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The Study of Antimicrobial Resistance among Salmonella enterica Strains Isolated from Children with Gastroenteritisin in Tehran, Iran

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ABSTRACT

Background: *Salmonella enterica* is one of the predominant causes of the food-borne salmonellosis in humans. The aim of this study was to determine antimicrobial resistance patterns of *Salmonella enterica* isolated from stool samples of children with gastroenteritisin in Tehran, Iran.

Methods: Stool samples of patients with diarrhea in a pediatric hospital in were collected from June 2017 to May 2018. Microbiological methods were used for identification of *Salmonella*. The identity of *Salmonella enterica* serotype *enteritidis* (*S. enteritidis*) was also confirmed by a multiplex-PCR. Antibiotic susceptibility testing was performed according to the standard procedure of the Clinical and Laboratory Standards Institute (CLSI).

Results: Of 800 samples, 24 were identified as *Salmonella*. The most prevalent serotype was S. *enteritidis* (n=10, 41.7%), followed by *S. paratyphi* C, (n=6, 25%), *S. paratyphi* B (n=4, 16.7%), S. *arizonae* 2 (n=2, 8.3%), and *S. paratyphi* A (n=2, 8.3%). The highest rates of antibiotic resistance were obtained for nitrofurantoin (100%), followed by nalidixic acid (45.8%), and tetracycline (16.7%). Of 24 *S. enteritidis*, 9 distinct antibiotypes (Abs) were observed. In this respect, 3 isolates (12.5%) were resistant to at least three or more antibiotics. The most prevalent antibiotype was AB1 (n=8, 33%), which was indicative of resistance to nitrofurantoin.

***Conclusion*:** Considering the constant changes in antibiotic resistance patterns among food-borne pathogens over time, continuous monitoring of multidrug-resistant Salmonella isolates would definitely improve infection control strategies.

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Introduction

Non-typhoidal *Salmonella* serovars are pathogens of both human and animals (1). According to the WHO reports, there are nearly 17 million cases annually of acute gastroenteritis or diarrhea due to non-typhoidal salmonellosis, which resulted in 3 million deaths (2). Both meat and eggs are known to be a major source of foodbrone pathogens such as *Salmonella* and *Shigella*. (3). Given the fact that Salmonella is one of the major causative agent of gastroenteritis, several typing methods have been used for the surveillance of foodborne in trace-back of infections to food sources (4). Diarrhea due to Salmonella is typically self-limited and usually does not require antibiotic therapy among immunocompetent persons, but some immunocompromised patients such as the HIV-positive, diabetics or patients with rheumatoid arthritis have an increased risk of developing bacteremia. These patients may benefit from antibiotics to minimize the occurrence of complications (5, 6).

Molecular techniques, especially PCR (polymerase chain reaction) assays, for detecting specific O and H antigen gene alleles are a rapid and cost-effective approach in comparison to classical serotyping. On the other hand, a number of different DNA-based typing methods such as REP-PCR (repetitive element sequence-based PCR), plasmid profiling, biotyping, ribotyping, pulsed field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) have been used to clarify various clones of *Salmonella* serovars. In fact, these approaches have provided useful insights into the evolutionary and epidemiological relationships of Salmonella strains (7, 8). However, each method has their own advantages and limitations (9). For instance, the basis of REP-PCR is on primers which are complementary to the short repetitive sequence elements along the bacterial genome. The amplification of these elements generate DNA fragments that produce fingerprints with high discrimination degree between bacterial strains (2, 10, 11).

There is a paucity of data with regard to different serovars as well as antimicrobial resistance of clinical *Salmonella* isolates in Iran. Therefore, the aim of this study was to assess the prevalence and antibiotic resistance patterns of different *Salmonella* serovars isolated from children with diarrhea in Tehran, Iran.

Materials and Methods

Totally, 800 rectal swabs samples were obtained from children under 5 years of age admitted to the Children's Medical Centre Hospital in Tehran from June 2017 to May 2018. In brief, after overnight incubation into Hektoen enteric agar (HEK) and Xylose Lysine Deoxycholate (XLD) (Himedia Laboratories Corporate Office, Mumbai, India) at 37°C, suspected colonies were subjected to routine biochemical tests, as mentioned elsewhere [11,12].

Since there was no pre-designed primer for *Salmonella paratyphi* A, B, C, serotyping according to the slide and tube agglutination tests was performed [14]. Serotyping of the isolated Salmonella strains was performed by commercially reliable antisera (Difco, Detroit, USA), and the results were interpreted according to the Kaufmann-White scheme (Popoff and Le Minor, 1992) [13]. We also used multiplex PCR for further confirmation of *S. enteritidis* [8]. The primer sequences used for multiplex PCR amplification and predicted amplicon sizes are listed in Table 1.

Antimicrobial susceptibility was determined by disc diffusion method according to CLSI guidelines. *S. enteritidis* ATCC 23564 was also used as quality control [15]. The following antibiotics (Mast Diagnostics, Mast Group Ltd, Merseyside, UK) were used: amoxicillin (20 µg), cefotaxime (30 µg), ceftazidim (30 µg), ceftriaxone (30 µg), cephalexin (30 µg), gentamicin(10 µg), streptomycin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), colistin sulfate (25 µg), chloramphenicol(30 µg), nitrofurantoin (300 µg), co-trimoxazole(25 µg), tetracycline(30 µg), imipenem (10 µg) and meropenem (10 µg).

Bacterial colonies were harvested from Tripticase soy agar after overnight inculation. Afterwards, template DNA was purified using a commercial kit (Bioneer, Seoul, Korea) according to manufacturer's instructions. For molecular detection of S. enteritidis, multiplex PCR was carried out in a PEQLAB thermal cycler (Germany) in a total volume of 25 µL using following cycling program: 5 min of initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30s, and extension at 72°C for 60 s, with a final extension at 72°C for 5 min (8). In order to confirm the expected sizes of the PCR amplicons, the PCR products were subjected to electrophoresis in 2.0% agarose gel, were stained with DNA Green ViewerTM (GeneCopoeia, Rockville, MD) and were visualized under ultraviolet light.

Results

Out of 800 patients, 24 (3%) *Salmonella* isolates were recovered from stool specimens. The results of multiplex PCR amplifications are shown in Fig 1. There were 15 males and 9 females. The frequency of various clinical symptoms among different *Salmonella enterica* serovars are shown in Table 2. The mean age of the patients with diarrhea was 3.2 ± 1.4 years. The most common serotype was *S. enteritidis* (n=10, 41.7%), followed by *S. paratyphi* C, (n=6, 25%), *S. paratyphi* B (n=4, 16.7%), *S. arizonae* 2 (n=2, 8.3%), and *S. paratyphi* A (n=2, 8.3).

The highest rates of antibiotic resistance were obtained for nitrofurantoin (100%), followed by nalidixic acid (45.8%), and tetracycline (16.7%). All of the isolates (100%) were susceptible to co-trimoxazole, gentamicin, ceftriaxone, cefotaxime, ceftazidim, ciprofloxacin, cephalexin, colistin sulfate, imipenem, and meropenem. Of 24 *S. enteritidis*, 9 distinct antibiotypes (Abs) were observed. In this regard, 3 isolates (12.5%) were resistant to at least three or more antibiotics. The most common antibiotype was AB1 (n=8, 33%), which was indicative of resistance to nitrofurantoin. The second prevalent antibiotype was AB2 (n=6, 25%), which characterized by simultaneous resistance to nitrofurantoin and nalidixic acid, as depicted in Table 3.

Discussion

Over the past few years, *S. enteritidis* has become a major cause of gastroenteritis and food poisoning across the globe (18, 19). In the current study, the prevalence of enteritidis serovar in fecal samples of children with diarrheal disease who referred to Children's Hospital Medical Center was around 41%. These findings indicate a steep rise of the mentioned serovar in Tehran, coincided with the increasing prevalence around the world. Consistent with our results, some recent reports from India and Spain showed that *S. enteritidis* as the most frequent serotype with the incidence of 9.86% and 15.15 %, respectively (20,21).

Notably, high frequencies of resistance towards nitrofurantion and nalidixic acid were observed among *Salmonella* isolates. This poses a serious threat to global public health and measures should take seriously for the use of these antibiotics. Generally, indiscriminate use of antibiotics in livestock production and animal husbandry have contributed to emergence of drug- resistant pathogens. Indeed, food-producing animals (e.g., cattle, chickens, and turkeys) are the major reservoirs. Asymptomatic animals are of particular concern in the dissemination of Salmonella. (22, 23). Transmission of the pathogen is usually associated with contaminated food such as meat, poultry, and egg, may be resulted in nationwide outbreaks (24, 25). Furthermore, animals can shed billions of bacteria in their manure, even when they do not seem to be ill. Using fecal-based fertilizers has a potential danger in the spread of Salmonella. It has been shown that *Salmonella* can survive in the soil at least 14 days after the application of the slurry (2, 26).

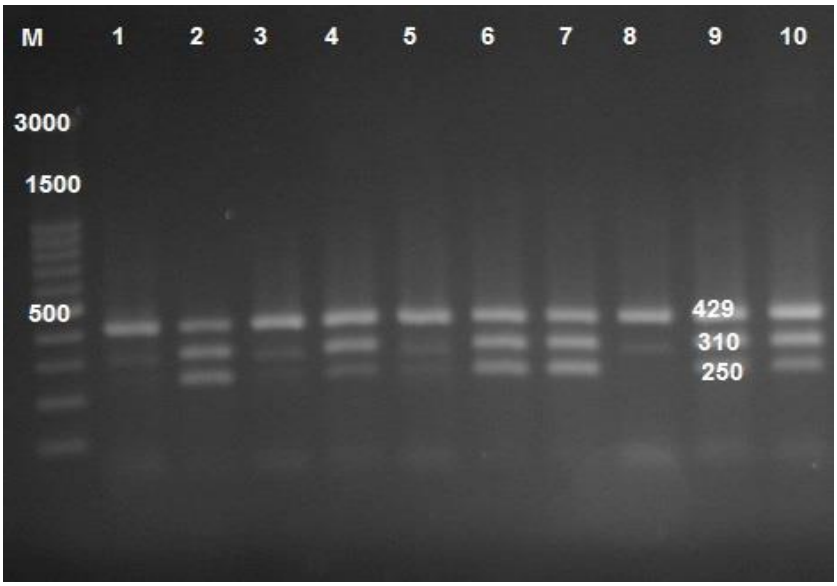

Figure 1. PCR amplifications of 16S rRNA, *spv*, and *sef-A* in *Salmonella* isolates. M represents DNA marker. Numbers above the lanes indicate the isolate code. The sizes relating to each band (bp) are also shown in lane 9.

Table 1. Primers used in the present study for multiplex PCR amplification.

Target gene	Primers	Sequences	Amplicon	Reference
16srRNA	ST11 ST14	GCCAACCATTGCTAAATTGGCGCA GGTAGAAATTCCCAGCGGGTACTGG	429	Fardsanei et al. (2016)
Spv	S1 S4	GCCGTAGATACACGAGCTTA ACCTACGGGGCACAATAAC	250	Fardsanei et al. (2016)
sef-A	SEFA2 SEFA4	GCAGCGGTTACTATTGCAGC TGTGACAGGGACATTTAGCG	310	Fardsanei et al. (2016)

Table 2. Frequency distribution of common *Salmonella enterica* serovars by clinical symptoms.

Clinical symptom	*S. enteritidis* Number(%)	*S. Paratyphi A* Number(%)	*S. Paratyphi B* Number(%)	*S. Paratyphi C* Number(%)	*S. arizonae* Number(%)	Total Number(%)	P value
dysentery	2(%20)[a]	0(%0)	0(%0)	0(%0)	0(%0)	2(%8.3)	0.183
Non-bloody diarrhoea	10(%100)[b]	1(%50)	3(%75)	3(%50)	2(%100)	19(%79)	0.001
Vomiting	5(%50)[c]	0(%0)	1 (%25)	1(%17)	1 (%50)	8(%33)	0.002
Nausea	5(%50)[d]	0(%0)	1 (%25)	2(%34)	1(%50)	9(%37.5)	0.031
Fever	4(%40)[e]	1(%50)	1 (%25)	2(%34)	0(%0)	8(%33)	0.003
Headache	4(%40)[f]	1(%50)	0(%0)	1(%17)	0(%0)	6(%25)	0.005
Abdominal cramps	6(%60)[g]	1(%50)	0(%0)	3(%50)	0(%0)	10(%42)	0.001

[a] represents the number and the percentage of patients involved with dysentery. [b] represents the number and the percentage of patients involved with Non-bloody diarrhoea.[c] represents the number and the percentage of patients involved with vomiting.[d] represents the number and the percentage of patients involved with nausea.[e] represents the number and the percentage of patients involved with fever.[f] represents the number and the percentage of patients involved with headache.[g] represents the number and the percentage of patients involved with abdominal cramps.

Table 3. Different antibiotypes observed in studied isolates.

Antibiotype (AB)	CTX	A	T	NI	TS	S	C	CRO	GM	CO	CAZ	CIP	CFX	NA	MEM	IMI	Resistance (%)
AB_1	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	33.3%
AB_2	S	S	S	R	S	S	S	S	S	S	S	S	S	R	S	S	25%
AB_3	S	S	S	R	S	S	S	S	S	S	S	S	S	I	S	S	8.33%
AB_4	S	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S	8.33%
AB_5	S	S	R	R	R	R	S	S	S	S	S	S	S	R	S	S	4.2%
AB_6	S	S	R	R	S	I	S	S	S	S	S	S	S	S	S	S	4.2%
AB_7	S	S	I	R	S	S	S	S	S	S	S	S	S	R	S	S	4.2%
AB_8	S	S	I	R	S	S	I	S	S	S	S	S	S	R	S	S	4.2%
AB_9	S	S	S	R	S	I	S	S	S	S	S	S	S	S	S	S	4.2%

CTX:Cefotaxime, A:Amoxycillin, T:Tetracyclin, NI: Nitrofurantion TS: Co-trimoxazole, S: Streptomycine, C: Chloromphenicol, CRO: Ceftriaxone ,GM: Gentamicine, Co: Colistin sulfate, CAZ: Ceftazidime, CIP: Ciprofloxacin, CFX: Cephalexin, NA: Nalidixic acid, MEM: Meropenem , IMI: Imipenem.

Despite widespread use of serotyping, it suffers from several shortcomings that limit its utility, including that it usually takes several days to generate a result and approximately 5 to 8% of isolates cannot be typed. Following the advent of molecular diagnostic methods PCR, it has been extensively used for detection of various genes among bacterial pathogens. The gene sequences which appear to be specific to serotypes can also be detected using specific primers (27). In the present study, we used a simplified multiplex PCR for detection *S. enteritidis*. The corresponding PCR primers targeted spv (*Salmonella* plasmid virulence genes) and *sef-A* (*Salmonella enteritidis* fimbrial antigen gene). During the past years, several studies successfully evaluated the effectiveness of PCR assays as a replacement for serotyping methods as well as identification of the pathogen in either clinical or food samples. For instance, different open reading frames (ORFs), several sequences specific to serovar Typhimurium (STM gene names) or Typhi (STY gene names), and 16S rRNA gene have been used as a target for specific detection of various serovars (27-30). On the whole, these studies demonstrated that PCR-based serotyping methods are fast, accurate, reliable, and cost-effective that can accurately discriminate between different *Salmonella* serotypes.

Conclusion

Taken together, our study provided valuable information on patterns of antibiotic resistance among different serovars of *Salmonella* isolated from children with diarrhea in Tehran, Iran. The implementation of epidemiological studies based on the use of both molecular and phenotypic assays should be standardized and encouraged. Undoubtedly, continuous monitoring of

multidrug-resistant *Salmonella* isolates would definitely improve infection control strategies.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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