Original Article

Genetic Characterization of *Salmonella Typhimurium* Isolates from Faeces of Children with Gastroenteritis Hospitalized in Baqiatollah-Azam Hospital, Tehran, Iran

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Introduction: In Iran, invasive nontyphoidal Salmonella (iNTS) disease causes severe bacteremic illness among children <5 years old. The global yearly incidence of iNTS disease in children was reported to be in the 3.4 (range 2.1-6.5) million cases, (overall incidence 49 cases (range 30-94) per 100,000 population), the iNTS case-fatality ratio (CFR) of 20% yielded 681,316 deaths annually. **Methods:** The microarray analysis enables identification of the strains that have the 90kb *Salmonella typhimurium* virulence plasmid, presence or absence of the *Salmonella* pathogenicity islands (SPIs), adherence factors and other virulence determinants. Twelve isolates of *S. typhimurium* obtained from faeces of children with gastroenteritis were analyzed by microarray technique. **Results**: The virulence plasmid was present in 83.33% of isolates and all the isolates contained the SPI-4 and SPI-5. None of the strains had the cytolethal distending toxin, *cdtB*. All strains were positive for *rck* and *mig-14*. The adherence genes were present in all the strains in the range of 51.55% to 73.20% of the adherence genes interrogated in the microarray. Two strains were the least pathogenic *S. typhimurium*. **Conclusion:** Microarray analysis proved to be a valuable tool in confirmation of serotyping results and genetic characterization of *S. Typhimurium. J Med Microbiol Infec Dis, 2015, 3 (1-2): 29-34.*

Keywords: *Salmonella typhimurium*, Gastroenteritis, Microarray Analysis.

INTRODUCTION

Salmonella is an enteric, facultative intracellular and ubiquitous pathogen widely distributed in food animals, pets, plants and the environment [1, 2]. Usually, the consumption of foods contaminated with *Salmonella* spp. may result in human infections [3]. Infections due to Salmonella may be asymptomatic or persist through gastric infections to potentially fatal systemic disease. Diseases in both human and animal hosts are caused by different serotypes and strains of the bacterial species *Salmonella enterica* and *Salmonella enterica subsp. Enterica serovar typhimurium* is the leading cause of human disease among Salmonella serotypes, world-wide [4]. Comparing to developed countries, the mortality and morbidity are much higher in third-world countries, where typhoid fever is a major killer due to poor sanitary conditions. In Iran, thousands of foodborne disease cases related to *Salmonella* are reported annually. Most of the *Salmonella enterica* isolates from animal products are found to be multi-drug resistant, and may cause serious health problems in humans [5].

The virulence potential of the *Salmonella* depends upon the genetic profile of the *Salmonella* strain and the susceptibility of the host. Virulence factors (VF) in Salmonella are necessary for adherence, invasion and replication inside the host cells [6]. Virulence factors are encoded by genes present on genetic elements, such as the bacterial chromosome, plasmids, prophages and *Salmonella* Pathogenicity Islands (SPIs). The major SPIs include SPI-1, SPI-2, SPI-3, SPI-4 and SPI-5. Some of these SPIs, *e.g.*, SPI-1 and SPI-2 are conserved throughout the genus [7].

S. typhimurium uses two type III secretion systems (T3SS), the secretion systems are intrinsic length control mechanisms for external bacterial cell projections: the hook of the flagellum and the injectisome needle [8]. SPI-1 allows delivery of effector proteins from the bacterial cytoplasm into the eukaryotic cytosol [9]. SPI-3 genes are involved in both gut colonisation and intracellular survival due to high-affinity magnesium transport encoded by *mgtABC*. SPI-4 genes are required for the intestinal phase of disease [10]. The genes within SPI-5 code for effector proteins translocated by the T3SSs of both SPI-1 and SPI-2 [11]. The SPI-1 T3SS triggers invasion into the fibroblasts and epithelial cells, inducing cell death [12].

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SPI-2 is mainly required for systemic disease, therefore assuring and enhancing *Salmonella* survival and replication inside macrophages [13]. Other SPI's are serotype specific (*e.g*. SPI-7 in *Salmonella typhi* [14]) and increase the virulence potential of the pathogen [7].

Effective epidemiological surveillance of *Salmonella* spp. and their outbreak investigations largely depend on efficient isolation, detection and typing methods [15]. DNA microarray is a powerful tool for the parallel, high throughput detection and quantification of many nucleic acid molecules and they enable the detection of up to several thousand microbial strains and genotypes in a single assay [16, 17]. The present study was performed for the serotyping, virulence and plasmid gene discovery in *S. typhimurium* isolates implicated in children with gastroenteritis referred to the pediatric department of Baqiatollah-Azam hospital, Tehran, Iran.

MATERIAL AND METHODS

Bacterial strains, growth conditions and DNA isolation. Faecal specimens were collected from feces of children suffering from gastroenteritis and suspected with salmonellosis. The isolation of *Salmonella* strains was carried out according to the ISO 6579:2002 [18], followed by biochemical confirmation, based on TSI agar test. All isolated *Salmonella* strains were then serotyped using Salmonella H Antisera and Salmonella O Antisera (Difco). *Salmonella* genomic DNA was isolated using AccuPrep 96 genomic DNA Extraction Kit (Bioneer Inc.) according to the manufacturer instructions. DNA was further purified by QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany) and its concentration was measured at 260 nm using a nanodrop spectrophotometer (ThermoScientific, USA).

Oligonucleotide design. The Salmonella microarray used in this study was designed and produced by NRC Biotechnology Research Institute (NRC-BRI, Canada) and Groupe de Recherchesur le Maladies Infectieuses du Porc (GREMIP, Canada). The oligonucleotide probe design was firstly based on the selection of target gene sequences related to housekeeping, serotype, fimbrial clusters, pathogenicity and plasmid associated genes specific for *Salmonella* spp. Also gene sequences of positive and negative controls and of pathogens other than *Salmonella* were chosen, with the purpose of identifying potential co-infections in a given sample. The protocol used for the selection of probe sequences were described previously [17, 19].

DNA labelling. Purified *Salmonella* DNA samples were labelled with Cy3-dCTP (GE Healthcare UK Limited, Buckinghamshire, UK) using BioPrime Array CGH Genomic Labeling System (Invitrogen, Carlsbad, CA) according to the manufacturer instructions. Unincorporated dyes were removed using QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany). The labelling efficiency was determined by measuring labelled samples with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware) and importing the obtained OD values to the % Incorporation Calculator (http://www.pangloss.com/seidel/Protocols/percent_inc.html). The samples were dried under vacuum in a rotary desiccator and stored at -70°C until hybridization.

Hybridization and washing. The microarray slides were incubated at 42°C for one hour in a pre-hybridization buffer (5x SSC, 0,1%SDS, 1%BSA), rinsed with filtered H_2O and 100% ethanol, and dried in a centrifuge at 1000g for 5 min. Dried labelled DNA samples were resuspended in 30 µl of hybridization buffer made of DIG Easy Hyb (Roche Applied Science, Penzberg, Germany), tRNA from baker's yeast (Sigma-Aldrich, Saint Louis, Missouri) and salmon sperm DNA solution (Invitrogen, Carlsbad, CA). The labelled samples were then denatured at 95°C for 5 min, cooled on ice for 1 min, were finally loaded onto the slides and incubated at 42°C overnight in the SlideBooster SB401/800 (Advalytix, Munich, Germany). The slides were subsequently washed in AdvaHyb AH 100 wash solutions (Advalytix, Munich, Germany) pre-heated at 42°C. The slides were then dried in a centrifuge at 1000 g for 5 min and stored in the dark until scanning.

Microarray data analysis. The slides were scanned using ScanArray G_x PLUS Microarray Scanner (PerkinElmer, Massachusetts, USA) at 543 and 633 nm. Fluorescence signal intensities were extracted by ScanArray Express software (PerkinElmer, Massachusetts, USA) and saved as Tiff format images, that were then processed by QuantArray software (Packard BioChip Technologies, Billerica, MA) for fluorescence background subtraction. Resulting fluorescence data were exported as *.txt* files and imported into a MS Excel spreadsheet, where the medians of each triplicate spot fluorescence values were performed, adjusted over signals from negative controls and log (base 2) transformed. Hierarchical clustering of serotyping and plasmid gene data sets and dendrogram visualization were respectively performed by Cluster 3.0 and JavaTreeview softwares (http://rana.lbl.gov/EisenSoftware.htm). The algorithm used was uncentered Pearson correlation complete distance.

RESULTS

Identification of isolates. The microarray data analysis of genes relevant to the various serotypes and plasmids confirmed the phenotype and serotype of isolates, all of the isolates were *S. typhimurium* as reported in Figure 1. The isolate 4 and 12 lacked the *S. typhimurium* plasmid.

Pathogenicity islands. In the SPI-1, the genes *invJ* , *invH* and *sopE* are lacking in isolate 12. Isolate 10 lacks *invJ* and *sopE*. The gene *sopE* is also lacking in the following isolates: 1, 2, 3, 4, 6, 8, 11.

In the SPI-2, isolate 7 lacks the following genes *ssaC, srfH, sseF, ssaL, sseL, sopD2* and *sseJ*. Isolate 12 lacks *srfH, ttrC, orf242, ttrB, sopD2* and *sseJ*. Isolates 1, 3, 4, 6, 8, 9, 11 lack the following genes: *srfH, sopD2* and *sseJ*. Isolate 2 lacks *srfH* and *sseJ*. Isolate 5 lacks *sopD2* and *sseJ*.

Isolate 12 lacks the genes *mgtB* and *mgtC*, both genes belonging to the SPI-3. All of the isolates contain all of the genes to the SPI-4 (except for isolate 12 which was

harbouring 94% of related genes) and SPI-5. Isolates 7, 9 and 11 had the complete set genes for the $T6SS_{SPI-6}$, isolates 2, 4, 5 and 6 had 92.86% of the genes for the $T6SS_{SPI-6}$, isolates 3, 8 and 10 had 83.71% of the genes, isolate 1 and 12 had 57.14% and 28.57% of the genes for the $T6SS_{SPI-6,}$ respectively. There are adherence genes present in all of the isolates in the range of 51.55% to 73.20% of the adherence genes interrogated by the microarray (Table 1).

The microarray also interrogates the presence of the effector proteins in the *S. typhimurium* isolates, isolate 12 has only 50% of the effector proteins. Isolate 12 resulted in having the least number of virulent genes and effector genes. None of the isolates were positive for *cdtB.*

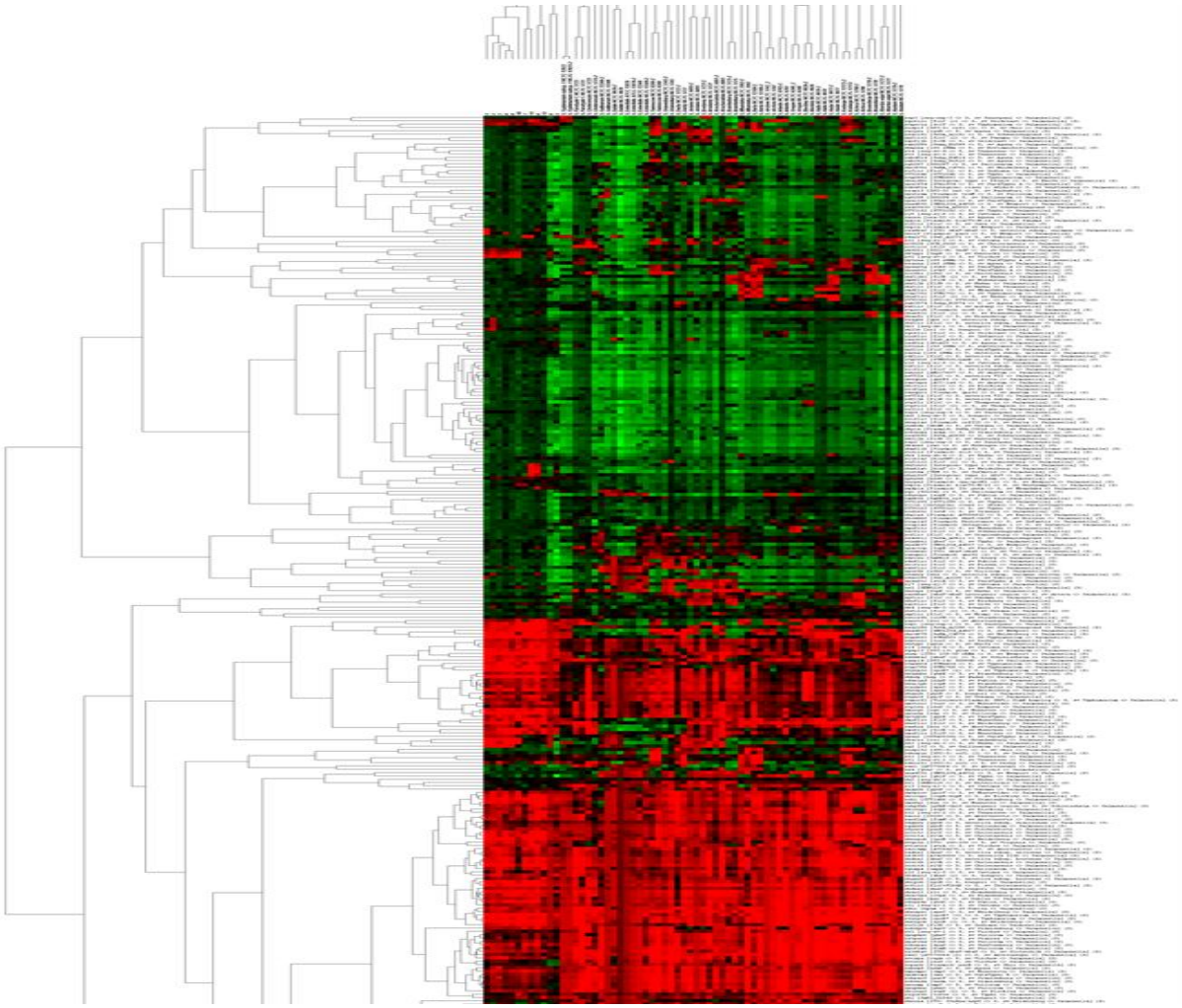

Fig. 1. Phylogenetic comparison and relationship of isolated *S. Typhimurium* strains from children gastroenteritis with standard strains of *Salmonella* enteric serotypes. Genetic elements monitored in the present study are listed at the right side of the figure, and phylogenetic relationships between tested and reference strains are showed at the left side.

Table 1. Percentage of known genes in SPI-s within Salmonella isolates in this study.

Isolate no.	Presence of *S. Typhimurium* plasmid	SPI-1	SPI-2	SPI-3	SPI-4	SPI-5	TFSS-SPI-6	Adherence genes	*cdtB*
1	+	97.96	94.55	100.00	100.00	100.00	57.14	63.92	neg
2	+	97.96	96.36	100.00	100.00	100.00	92.86	67.01	neg
3	+	97.96	94.55	100.00	100.00	100.00	85.71	68.04	neg
4	neg	97.96	94.55	100.00	100.00	100.00	92.86	64.95	neg
5	+	100.00	96.36	100.00	100.00	100.00	92.86	73.20	neg
6	+	97.96	94.55	100.00	100.00	100.00	92.86	67.01	neg
7	+	100.00	87.27	100.00	100.00	100.00	100.00	60.82	neg
8	+	97.96	94.55	100.00	100.00	100.00	85.71	58.76	neg
9	+	97.96	94.55	100.00	100.00	100.00	100.00	61.86	neg
10	+	95.92	92.73	100.00	100.00	100.00	85.71	58.76	neg
11	+	97.96	94.55	100.00	100.00	100.00	100.00	63.92	neg
12	neg	89.80	90.91	80.00	94.12	100.00	28.57	51.55	neg

DISCUSSION

Bacterial pathogenicity is one of the most important subjects in microbiology. The pathogenicity of bacteria depends on the ability to employ virulence factors [20]. Plasmids are frequently found in *Salmonella* serotypes and some are specific to the serotype [7]. The virulence plasmid pSLT is contained in the serovar Typhimurium and in the present study, two out of twelve tested isolates were negative for serovar typhimurium plasmid.

Virulence factors responsible for pathogenicity in enteric bacteria are often encoded by plasmids, and the importance of the virulence plasmid in septicemic diseases caused by non-typhoid serovars can only be inferred from indirect evidence. Plasmids are more frequently found in *S. typhimurium* and *S. enteritidis* strains isolated from blood and other extraintestinal sources than in strains isolated from feces [21]. The plasmid detection of *S. typhimurium* isolates from children with gastroenteritis in our study shows that 10 isolates harboring the virulence plasmid are capable to invade extraintestinal tissues and make problems like septicemia in children.

From the SPI-1, the translocated effector protein *sopE* allow*s S. typhimurium* to specifically activate different sets of RhoGTPase signaling cascades [22]. *SopB* has phosphatidyl inositol phosphatase activity and a *sopB*⁻ mutant is less invasive than wild type *S. typhimurium* [22, 23]. Phylogenetic analyses have shown that *sopE2* is present in all contemporary *Salmonella* lineages [2, 3]. In contrast, *sopE* is encoded in the genome of a bacteriophage, which is only present in very few *Salmonella* strains [22-25]. Nine Salmonella isolates within twelve *S. typhimurium* from children suffering gastroenteritis in our study were lacking the *sopE* gene confirming the results of Freibel *et al.* 2001, that *sopE* is not necessary for establishment of an infection and other effectors like sopE2 can play the role to onset the virulence progress [22].

Also the gene *invJ* from SPI-1 was absent in 2 strains of our study. The absence of *InvJ* results in bacterial cells exhibiting very long needles, indicating that this protein is involved in controlling the length of this structure. Certain structural features of *InvJ*, such as the presence of several glutamines at its carboxyl terminus, also are present in *FliK*, a protein involved in determining the length of the flagellar hook, suggesting that these two proteins may exert their functions in a similar manner and mutations in *InvJ* not only result in elongated needles but also in the complete absence of type III secretion [26, 27].

From the SPI-2, the genes *sseJ*, *srfH* and *sopD2* were the most absent loci in the tested isolates and only the isolate 10 is harboring all the genes related to SPI-2. The phenomenon by which a microorganism becomes adapted to its host involves the loss of genetic functions resulting in pseudogene generation, a process termed "reductive evolution". Some Salmonella serovars have accumulated mutations that enhance their survival within their respective hosts. SPI-2 regulated genes are related to bacterial intracellular trafficking and proliferation, and encode a protein complex known as the T3SS and in *S. typhimurium*. It has been concluded that *sseJ* inactivation in some serotypes like *S. Typhi* has an important role in the development of the systemic infection and it may suggest that most of our Typhimurium isolates are capable of forming systemic infections in children [28-30].

The *mgtCB* locus has been identified as part of SPI-3. Transcription of *mgtCB* is regulated by extracellular Mg^{2+} via the two-component PhoPQ regulatory system important for virulence [31]. Organisms have evolved different strategies to maintain an almost constant Mg^{2+} concentration in the cytoplasm despite wide fluctuations in the environmental levels of this divalent cation. One of the *S. typhimurium* isolates in the present study is lacking *mgtB* and *mgtC* loci and it seems there may not be any interruption in the progress of gastrointestinal stage of the disease in children.

Five phylogenetically distinct T6SS loci are described in the genus Salmonella, two of these clusters, $T6SS_{SPI-6}$ and $T6SS_{SPI-19}$, have been linked to Salmonella pathogenesis. Several authors have linked T6SS to antibacterial killing through delivery of toxins to susceptible Gram-negative bacteria [32], other authors proposed that T6SS could contribute to bacterial adaptation and competition for new niches, including animal hosts [33]. The $T6SS_{SPI-6}$ might have a role in both competition with the normal intestinal flora and survival within phagocytic cells. The *S. typhimurium* isolates in our study were harbouring 28.57 to 100% of genes cluster belonging to $T6SS_{SPI-6}$ locus showing diversity in the genes set of the mentioned locus of strains from children suffering from gastroenteritis and the adherence genes and effector proteins present in all of the tested isolates were interrogated by microarray technology, ranging from 51.55% to 73.20%.

DNA microarray is a powerful tool for the parallel, high throughput detection and quantification of many nucleic acid molecules and they enable the detection of up to several thousand microbial strains and genotypes in a single assay this methods can indicate close relationships between isolates from a distinct geographical region in a limited time [19]. There is no comprehensive data available about genetic characteristics and distribution of virulence genes related to different stages of infection in the hosts in *S. typhimurium* isolates from children with gastroenteritis in different regions of Iran and this study shows that microarray analysis can define the exact genetic contents of S. *typhimurium* strains and their genetic relationship.

In conclusion, keeping in mind the members of *S. typhimurium* serotype as zoonotic bacteria from different sources and circulation of virulence genes and their ability to transmit these factors vertically and horizontally, DNA microarray genotyping interpretation is an essential tool for epidemiology considerations.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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