. Following culturing the samples, isolates went through biochemical tests for phenotypic identification. Results were confirmed through PCR technique by targeting *invA* gene. Antimicrobial susceptibility test was conducted by means of disk diffusion method.

Results: The rate of contamination with *Salmonella* bacteria was 6.57% in all samples. The PCR assay of all isolates showed that 23 isolates (100%) carried the *invA* gene. No significant association between the frequency of *salmonella* spp. and types of dairy and their origin was reported (P>0.05). The highest antibiotic resistance rate among the isolates belonged to tetracycline (34.8%) and the highest sensitivity was seen to imipenem, cefepime, and cefotaxime (each 91.3%).

Conclusion: According to our results there has been a rise in multiple drug resistance and contamination rate in traditional dairy products in Yazd province.

Keywords: Dairy products; *invA*; Cow's milk; *Salmonella*; Antimicrobial resistance

INTRODUCTION

Milk and dairy products serve as a vital source of nutrition in human diet. They contain high levels of protein, calcium, trace elements, macronutrients and micronutrients as well as essential vitamins (1). Dairy products are known to be susceptible to microbial contamination providing a favourable growth medi-

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um for pathogenic bacteria (2). Bacteria may contaminate milk through an adulterant, such as contaminated water, or they can originate from the animal, the environment, milking equipment, or milk handlers. Also, chemical contaminants may enter milk through the food or veterinary treatments of the animal or through later accidental or deliberate contamination (3). *Listeria monocytogenes, Salmonella* spp., *Escherichia coli,* and *Staphylococcus aureus* are among the most common pathogens isolated from milk (4). Among these pathogens *Salmonella* spp., as a widespread pathogen, constitutes a threat to public health as well as global food safety, which can lead to food-borne diseases and considerable economic losses (5). It is the most significant member of a huge genus with global public health implications (6). *Salmonella* is a genus of Gram-negative, motile, facultative anaerobic rod-shaped bacteria from the family of Enterobacteriaceae (7). There are two species including *Salmonella enterica* and *Salmonella bongori* in the *Salmonella* genus (8). Currently, over 2,600 serovars have been identified in this genus (9), and many of these serovars can lead to both human and animal infection (10). Salmonellosis is the most frequently reported cause of food-borne diseases caused by various strains of the *Salmonella* (11). which can be transmitted by contaminated foods including beef, chicken, broilers, eggs, and pork. It can contaminate vegetables, fruits, milk and water through human and animal fecal contamination (12).

In most cases, salmonellosis causes self-limiting gastroenteritis. The symptoms of salmonellosis typically appear within 12 to 72 hours after infection, including diarrhea, abdominal pain, and vomiting which may last for 4-7 days (13, 14). Immunocompromised individuals, infants and the elderly are the most susceptible ones who may show severe infections (15).

Salmonella-related infections are mostly invasive infections or severe diarrhea which could be life-threatening and need antibiotic therapy (16). Unfortunately, due to the indiscriminate antibiotic use, *Salmonella* strains have developed multidrug-resistant (MDR), and they have spread far and wide (17). The problem with emergence of multidrug-resistant strains not only lowers the chance of treatment, but also results in antibiotic resistance (18). The rapid spread of resistant *Salmonella* strains in food products is a serious global health issue, (19) and some studies have predicted that, antimicrobial-resistant pathogens will cause at least 10 million death worldwide (20). Antimicrobial resistance has been classified as a global public health crisis, requiring immediate attention by World Health Organization (19). Therefore, this study was aimed at identification *Salmonella* spp. in raw milk and dairy products in Yazd province by means of conventional as well as molecular techniques, and also, determination of their susceptibility to a variety of antibiotics.

MATERIALS AND METHODS

Sampling. In the present cross-sectional study, 350 samples of raw milk and traditional dairy products were randomly collected from retail markets in five regions of Yazd City (north, south, east, west, and center) from July to October 2018. The collected samples included 125 raw milk, 75 samples of traditional cheese, 75 samples of traditional ice cream and 75 samples of traditional cream. The samples were collected aseptically and transferred to the laboratory in a cold box. Sampling was performed in accordance to Iran standard (21).

***Salmonella* isolation.** *Salmonella* was isolated from dairy products as per Institute of Standards and Industrial Research of Iran NO.4413 (22). Initially, the samples were homogenized in the lab blender BagMixer 400 W (MIXWEL, France). Subsequently, milk (25 ml) and dairy samples (25 g) were added into 225 ml buffered peptone water (Merck, Germany) and incubated at 37°C for 24 h. This was followed by transferring 1 ml of pre-enriched broth into tubes containing 10 ml Selenite F Cysteine Broth (Liofilchem, Italy), and they were incubated at 37°C for 24 h. Moreover, 0.1 ml of pre-enriched broth was transferred into tubes including 10 ml Rapoport Vasiliadis Broth (Quelab, Canada), and incubated overnight at 41.5°C. A loopful of the enriched broths was streaked onto plates of Xylose Lysine Deoxycholate agar (XLD; Merck, Germany) and Brilliant Green Agar (BGA; Merck, Germany). The plates were incubated at 37°C for 24 h while the suspected *Salmonella* colonies (red colonies with a black center on XLD and pink colonies growing on BGA) were streaked onto nutrient agar (Merck, Germany) slopes and incubated at 37°C for 18-24 h. Identification of the isolates was performed by Gram staining and biochemical media such as Simmons citrate agar,

urea agar, lysine-iron agar, Sulfide Indole Motility agar, Triple Sugar Iron (TSI) agar, Methyl Red-Voges Proskauer (MR-VP) broth (Merck, Germany). The confirmed isolates were kept in Trypticase Soy Broth including 20% glycerol at -20°C until molecular experiments.

Molecular confirmation the isolates. Bacterial DNA extraction was performed by means of boiling method (23). The target gene of *invA* was used for species verification. Amplification was performed using a specific primer pair of F: 5′-GCTGCGCGCGAACGGCGAAG-3′ and R: 5′-TCCCGGCAGAGTTCCCATT-3' to amplify 389 bp fragment by thermocycler (ABI, SimpliAmp, Singapore) (24). PCR solution contained 20 µl of 1 X master mix (Amplicon, Denmark), 0.5 µM of each primer, and 100 ng of the DNA templates. The temperature conditions included 94°C for 5 min for the first denaturation, followed by 25 cycles of 94°C for 20 sec, 59°C for 30 sec, 72°C for 20 sec, and the last extension was performed at 72°C for 5 min. After agarose gel electrophoresis, the gel was visualized using the Gel Documentation system (ATP, Compact, Iran) alongside with 50 bp DNA ladder. The *Salmonella enterica* serovar Enteritidis strain (ATCC-13076, Institute Pasteur of Iran) and sterile deionized water were used as positive and negative controls, respectively.

Sequence analyzing. For verification, two amplicons from the target gene of *invA* were purified and sequenced using BLAST and multiple alignments and submitted in GenBank, NCBI.

Antimicrobial resistance test. Antibiotic resistance testing of isolates to various antibiotics was performed using the disk diffusion method as per the Clinical and Laboratory Standards Institute (CLSI 2017) protocols.

Antibiotic disks (Padtan Teb, Iran) were used including Imipenem (10 µg), Cefepime (30 µg), Ciprofloxacin (5 µg), Trimethoprim/Sulfamethoxazole (1.25/23.75µg), Chloramphenicol (30 µg), Tetracycline (30 µg), Cefotaxime (30 µg), Ceftazidime (30 µg), Amoxicillin (25 µg), and Amoxicillin/Clavulanic acid (20/10 µg). A suspension was prepared from fresh (18-24 h) bacterial colonies in physiological saline having turbidity of (0.5 McFarland) and cultured on Mueller Hinton agar (Liofilchem, Italy). After placing the antibiotic disks on the medium and incubating them at 35°C for 18 hours, the bacterial inhibition zone was measured and reported as Sensitive, Intermediate, and Resistant according to the CLSI 2017 protocols and *Escherichia coli* ATCC 25922 standard strain was applied as a control.

Extended-spectrum beta-lactamase enzymes (ESBLs) Confirmatory test. The ESBL production was initially detected by confirmatory combination disk test according to CLSI 2017 guidelines using Ceftazidime (30 mg) and Cefotaxime (30 mg) disks separately and in combination with clavulanic acid (10 mg) (Padtan Teb, Iran). The increase of ≥5 mm in the inhibition zone diameter around the antibiotic disk along with clavulanic acid, compared with the antibiotic disk alone, was considered as ESBL production (25).

Statistical analysis. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS, version 23) software. Descriptive statistics were used to describe the percentage of contamination and antibiotic resistance. Also, chi-square test and Fisher's exact test were run to compare the percentage of bacterial contamination among dairy products and also the percentage of antibiotic resistance between bacterial isolate. The differences were considered significant at a P-value of $p<0.05$.

RESULTS

Microbiological and biochemical tests showed that out of the 350 samples, 23 samples (6.57%) were positive for *Salmonella* spp. (Table 1). All 23 isolated *Salmonella* spp. were confirmed by PCR method through identification of *invA* target gene (Fig.1). Contamination with *Salmonella* spp. was observed

Table 1. Prevalence of *Salmonella* spp. in milk and dairy products in Yazd province

Type of Sample	No. Samples	Positive samples *Salmonella* No. (%)
Raw milk	125	7 (5.6)
Ice cream	75	6 (8)
Cheese	75	6 (8)
Cream	75	4 (5.3)
Total	350	23 (6.57)

in 5.6% of raw milk, 5.3% of traditional cheese, 8% of traditional cream, and 8% of traditional ice cream samples. The percentages of samples contaminated by *Salmonella* in the northern, eastern, southern, western, and central areas of Yazd Province were 26.1%, 21.7%, 17.4%, 26.1%, and 8.7%, respectively. The results of the Chi-square test showed that the frequency of *salmonella* had no significant association with the type of dairy and area of investigation (p>0.05). Antibiotic resistance pattern of 23 *Salmonella* spp. isolates is represented in Table 2. The highest resistance was seen against tetracycline (34.8%) and the most sensitivity was against imipenem, cefepime, and cefotaxime (each 91.3%). Out of 23 *salmonella* spp. Isolates, 2 (8.69%) isolates were ESBL producer. One of the ESBL-producing *Salmonella* spp. isolates were from raw milk and the other from traditional cheese.

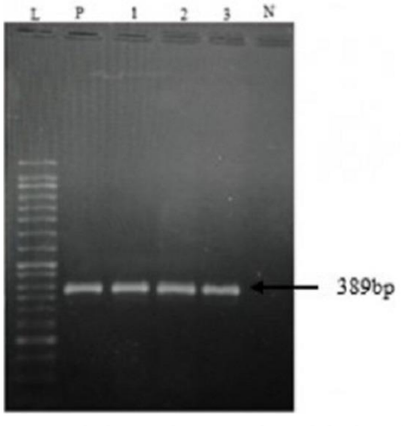

Fig. 1. Agarose gel electrophoresis of amplified *invA* gene. L: 50 bp DNA ladder, P: positive control (*S. enterica* serovar Enteritidis strain), N: negative control (ddH_2O), lanes 1 to 3: samples with *invA* gene (389 bp).

Sequencing. After sequencing and analyzing using BLAST and multiple alignments, the sequences were submitted in GenBank, NCBI with the accession numbers of MK754231 and MK754232. BLAST analysis showed the homology of 100 percent between the gene in our studied isolates with the one in GenBank, NCBI.

DISCUSSION

The overall *Salmonella* spp. frequency in this study was reported as 6.57% in raw milk and traditional dairy products. Various studies have investigated this issue. In a similar study conducted in Egypt, contamination rate of raw milk and bulk tank milk samples with *Salmonella* was reported as 6.66% (26). *Salmonella* prevalence in raw milk in various regions such as Dire Dawa Town, Pakistan, Henan province and Iran have been reported as 16%, 10.42%, 21.09% and 21%, respectively which is higher than the prevalence reported in our study (2, 4, 27, 28). The prevalence rate is influenced by a variety of factors including farm size, number of animals on the farm, milking hygiene, farm management and season, technical details concerning sampling methods, and transportation conditions (29).

In the present study, the prevalence of *Salmonella* in traditional cheese was 5.3%. In 2023, Bedassa and his colleagues reported *Salmonella* prevalence in tested cottage cheese samples collected in Ethi-

Table 2. Antibiotics resistance pattern of *Salmonella* spp. isolated from raw milk and traditional dairy products in Yazd province

Antibiotics	Sensitive No. (%)	Intermediate No. (%)	Resistant No. (%)
Ciprofloxacin (5 µg)	19 (82.6)	1 (4.3)	3 (19)
Chloramphenicol (30 µg)	20 (87)	0 (0)	3 (19)
Tetracycline (30 µg)	15 (65.2)	0 (0)	8 (34.8)
Trimethoprim/sulfamethoxazole (1.25/23.75 µg)	18 (78.3)	2 (8.7)	3 (19)
Cefepime (30 µg)	21 (91.3)	1 (4.3)	1 (4.3)
Imipenem (10 µg)	21 (91.3)	1 (4.3)	3 (19)
Ceftazidime (30 µg)	20 (87)	1 (4.3)	2 (8.7)
Cefotaxime (30 µg)	21 (91.3)	0 (0)	2 (8.7)
Amoxicillin (30 µg)	19 (82.6)	3 (19)	1 (4.3)
Amoxicillin/Clavulanic acid (20/10 µg)	20 (87)	0 (0)	3 (19)

opia as 6.3%, which is consistent with our findings (30). In Colombia and Egypt, 62.9% and 0.5% of cheese samples were contained with *Salmonella*, respectively (31, 32). In Japan, 66 natural cheeses were examined but no infection with *Salmonella* spp. was observed (33). A study from Morocco showed that 5.9% of *Salmonella* contamination was found in cheese samples whose prevalence is similar to the one in our study (9). It was indicated that traditional ice creams were one of the main sources of *Salmonella* spp. (8% contamination). Since *Salmonella* does not survive typical minimum pasteurization processes, its presence may indicate that either the pasteurization process was improper or that the contamination occurred after pasteurization (2, 34). The prevalence rates reported from Cambodia (1.9%) and from India (0%) were much lower than those observed in the current investigation (35, 36). These differences could be due to variations in sample types, the source of the samples, testing methods and seasonality which most likely all affected the results (37).

Cream is a high-fat dairy product which contains high levels of milk fat globules dispersed in a continuous phase of skim milk. It is obtained by mechanical separation from the milk (38). Cream is a rich nutrient media for microbial growth (39). In our study, 6 of the 75 samples of cream showed *Salmonella* spp. growth lower incidence was observed by McLauchlin et al. (2020), finding that 0% of *Salmonella* spp. were isolated from cream samples in England (40). Busani et al. detected *Salmonella* in only 0.1% of cream samples from Italy (41). Rios-Muniz et al. in Mexico City reported a low prevalence (0%) of *Salmonella* among cream samples (42). The difference in prevalence between different studies might be associated with difference in equipments, method of cooking/boiling, and the detection methods employed in different studies (43).

Multidrug-resistant bacteria may contact humans directly through their food (e.g., meat, fish, dairy products, and egg) or exposure to animals or indirectly via environmental pathways (44). As specified in our study, all *Salmonella* spp. isolated from the study samples were highly resistant to tetracycline (34.8%); on the contrary, they showed high sensitivity to cefepime, imipenem, and cefotaxime (each 91.3%). Likewise, some other studies on raw milk and dairy products worldwide reported resistance to the mentioned antibiotics. according to Ejo et al. (2016) in Gondar, Ethiopia the highest rate of resistance was attributed to nalidixic acid (78.57%), tetracycline (42.58%), and chloramphenicol (21.42%) (45). As Sobur et al. (2019) reported, the resistance of *Salmonella* spp. isolated from raw milk to tetracycline was 88.89%. This finding contradicted the results achieved by the present study in which 34.8% of the isolates were resistant to tetracycline (46). In contrast, a study conducted in the United States revealed that 12.2% of *Salmonella* isolates were resistant to tetracycline indicating a lower rate of resistance in comparison with our study (47). Furthermore, a study in Jordan reported a high resistance rate for *Salmonella enterica* isolated from cattle dairy to Chloramphenicol (35.7%) and ciprofloxacin (32%) (48). Addis et al. (2011) noted that the highest resistance rates were attributed to tetracycline (50%) and gentamycin (33.3%), respectively. The lowest resistance rates were related to chloramphenicol (16.7%) (49). In the US, Van Kessel et al. (2013) stated that the highest rates of resistance were against tetracycline (15.3%), chloramphenicol (13.1%), and amoxicillin-clavulanate (11.4%); these rates are lower than those reported in our study (50).

According to the results of this investigation, isolates of *Salmonella* spp. are resistant to the majority of the studied antimicrobial agents. The high resistance rate to tetracycline noted in the present investigation could be due to tetracycline used in veterinary medicine to treat infected animals in Iran over the past decade.

CONCLUSION

This was the first study on determining the presence of *Salmonella* spp. in raw milk and traditional dairy products in Yazd province. Results of current study indicated that unpasteurized dairy products can serve as a rich growth medium for food-borne pathogens like *Salmonella* spp. leading to human infection. Study limitations included not identifying the *Salmonella* species, lack of direct sampling from cattle and livestock farmers and limited number of samples. This study needs to be perused further on larger number of samples by employing different isolation methods. Also, this study highlighted the need for implementing control measures so as to reduce the spread of the pathogen in the production processes.

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