Short Communication

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Detection of bla TEM, bla CTX-M and bla SHV in Salmonella Species Isolated from Children with Acute Infectious Diarrhea by Multiplex-PCR and Their Antibiotic Resistance Profile

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ABSTRACT

BACKGROUND AND OBJECTIVE: Treatment for *Salmonella* gastroenteritis is important in children and people with immune system weakness. The aim of this study was to investigate the presence of *bla SHV, bla TEM* and bla *CTX-M* genes in antibiotic resistance to isolated *Salmonella* strains from children with acute bacterial diarrhea and to determine their resistance pattern.

METHODS: In this descriptive cross-sectional study, 300 stool specimens from children with diarrhea referred to the Tehran Medical Center Hospital were collected. The antibiotic susceptibility test was determined using the disk diffusion method agreeing with CLSI guideline. Then, M-PCR was achieved for determination of β–lactamase genes by specific oligonucleotides primers. Data were analyzed by SPSS software version 17 and descriptive statistics.

FINDINGS: Of the 300 stool samples collected, 18 (6%) of Salmonella were identified, of which 11 (61.1%) were salmonella typhi, 5(27.7%) were *Salmonella enteritidis* and 2(11.1%) *Salmonella typhimorium* cases. Resistance to isolates showed that the highest and lowest resistance was related to imipenem, ceftriaxone (100%) and onloxacin (54.5%) respectively. The results of the molecular analysis indicated that 7 strains (38.8%) *CTX-M* and 8 isolates (44.4%) had TEM genes respectively, while 2 strains (11.1%) contained the *SHV* gene.

CONCLUSION: accurate detection and fast identification of *Salmonella* producing ESBLs is important from the source of infection such as, foods, animals and its products and carriers.

KEY WORDS: *β-Lactamase Genes, Salmonella, Diarrhea, Antibiotic Resistance*

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Introduction

Salmonella is a member of the Enterobacteriaceae family, which are gram negative, facultative anaerobe, and non-acid fast, and non-sportive and mobile (1). These intestinal organisms have more than 2500 different serotypes that are divided into three distinct species: Salmonella typhi, salmonella choleraesuis and Salmonella enterica (2). Salmonella enterica, the subtype of Enterica serotype enteritidis, Salmonella enteritidis, is the most important cause of salmonellosis.

It is one of the most important infectious diseases between humans and animals, mostly related to the consumption of meat, poultry, eggs and milk (3). Therefore, this bacterium is a food-borne pathogen. Salmonellosis can be a form of typhoid fever (typhoid), septicemia, and gastrointestinal infections (gastroenteritis).

The common serotypes involved in Salmonella gastroenteritis, are Salmonella typhimurium and Salmonella enteritidis (4). The onset of these sudden symptoms varies from 12 hours to a week, and many people recover without the need for antibiotics (5). Due to the addition of antibiotics to animal diets, improper, excessive and arbitrary use of antibiotics and lack of proper monitoring of drug administration has led to the development of antibiotic-resistant strains (6). The main problem in treating infections caused by these organisms is the emergence of multidrug resistance (MDR), which often leads to prolonged hospitalization and increased therapeutic costs compared with antibiotic susceptible microbes and Finally, Drug Therapeutic Failure (DTF) (7, 8).

Beta-lactamases are enzymes that deactivate these antibiotics by hydrolyzing the core of the β-lactam ring. Ambler classified these enzymes into four groups (AD), based on their initial structure, which are type B metalloalbelactamase (MBLs), type C is cephalosporinase, and type A is the broad spectrum beta-lactamase (ESBL)(9). In group A of Ambler beta-lactamase, and be2 and b2 classes of the bush classification, beta-lactamase such as TEM (Temoneira) and SHV (sulfhydryl variable) are located and are the leader of the ESBLs enzymes and altering the sequence of one or more amino acids in the two enzymes above, Other enzymes are obtained (7). The most abundant type of ESBLs in clinical specimens is SHV, whose encoding gene is based on a transferable plasmid and easily distributed among bacterial strains (10). SHV beta-lactamases are inhibited by clavulanic acid but not controlled by EDTA. TEM-1 was the first broad-spectrum β-lactamase encoded by the plasmid in Enterobacteriaceae, which other bacteria such as Pseudomonas aeruginosa were able to produce (11). ESBLs not belonging to the TEM family or SHV are classified as 1-CTX-M. This gene is also plasmid and is effective on cefotaxime.

These enzymes have the ability to hydrolyze cephalosporins and are inhibited by clavulanic acid, sulbactam and tazobactam (12). In recent years, production of broad-spectrum beta-lactamase (ESBL) enzymes and bacterial resistance, followed by bacterial failure, have become prevalent among human and animal-derived bacteria, especially salmonella, and this issue is important in terms of public health. Boisram-Gastrin et al. reported a study on salmonella isolated from children that all ESBL-derived isolates contained the blaTEM-1 gene (13).

Regarding the high prevalence of resistance to antibiotics used in the population, the aim of the study was to detect beta-lactamase resistance genes (bla TEM, bla SHV and bla CTXM) in different salmonella strains isolated from clinical specimens and to determine the resistance pattern of these strains.

Methods

In this cross-sectional study, which was conducted during a 9-month period (from the beginning of June to the end of March 2015), 300 stool specimens were collected in plastic and sterile containers from suspected cases of acute diarrhea and had been referred to the Tehran Pediatrics Center Hospital in Tehran . All specimens were transferred to laboratory under sterile conditions and transferred to culture medium Selenite F (Merck, Germany) and incubated at 37 ° C for 8-12 hours.

The stool samples were cultured in selenite medium, then cultured on a XLD Agar medium and SS Agar (Merck, Germany) and incubated at 37 ° C for 24 hours. Grown and suspected colonies were identified by using biochemical standard tests such as TSI, SIM, MR-VP, Simon Citrate, phenylalanine deaminase, urease, H2S production, sugar fermentation test and nitrate recovery. Serotyping test was performed to determine the somatic (O), flagellum (H) and capsule (VI) antigens using MAST by slide agglutination method. The standard strain of Salmonella enteritidis 13076 ATCC and Salmonella typhimurium ATCC 14028 were used. Antibiotic susceptibility of strains

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was carried out using a disk diffusion method and on the Muller Hinton Agar (MHA) (manufactured by Merck, Germany) and according to the CLSI (14) and for the Seftazidime disc (30 µg), aztreonam disc (30 µg), imipenem disc (10 µg), cefotaxime disc (30 µg), Ofloxacin disc (5 µg), amikacin disc (30 µg) and tetracycline disc (30 µg) (prepared by HiMedia-India). Combined disk test (CDT) was used to screen strains of ESBLs for β-lactamases. After preparing the Muller Hinton Agar (Merck, Germany), and suspensions equivalent to the MacFarland half, microbial culture was performed. Then, ceftazidime disk (30 µg), ceftazidime-clavulanic acid disk (10-30 µg), cefotaxime disk (30 µg) and cefotaxime-clavulanic acid disk (30 µg/10) (Himedia, India) at a distance of at least 2.5 cm from the each other on medium and incubated for a full day at 37°C. Considering the principles of CLSI, whenever the growth inhibition zone around the Ceftazidime-Clavulanate disk is 5 mm higher than Seftazidime alone or if the cefotaxime - clavulanate growth inhibition zone is 3 mm higher than cefotaxime alone, then the isolate of the ESBLs will be taken. The cell DNA was extracted using Sinagene DNA kits (Cell culture, Tissues, Gram negative bacteria, and CSF). M-PCR was performed using specific primers for the detection of beta-lactamase genes bla SHV (15), bla TEM and bla CTX-M (16) (table 1). The PCR reaction was carried out in a volume of 25 µL. Each PCR reaction contains 200 µmol dNTP, 10 picomoles per primer, 1.5 mmol/L MgCl2, 0.5 units Taq enzyme, and 50 ng DNA pattern. The PCR reaction was performed on Thermocycler device. A 10-minute cycle at 95°C (initial denaturation) then 35 cycles, including a 60 second denaturation at 94°C, a 60 second anneling at 60°C, a 1 minute extension at 72°C, and a 10-minute cycle at 72°C. PCR products were examined for the presence of the genes by electrophoresis on 1% agarose gel and after staining with ethidium bromide. The standard strain of Escherichia coli ATCC 25923 was used as a qualitative control. Data were analyzed using SPSS 17 soft ware and descriptive statistical tests.

Table 1. Oligonucleotide sequence of primers used in this study

Primer sequence	Oligo-nucleotide sequence (5'→3')	Piece length (bp)
bla $_{SHV}$	F=5'-ATGCGTTATATTCGCCTGTG-3' R=5'-TGCTTTGTTATTCGGGCCAA-3'	747
bla $_{TEM}$	F=5'-TCGCCGCATACACTATTCTCAGAATGA-3' R=5'-ACGCTCACCGGCTCCAGATTTAT-3'	445
bla $_{CTX-M}$	F=5'-ATGTGCAGYACCAGTAARGTKATGGC-3' R=5'-TGGGTRAARTARGTSACCAGAAYCAGGG-3'	593

Results

A total of 300 stool specimens collected, in 18 samples (6%) Salmonella were detected. After biochemical and serotyping tests on 18 detected samples, 11 cases (61.1%) of Salmonella Typhi, 5 cases (27.7%) of Salmonella enteritidis and 2 cases (11.1%) of Salmonella typhimurium were identified. Investigating the resistance of isolates to the antibiotics by disc diffusion method showed that the highest resistance among Salmonella typhi isolates was related to cefotaxime antibiotic (90.9%) and the highest sensitivity ratio was about imipenem and ceftriaxone (100%).

Among the isolates of Salmonella enteritidis, the highest resistance to ceftazidime and Azteronam antibiotics (80%) and the highest susceptibility to imipenem, ceftriaxone and ciprofloxacin (100%) were reported. Salmonella typhimurium isolates showed the highest resistance to ceftazidime, cefotaxime, amikacin and tetracycline antibiotics (100%) and the highest sensitivity to imipenem (100%). The results also showed that the incidence of resistance to Salmonella typhimurium is far more than the two other. The results of simultaneous strain resistance (MDR phenotype) showed that of 18 isolates of salmonella, 5 isolates were resistant to 4 antibiotics and 3 isolates were resistant to 3 different antibiotics. The results of the combined disk test showed that out of a total of 18 salmonella, 8 isolates (44.4%) in terms of the phenotype were ESBLs productive.

The results of molecular analysis for all isolates of Salmonella showed that 7 strains (38.8%) had CTX-M gene and 8 isolates (44.4%) had TEM gene, while only 2 strains (11.1%) carrying the SHV gene (Fig. 1) (Table 2). By examining the presence of the genes studied in the samples, only one sample (9.1%) of the Salmonella typhi isolates was found to have all three beta-lactamase resistance genes.

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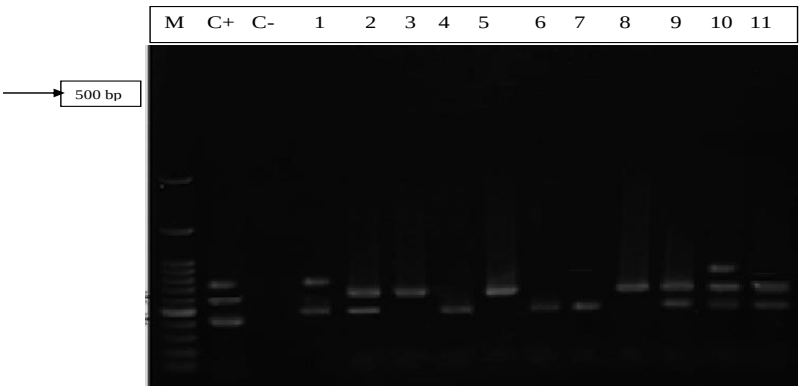

Figure1. The results of M-PCR test from left to right: M; (Sinagen Co., Iran) bp100Ladder, positive control (Salmonella enteritidis 13076 ATCC), negative control (Escherichia coli, 25923ATCC), bla SHV (bp747), bla TEM (bp455) and bla CTX-M (bp593), 11-1; Clinical strains of Salmonella

Table2. Distribution of studied genes in different salmonella strains in the forthcoming study

Microbial isolate	Salmonella typhi(N=11) N(%)			Salmonella enteritidis (N=5) N(%)			Salmonella typhi morium (N=2) N(%)		
Target gene	*SHV*	*TEM*	*CTX-M*	*SHV*	*TEM*	*CTX-M*	*SHV*	*TEM*	*CTX-M*
	1(9)	4(36.4)	4(36.4)	1(20)	2(40)	3(60)	0	1(50)	1(50)

Discussion

The results of this study showed that resistance to salmonella typhimurium isolates was more than typhi and enteritidis strains and from 18 isolates of salmonella, 5 isolates were resistant to 4 antibiotics and 3 isolates were resistant to 3 antibiotics. In molecular results, it was found that 38.8% had CTX-M and 44.4% had TEM gene, while (11.1%) were identified as carriers of SHV gene.

Diarrhea caused by Salmonella strains is a health problem around the world. Antibiotic resistance to beta-lactam antibiotics is reported abundantly, so monitoring information on antibacterial resistance should be updated and used in clinical management and treatment guidelines (5, 17). In this study, at total of 300 stool specimens from children with acute bacterial diarrhea referring to the pediatric medical center about 18(6%) Salmonella strains were found, of which is consistent Dallal et al. (18) but is not consistent with Rezaee et al (2%) (19) and Amiri et al (11.1%) (20).

This contradiction can be due to the number of samples and the time of sampling and the year of the study. The results of serotyping (MAST) on the basis of agglutination method on the lam showed that 11 (61.1%) cases were Salmonella typhi, 5 cases (27.7%) were Salmonella enteritidis, and the 2 cases (11.1%) were Salmonella typhi morium. A small difference was observed in the prevalence of serotypes identified by Dallal et al. (18) and Rezaee et al. (19) as a result of distribution of serotypes at different treatment centers. The results of the Kirby-Baer test showed that all strains (100%) were sensitive to imipenem and ceftriaxone, and six strains (54.5%) of all isolates had the most resistance to ofloxacin. These findings confirm the results of the study by Eshraghi et al. (21). In this study, 26 strains of Salmonella were obtained from 1950 stool specimens, all of which were sensitive to ciprofloxacin and imipenem.

These researchers showed that quinolones are well-suited to treatment with cephalosporin family. These results are consistent with studies conducted in Saudi Arabia (22), America (23), and the United Kingdom (24). Spiliopoulou et al., In Greece (25), showed that all isolates were sensitive to ceftriaxone and ciprofloxacin, which is consistent with the present study. However, only one isolate had a multiple resistance pattern, while in our study, 6 isolates had multiple resistance patterns had at least 5 different antibiotic types. In the study of Amiri et al., 60 isolates of Salmonella typhimurium were obtained from 28200 stool specimens for ciprofloxacin 100% sensitivity (20). The results of Imipenem's resistance to the study are consistent with White et al. (26), Eshraghi et al (21) Diniz-Santos et al (27) and Shahcheraghi et al (28). The results of molecular analysis for all isolates of Salmonella showed that 7 strains (38.8%) and 8 isolates (44.4%) had CTX-M and TEM genes respectively, while 2 strains (11.1%) carrying the SHV gene. These results contradict with Abdollahi et al study (29). In the study, all 174 Salmonella were negative in terms of the presence of TEM, CTX-M and

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SHV genes, which could be due to the years of research and time intervals, the common mechanisms of resistance in this group of bacteria, such as in-efflux pumps, purine pumps, and there are several resistance mechanisms at the same time.

However, this result suggests that resistance genes are rapidly spreading among salmonella strains, which could be a serious warning to the health system in the future. The results of the TEM gene prevalence among E. coli strains are consistent with Shahcheraghi et al. (28), but the lower incidence of SHV gene (6%) compared with the current study (11.1%) can be due to differences in the type of isolate Microbial (Escherichia coli compared to Salmonella). In the study of Abdollahi et al. (29), 60 cases of Salmonella enterica, 45 isolates had MDR phenotype, 5 isolates had resistance to cefotaxime (ESBLs), and 2 isolates also had the bla-CTX-M-type gene. This difference can be due to the difference in the type of microbial isolate and the site of sampling. The results of the disk diffusion test for the antibiotics of Imipenem and ciprofloxacin are consistent with the present study. In Yang et al., 2 isolates of Salmonella enteritidis contained the blaTEM gene (30).

The results of the , Boisrame-Gastrin et al study, showed that all isolates producing ESBL contained blaTEM-1 gene, and 21 isolates containing the blaSHV gene and 5 isolates containing the blaCTX-M gene (13). Transmission of resistance inside and between different species of bacteria and ultimately contamination of humans with highly resistant bacteria is very important. Treatment for infections caused by resistant salmonella causes not only high health risks, but also severe economic losses, as frequent and continuous use of conventional antibiotics that have been shown to be resistant to these causes the selection of resistant organisms and the spread of this organism in societies.

Accurate diagnosis and rapid identification of ESBLs producing salmonella strains from sources of transmission such as food (especially eggs), animals and their products (such as poultry and meat products) and humans that are considered chronic carriers and salmonella through feces are emitting into the environment, is very important. Therefore, the continuous evaluation of this resistance, especially at the national and regional level, is obviously preventing the spread of resistance to other strains, reducing the cost of treatment, reducing mortality in veterinary and reducing the contamination of food sources.

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