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Isolation, Identification, and Antibiotic Susceptibility Testing of *Salmonella* Isolated from Foodborne Outbreaks

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Abstract

Background: Foodborne diseases are a major problem worldwide. The epidemiological investigations in many parts of the world have shown an increase in infections caused by *Salmonella* serovars. Furthermore, the emergence of drug resistance among them has become a major global concern–and awareness of the resistance patterns of *Salmonella* could be very useful in treatment of diseases.

Objective: This study aimed to investigate *Salmonella* serotypes in foodborne outbreaks by sequencing of ITS region of 16S-23SrRNA gene and to determine their antimicrobial susceptibility pattern.

Materials and Methods: A total of 614 diarrheal stool samples were collected from 173 foodborne outbreaks in different provinces of Iran during one year. Identification of *Salmonella* was carried out by phenotypic and molecular (16s-23srRNA gene detection) methods and antibiotic susceptibility was performed using disc diffusion method.

Results: Out of 614 samples, 18 isolates were identified as *Salmonella* of which 16 (88.9%) isolates were *Salmonella* Enteritidis and 2 (11.1%) isolates as *Salmonella* Paratyphi A. All isolates were sensitive to ceftazidime, and high resistance was seen with nalidixic acid with 14 (77.8%) isolates.

Conclusion: Increasing antibiotic resistance in many bacterial pathogens such as *Salmonella* has been a major threat for human health. Therefore, identifying the antibiotic resistance patterns of *Salmonella* serovars may help in treatment of the associated infections.

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Background

Today, antibiotic resistance is growing rapidly; the excessive or inappropriate use of antibiotics led to the emergence of resistant strains of *Salmonella*.[1,2] Extending resistance to antimicrobials may be created through direct or indirect consumption of antibiotic-containing foods. Animal foods can act as reservoirs of resistance genes. The prohibition of unnecessary use of antibiotics not only helps to prevent the spread of resistant strains but also prevents losing therapeutic effect of them.[3] Hence, the community health systems should also consider these issues in animal husbandry and agriculture.[1] However, with increasing awareness, more countries have banned the misuse of antibiotics, but the resistance of pathogens has not diminished so far, and now the main concern for food safety is inhibitory activity against such resistant bacteria.[4] *Salmonella* is a gram-negative rod-shaped bacterium in the *Enterobacteriaceae* family with more than 2500 serovars. This bacterium causes salmonellosis, one of the most common food-borne diseases transmitted to human by contaminated foods such as poultry, beef, pork, egg, milk, cheese, seafood, fruits, juice, and vegetables.[5] It is estimated that about 94 million cases of gastroenteritis and 155 000 deaths occur because of *Salmonella* each year worldwide, of which 85% are food related (6). Nontyphoid *Salmonella* spp., such as *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis, are important agents of food-borne outbreaks worldwide.[7] Unfortunately, the outbreak of *Salmonella* even in developed countries has increased recently. In addition, the emergence of

antibiotic resistance of *S. enterica* serovars (Typhimurium, Enteritidis, and Paratyphi A) to common antibiotics and their spread has been an issue for the health system.[8] The overuse of antibiotics in animals for prevention or treatment of diseases and growth promotion has increased the antibiotic resistance in zoonotic food-borne pathogens such as *Salmonella*.[9] Of particular concern is development of resistance to effectual drugs such as fluoroquinolones and β-lactams. Today, the number of *Salmonella* isolates with high level of resistance to several antibiotics (multiple drug resistance [MDR]) is growing. MDR *Salmonella* may be transmitted to human throughout the production chain and fecal-oral route.[8,10] This study aimed to isolate and determinate the *Salmonella* serotypes in foodborne outbreaks by evaluating phenotypic characteristics and sequencing of ITS region of 16S-23 SrRNA gene followed by the antimicrobial susceptibility testing (AST).

Materials and Methods

Sample Collection, Isolation, and Phenotypic Identification

In this descriptive cross-sectional study, from October 2013 to September 2014, a total of 614 diarrheal stool samples were collected from 173 foodborne outbreaks from different provinces of Iran. Exclusion criteria were history of gastrointestinal disease in the past one month, diarrhea after medication, and viral diarrhea. According to the standard methods, stool samples were transferred to the Kerry Blair transfer medium (Merck, Germany), then transferred to the Selenite F (Merck, Germany) and cultured on Hektoen Enteric Agar medium (Merck, Germany) for 8 to 12 hours in the microbiology laboratory of the Department of Pathobiology of Tehran University of Medical Sciences, Tehran, Iran. After incubation for 24 hours at 37°C, the black colonies suspected to *Salmonella* were chosen and then tested for phenotypic characteristics and biochemical tests such as indole, lactose, H_2S, lysine, citrate, Methyl Red-Voges Proskauer (MR-VP), Urea, and motility.[11]

Antimicrobial Susceptibility Testing

AST was performed using disc diffusion method on Mueller-Hinton agar as recommended by the Clinical and Laboratory Standards Institute (CLSI).[6] The used antibiotic discs (MAST, UK) for AST were amoxicillin (20 µg), chloramphenicol (30 µg), tetracycline (30 µg), ampicillin (10 µg), trimethoprim-sulfamethoxazole (23.75/1.25 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), cefotaxime (30 µg), ceftazidime (30 µg), and meropenem (10 µg). After incubation for 18 hours, based on the diameter of created inhibition zone around the antibiotic discs, *Salmonella* isolates were considered as sensitive (S) or intermediate (I) or resistant (R).[12]

Genotypic Identification

Identification and determination of the *Salmonella* strains was performed using sequencing of the 16S–23S rRNA gene internal transcribed spacer region. The primer sequences were as follows: reverse primer 5'-TATAGCCCCATCGTGTAGTCAGAAC-3' and forward primer 5'-TGC GGC TGG ATC ACC TCC TT-3 .[13] Genomic DNA of the *Salmonella* isolates was extracted using boiling method from purified cultures.[14] In short, using sterile loop, a few colonies of bacteria were suspended in 50 µL of Sodium Chloride-Tris-EDTA (STE) Buffer in 1.5 mL microtube and then boiled for 10 minutes. After centrifugation at 13300 rpm for 3 minutes, the supernatant was removed and finally concentration of DNA was measured by NanoDrop 3300. The polymerase chain reaction (PCR) reaction was carried out in a total volume of 20 µL containing 15.1 µL of sterile distilled water, 2 µL of PCR-buffer (10×), 0.6 µL of MgCl2 (50 mM) 0.4 µL of dntp-mix (10 mM), 0.3 µL of Primer F, 0.3 µL of Primer R, 0.3 µL of Taq polymerase (5 u/µL) (Pishgam Co., Iran), and 2 µL of DNA. The PCR temperature conditions included initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 71°C for 30 seconds and extension at 72°C for 50 seconds. Finally, the final extension was allowed at 72°C for 10 minutes. After amplification, to analyze the PCR products, electrophoresis was done using a 1% agarose gel containing safe stain and a TBE 0.5X for electrophoresis buffer, as well as a 100-bp ladder and a voltage of 80-100 V.

Results

Phenotypic Identification and Antimicrobial Susceptibility Testing

Out of 614 samples, 18 (2.93%) isolates were identified as *Salmonella*. The most abundant serotype was *Salmonella* Enteritidis with a frequency of 16 (88.89%), while the remaining 2 (11.11%) isolates were *Salmonella* Paratyphi A.

Of the total 18 isolates of *Salmonella* collected from different provinces, Alborz province with 5 (27.78%) isolates and Mazandaran with 4 (22.22 %) isolates had the highest rate of *Salmonella* outbreak. Moreover, 15 (83.33 %) isolates belonged to the samples collected in the spring and summer seasons. Women were the major origin of *Salmonella* with the frequency of 15 (83.33 %) isolates, while only 3 (16.67 %) isolates were obtained from men. The dominant age groups were 7-12-year-old and 25-35-year-old subjects, each with 4 (22.22%) isolates.

According to the results of AST, the highest rate of resistance was related to nalidixic acid (14 (77.77%)) and the highest susceptibility was seen in ceftazidime (18 (100%)), followed by amoxicillin and chloramphenicol (17 (94.44%)) (Table 1).

Table 1. Antimicrobial Susceptibility Pattern of 18 *Salmonella* Isolates

Antibiotic	Abbreviation	Sensitive No. (%)	Intermediate No. (%)	Resistant No. (%)
meropenem	MEM	2 (11.11)	13 (72.22)	3 (16.66)
Cefotaxime	CTX	14 (77.77)	4 (22.22)	0 (0)
Chloramphenicol	C	17 (94.4)	1 (5.55)	0 (0)
Co-trimoxazole	TS	13 (72.22/)	1 (5.55/)	4 (22.22)
Ciprofloxacin	CIP	16 (88.88)	2 (11.11)	0 (0)
Nalidixic Acid	NA	3 (16.66)	1 (5.55)	14 (77.77)
Amoxicillin	A	17 (94.4)	0 (0)	1 (5.55%)
Tetracycline	T	14 (77.77)	0 (0)	4 (22/22)
Ampicillin	AP	14 (77.77)	4 (22.22)	0 (0)
Ceftazidime	CAZ	18 (100)	0 (0)	0 (0)

Genotypic Identification

After amplification of target gene and electrophoresis of the PCR products, 312 kb bands were observed in gel, which confirmed *Salmonella* genus (Figure 1). After confirmation, PCR products were sent to Takapoozist company to sequence it for determination of the serotypes of *Salmonella*. After sequencing and alignment by BLAST (NCBI tool), it was found that 16 isolates belonged to *Salmonella* Enteritidis and two isolates belonged to *Salmonella* Paratyphi A.

Discussion

The emergence of foodborne pathogens, along with their antibiotic resistance and effects on the general health of the community is one of the most important challenges to the healthcare system.[15] Kozak et al showed that Canada, with the highest consumption of vegetables and fresh fruits, had the highest rates for the most common causes of food-borne outbreak and *Salmonella* had the highest morbidity rate (about 50%).[16] This study showed that vegetables and fruits were the most frequent sources of *Salmonella* isolates causing foodborne outbreak. Of the total 18 isolates, 6 (33%) isolates belonged to the patients with consumption of fruits and vegetables, and 5 (27%) isolates were related to vegetables and meat, which are similar to the study by Kozak et al.

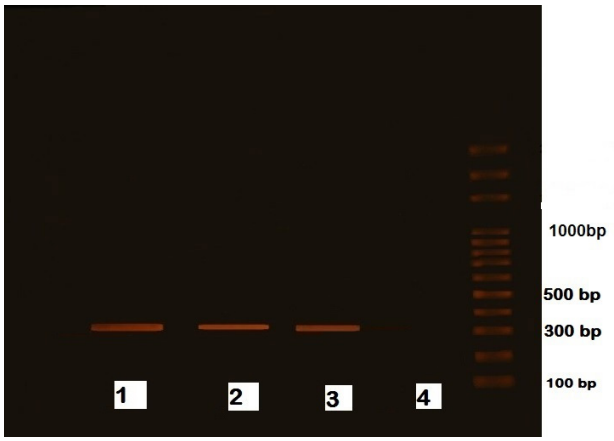

Figure 1. PCR Agarose Gel Electrophoresis Results for Detection of the 16s-23srRNA Gene in *Salmonella* Isolates (Product Size = 312 bp). 1, 2, and 3: *Salmonella* Isolates. 4: Negative Control.

A study in Turkey on the antimicrobial resistance of non-typhoid *Salmonella* in 2000-2002 showed that there is a decrease in sensitivity to ciprofloxacin especially in *Salmonella* C-type, which is slightly different with the results of our research because isolated serotypes in our study were all sensitive to ciprofloxacin, except for 2 (11%) cases that were semi-sensitive.[17] In a study conducted in Palestine between 1997 and 2002, 53.3% non-typhoid *Salmonella* was isolated, and the highest resistance rate was related to nalidixic acid (79%), which was similar to our study (77%).[18] In a study by Hur et al in Korea in 2011, 46 isolates of *Salmonella* obtained from different sources were investigated to detect antibiotic resistance genes, and the highest resistance rate was related to nalidixic acid and sulfamethoxazole. Of the 46 isolates, 21 isolates were resistant to ampicillin, piperacillin, and ticarcillin, of which 19 isolates had the *bla*-TEM gene and only one had the *bla*-CTX-M gene.[19] In our study, only 5 (27%) and 1 (5%) isolates of all isolates were semi-sensitive to ampicillin and chloramphenicol, respectively; in addition, no resistance was observed in the mentioned antibiotics.

Although the antibiotic resistance of *Salmonella* enteritidis is significantly lower than *Salmonella* typhimurium,[20] this is important since *Salmonella* enteritidis is the most important cause of salmonellosis in food-borne outbreaks.[15,21,22] Therefore, it is anticipated that the information obtained from this study could be helpful in detection of *Salmonella* and its antibiotic resistance pattern in order to reduce the therapeutic costs and take the necessary control and prevention measurements.

Authors' Contributions

MMSD, Conceived and designed the experiments; MK, ZR, carried out all the experiments; MA, writing the first draft of manuscript; SY, RB, contributed in literature survey and help in writing the manuscript; SMAL, designed preparation of experiments; MKSY, writing the final draft of manuscript.

Ethical Approval
Not applicable.

Conflict of Interest Disclosures
The authors declare that they have no conflict of interests.

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