Short Article

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Molecular Identification of Virulence Genes (*agfA* and *mgtC*) in *Salmonella Typhimurium* Strains Isolated from Children with Gastroenteritis Using Multiplex PCR Method and Determination of Their Antibiotic Susceptibility Pattern

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ABSTRACT

BACKGROUND AND OBJECTIVE: Gastroenteritis caused by *salmonella* is common in humans and is regarded as a global public health issue. The aim of this study is to identify *agfA* and *mgtC* genes in *salmonella typhimurium* strains isolated from stool samples using multiplex PCR method and to determine the resistance patterns of these strains.

METHODS: This cross-sectional study was conducted on *salmonella typhimurium* isolated from children with gastroenteritis admitted to Children's Hospital Medical Center in Tehran. Frequency of *agfA* and *mgtC* genes was evaluated using multiplex PCR method. In addition, antibiotic susceptibility of these isolates was studied using gel diffusion method and according to CLSI guidelines.

FINDINGS: Of total 200 stool samples, 60 *salmonella typhimurium* isolates were obtained. Molecular analysis showed that 24 isolates contained both *agfA* and *mgtC* genes at the same time, while 52 isolates contained *mgtC* gene and 40 isolates carried *agfA* gene. All strains (100%) were susceptible to ciprofloxacin and 85% of strains were resistant to nitrofurantoin.

CONCLUSION: Results of the study demonstrated that most frequent virulence gene in these strains was *mgtC* (86.6%) and the least frequent virulence gene was *agfA* (66.6%). Moreover, it was concluded that these isolates were 100% susceptible to ciprofloxacin and were the most resistant to nitrofurantoin (85%).

KEY WORDS: *Salmonella typhimurium, mgtC and agfA genes, Antibiotic susceptibility*.

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Introduction

The first stage in pathogenesis is the formation of organisms in tissue and their struggle to attach to the target tissue (1, 2). Fimbriae are superficial filamentary organelles composed of thousands of copies of the main subunit called “fimbrillin” as well as one or a few copies of adhesive subunits usually located at the tip of the filament (3, 4). One *salmonella* cell may express 200 to 300 copies of fimbriae, which are affected by phase change (5). Enterobacteriaceae fimbriae are generally divided into two groups of mannose sensitive (MS) and mannose resistant (MR) (6).

Accordingly, there are various types of fimbriae and one of them is type IV fimbriae or GVVPQ, which was detected in *escherichia coli* as fimbriae cross-reactive with SEF 17 (*salmonella enteritidis* fimbriae with a fimbrin molecular mass of 17 kDa) for the first time. SEF17 subunits are encoded by *agfA* (aggregative fimbriae) and can be detected in some (not all) *salmonella serovars.* Existence of *agfA* can be varying in a serovar; for instance, only 50% of *salmonella typhimurium* strains are positive in terms of antigen *AgFA*. Curli fimbriae are type of fimbriae that are gathered with extracellular cycle of accumulation/deposition (7-9).

Protein *CsgA* is the main component of curli fimbriae in *escherichia coli*, which is 86% similar to its equivalent in *salmonella typhimurium* (*AgfA*) (10%). After attachment and colonization, survival and proliferation of *salmonellas* inside macrophages is their key to succeed in pathogenesis. Most of the genes involved in the pathogenesis of these bacteria are codified in locations called Salmonella Pathogenicity Island (SPI) (8). There are three transportation systems for magnesium bivalent (Mg^{2+}) in *salmonella*, which are called *CorA*, *MgtA* and *MgtB*. Two bivalent magnesium absorption systems are codified by *mgtA* and *mgtCB* loci in SPI-3 (11).

The *mgtCB* locus codifies proteins *MgtC* and *MgtB*. Nikbakht et al. indicated that *MgtC* might play a role in regulation of ion homeostasis (12). Based on studies by Nikbakht et al., *MgtC* acts as Sodium-Potassium Pump (Na^+/K^+ ATPase) and is thus involved in regulation of membrane potential (12). Adding antibiotics to livestock ration, improper, excessive and arbitrary use of antibiotics and the absence of precise monitoring of drug administration have created antibiotic-resistant strains (6). Considering the similarity between *mgtC* in *salmonella typhimurium* and Sodium-Potassium Pump *agfA* and *csgA* in curli fimbriae, this study was conducted to detect *agfA* and *mgtC* genes in *salmonella typhimurium* strains isolated from stool samples using multiplex PCR method and to determine the resistance patterns of these strains (7,8).

Methods

This cross-sectional study was conducted within a 7 months period (from May 2015 to December 2015). 200 stool samples were isolated from children with suspected *salmonella* infection admitted to Children's Hospital Medical Center in Tehran and were kept in sterile plastic containers.

All samples were transferred to laboratory environment, were transferred to Selenite-F culture medium (Merck, Germany) for enrichment, and were incubated at 37°C for 8-12 hours. In the next step, stool samples cultured in SF medium were cultures in Xylose lysine deoxycholate agar (XLD) and Salmonella-Shigella (SS) agar (Merck, Germany) and were incubated at 37°C for 24 hours. Grown and suspicious colonies were detected using biochemical and microbiological routine and standard tests such as Triple sugar iron agar (TSI), Sulfide Indole Motility (SIM), Methyl Red-Voges Proskauer (MR-VP), Simmon's Citrate Agar and Hydrogen Sulfide (H2S) Production Test. Serotyping test was used to detect somatic (O), flagellar (H) and capsular (Vi) antigens using monovalent (MAST) and polyvalent antiserums prepared from Bahar Afshan Company using slide agglutination method. The standard strain of *salmonella typhimurium* (ATCC $_{14028}$) was used as positive control in all steps.

Antibiotic susceptibility of strains was studied using disk diffusion method in Mueller Hinton Agar (MHA) (Merck, Germany) based on The Clinical & Laboratory Standards Institute (CLSI) guidelines (13). For molecular analysis of genes under study, genomic DNA was extracted using CinnaGen DNA extraction kit (Cell culture, Tissues, Gram negative Bacteria and CSF). Multiplex PCR test was used to detect *agfA* and *mgtC* genes by specific primers (table 1).

PCR reaction was done in a volume of 25 µl. Each PCR reaction contained 200 µmol dNTP, 10 pmol of each primer, 1.5 mmol/L $MgCl_2$, 0.5 unit Taq enzyme and 50 ng of pattern DNA. Multiplex PCR reaction in thermocycler (Eppendorf, Germany) was done as follows: First, a 10-min cycle at 95°C (The initial denaturation). Then, 35 cycles including a 30-sec

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denaturing phase at 94°C, a 60-sec attachment phase at 59°C and a 1-min expansion phase at 72°C and finally a 5-min cycle at 72°C. Multiplex PCR products were analyzed regarding the presence of target genes using electrophoresis on 1% agarose gel and they were compared with standard strain of *salmonella typhimurium* (ATCC $_{14028}$) and standard strain of *escherichia coli* (ATCC $_{25923}$) as quality control (14).

Table 1. Oligonucleotide sequence of primer sequences used in this study

Primers	Primer sequences (5→3)	Product Length(bp)
agfA	F=5′-TACAAGGGATTCGGCATCG-3′ R= 5′-TAATGGCCTGTTCCCATGTG-3′	261
mgtC	F= 5′-AAAATCTGGGTACGCAAACG-3′ R=5′-ACATTATCCGCTGGAACAGG-3′	655

Results

Results of the study showed that 60 (30%) *salmonella typhimurium* isolates were obtained from the total 200 stool samples. According to antibiotic susceptibility test, highest resistance to nitrofurantoin and nalidixic acid was 85% and 70%, respectively. In addition, all 60 isolates (100%) were susceptible to imipenem, ciprofloxacin and cephalexin (table 2). The molecular analysis of target genes showed that 52 isolates (86.6%) contained *mgtC* gene and 40 isolates (66.6%) contained *agfA* gene (Fig 1). Simultaneous amplification of genes in Multiplex PCR reaction showed that 24 isolates (40%) contained both *agfA* and *mgtC* genes at the same time.

Table 2. The percentage of antibiotic susceptibility and resistance of strains under study

Antimicrobial factor (µg)	Salmonella typhimurium (n=60)		
	S(Susceptible) N(%)	I(Semi-susceptible) N (%)	R(Resistant) N(%)
Amoxicillin 25 µg	55(91.6)	5(8.4)	0(0)
Nitrofurantoin 300 µg	6(10)	3(5)	51(85)
Imipenem 10 µg	60(100)	0(0.0)	0(0.0)
Nalidixic acid 30 µg	13(21.6)	5(8.4)	42(70)
Ciprofloxacin 5 µg	60(100)	0(0)	0(0)
Cephalexin 30 µg	60(100)	0(0)	0(0)
Meropenem 10 µg	59(98.4)	1(1.6)	0(0)
Trimethoprim/ sulfamethoxazole 15 µg	47(78.3)	0(0)	13(21.7)

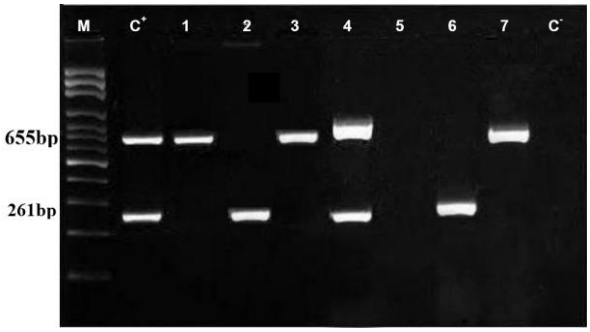

Figure 1. Multiplex PCR test results. From left to right: Ladder 100bp (CinnaGen Company, Iran), Positive control (*salmonella typhimurium* ATCC $_{14028}$), Negative control (*escherichia coli* ATCC $_{25923}$), *agfA* (261bp), *mgtC* (655bp), 1-7: Clinical isolates of *salmonella*.

Discussion

According to this study, the target genes codify the proteins that are involved in the reaction between the host and the bacteria and these effective proteins (effectors) play a key role in survival and proliferation of *salmonella*. The results of this study are in line with the results of Alphons et al. (15), which demonstrates the role of virulence genes in host-parasite interactions. The product of *agfA* gene plays a role in gliding motility, attaches the bacteria to epithelial cells, and ultimately causes bacterial accumulation.

There is another gene in SPI3 region of the main bacterial chromosome that codifies a protein called *mgtC*. This protein is created in low concentrations of mg^{2+} and growth conditions within macrophage and is involved in transmission of magnesium ions into bacteria. In this study, 24 isolates (40%) carried *mgtC* and *agfA* genes at the same time. These results are not in accord with the study of Amini et al., which might be due to the type of samples used (animal or human) (16). Gritli et al. (17) found that all *salmonella enteritidis* strains (100%) isolated from chicken carried *mgtC* gene. In the present study, highest level of resistance pertained to nitrofurantoin (85%) and nalidixic acid (70%), which was in line with the study of Banisaeed et al. (18). Moreover, all 60 isolates (100%) in this study were susceptible to imipenem, ciprofloxacin and cephalexin. Therefore, the aforementioned antibiotics are suggested to be used as the first choice for the treatment of infections caused by these strains. These results are in line with the studies conducted in United States (19) and England (20). According to a study by Spiliopoulou et al. (21), all isolates were susceptible to ceftriaxone and ciprofloxacin, which is in line with the present study.

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The results regarding resistance to imipenem in this study are in line with the studies conducted by Ranjbar et al. (22), Soltan Dallal et al. (23) and Diniz-Santos et al. (24). Ranjbar et al. (22) confirmed that excessive use or transmission of resistance elements such as plasmid among human species of *salmonella* might cause resistance.

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