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Comparative Virulence Genotyping and Antimicrobial Susceptibility Profiling of Environmental and Clinical *Salmonella enterica* from Cochin, India

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Abstract *Salmonella enterica* serotype Newport is an important cause of non-typhoidal salmonellosis, a clinically less severe infection than typhoid fever caused by *S. enterica* serotype Typhi. In this investigation, the virulence genotypes of *S. enterica* Newport isolated from a backwater environment were compared with *Salmonella* Typhi from clinical cases in the same region where salmonellosis is endemic. Genotyping was done by PCR screening for virulence markers associated with *Salmonella* pathogenicity islands (SPIs) and plasmids. The virulence genes associated with SPIs I–VI were detected in 95–100% of all the isolates, while the *viaB* locus representing SPI-7 was detectable in 66 and 73% of the environmental and clinical isolates, respectively. A significant number of *Salmonella* Newport lacked virulence genes *shdA* and *sopE* compared to *S.* Typhi. All *S.* Typhi and *S.* Newport isolates lacked large plasmid-borne virulence genes *spvR* and *pefA*. Further investigations into the antimicrobial resistance of *S.* Newport revealed multiple drug resistance to ampicillin, amoxicillin/clavulanic acid, trimethorprim-sulfamethoxazole, chloramphenicol, tetracycline, cephalothin, and cephalexin. In comparison, *S.* Typhi were susceptible to all clinically relevant antimicrobials. The results of this study will help in understanding the spread of virulence genotypes and antibiotic resistance in *S.* Newport in the region of study.

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Introduction

Salmonella enterica subspecies I comprises of more than 2,000 serotypes capable of causing a range of intestinal and extraintestinal infections in humans from life-threatening septicemic typhoid fever to mild self-limiting gastroenteritis [6, 26]. The diverse serotypes of *Salmonella* vary in their host adaptation and virulence [2]. *Salmonella* serotype Typhi is a highly host-adapted serotype responsible for lethal invasive typhoid fever in humans characterized by high morbidity and mortality. In contrast, non-typhoidal serotypes exemplified by *Salmonella* Typhimurium have a broad host range including birds, reptiles and mammals and cause mild gastroenteritis in humans. When antibiotic treatment is desired in severe cases of infections, the drugs of choice are usually ampicillin, third-generation cephalosporins (ceftriaxone) or fluoroquinolones (ciprofloxacin). Lately, emergence of *S. enterica* with decreased susceptibilities to both classes of the fluoroquinolones and the cephalosporins has complicated treatment of salmonellosis [15, 16].

Salmonella possesses a myriad of genetic factors contributing for its success as an intracellular human pathogen that participate at various stages of invasion, intracellular replication and survival within the host. The virulence genes are distributed on large genomic regions of 10–200 kb known as *Salmonella* pathogenicity islands (SPIs) [17, 22]. Some virulence genes not located on SPIs such as the chromosomally encoded *stn* (*Salmonella* enterotoxin gene), *phoP/Q* (two component global regulator) and *iroB* also play important roles in the virulence of *Salmonella* [3, 28]. Many *Salmonella* serotypes harbor large plasmids of varying sizes that carry genes responsible for virulence in mouse models [13, 14].

This study was designed to compare *Salmonella* isolates from a backwater environment with clinical isolates of

S. Typhi with focus on virulence genotypes and antimicrobial resistance. Salmonellosis is endemic in the region, though the relative contribution of typhoidal and non-typhoidal *S. enterica* to the disease burden is unknown. The evolution of virulence and antimicrobial resistance capabilities of pathogenic bacteria takes place by horizontal acquisition of genes and the aquatic environment is arguably an ideal ecosystem for such interactions among various groups of bacteria. The isolates were screened for genes associated with seven known SPIs and plasmids that are known to contribute significantly to establishment of infections and consequently the success of *Salmonella* as an intracellular pathogen. Such a study will help to understand the infection potentials and the evolution of virulence and antimicrobial resistance in *S. enterica* introduced into the environment, and lead towards developing suitable preventive strategies to counter the spread of this enteric pathogen in the community surrounding this backwater ecosystem.

Materials and Methods

Isolation and Identification of *Salmonella*

Ninety isolates of *Salmonella* were used in this study, of which 60 were *Salmonella* serotype Newport isolated from Cochin backwaters. Thirty clinical isolates of *Salmonella* serotype Typhi were provided by a local general hospital and private clinical laboratories. Environmental *Salmonella* Newport were isolated from water, shrimp and crabs following the method described in Bacteriological Analytical Manual, U.S. Food and Drug Administration [11]. Colonies typical of *Salmonella* were sub-cultured onto tryptic soya agar (TSA) slants and subjected to a series of biochemical tests for identification of *Salmonella* spp. The isolates were archived at −80°C in LB broth containing 15% glycerol. Serotyping of the isolates was done at the National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, India.

Virulence Genotyping of Isolates by PCR

The primers designed in this study for PCR detection of various virulence genes present on SPIs are listed in the Table 1. Pure genomic DNA from the isolates was extracted following the protocol of Ausubel et al. [1]. PCR amplifications of *invA*, *pefA*, *shdA*, *hilA, iroB, agfA, sopE*, *stn, ttrC, spi4D, pipA, spvC* and *spvR* were performed as described previously [27, 29, 35, 36]. In addition, primers were designed in this study for the amplification of *mgtB, ttrC, viaB, pagN, pipA, spi4R, spi4D, spiC*, *fliC, pefC, spvC, spvR, shdA* and *sopB* genes. The PCR cycling conditions consisted 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C. *S.* Typhimurium (ATCC14028) was used in *invA*, *hilA*, *spvR*, *iroB*, *agfA*, *shdA* and *stn* PCR assays, while *S.* Typhi (Presque Isle Cultures, Erie, PA) was used in *pefA* and *sopE* PCR as reference strains.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of *Salmonella* isolates of this study was determined by standard agar disc diffusion technique in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines [10] on Mueller Hinton agar using commercial discs (HiMedia, Mumbai, India). The antibiotics used were ampicillin (10 μg), amoxicillin (30 μg), bacitracin, cephalexin (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), nitrofurantoin (100 μg), norfloxacin (10 μg), penicillin G (10 U), streptomycin (10 μg), sulfamethoxazole (300 μg), tetracycline (30 μg), tobramycin (30 μg) and trimethoprim (10 μg).

Plasmid Extraction

For extraction of large plasmids associated with *Salmonella*, a previously described protocol was used [37]. *S.* Typhimurium 14028 was used as the positive control. The plasmids preparations were separated on a 0.7% agarose gel and visualized by staining with ethidium bromide.

Results and Discussion

The facultative intracellular bacterium *S. enterica* is the most diverse of all the Gram negative pathogenic bacteria considering its host range and the genetics of virulence. The virulence determinants of *S. enterica* are clustered into characteristic genomic regions known as SPIs (*Salmonella* pathogenicity islands), while certain genes located on large plasmids are also known to be important in the virulence of this bacterium [17, 22]. Variations reportedly exist among serotypes with respect to the presence or absence of SPIs, a feature predicted to be responsible for host adaptation, expansion of host range and differences in severity of infections by different serotypes [7, 12, 31].

Isolation of *Salmonella* and PCR Genotyping

A total of 60 *S.* Newport were isolated, of which 36 were from shrimp, 15 were from backwater and 9 were from crabs. All *S.* Newport isolates and 30 clinical isolates of *S.* Typhi were subjected to PCR genotyping. The results are

Table 1 Primers designed in this study for genotyping of *Salmonella enterica*

Target gene	Gene function/location on SPI	Primer sequence (5′–3′)	Coordinates	Product size (bp)
sopE	Effector/SPI-1	gcttcctcggtgaaagtgtg	514–534	200
		taactttatttccccataactttagaa	687–713	
spiC	Effector/SPI-2	ccgaaggtaatagccgatcc	47–67	334
		taccccacccgaataaagtt	362–381	
ttrC	SPI-2	tattcctgccgctgtttacc	299–318	241
		agtgcgaagaaggcctgtaa	520–540	
mgtB	Mg^{2+} uptake /SPI-3	ggcaggagtttcgcactaac	401–420	445
		gcgtacccacaatggatttc	825–846	
spi4R	Type I secretion/SPI-4	ttgtctctggccgtatttcc	965–985	461
		gcggttttaacgcgaaatta	1407–1426	
		gcgcttcggtttcatcttta	284–303	200
		cacaaatgctgatgacatgg	464–483	
Spi4D	Type I secretion/SPI-4	gttcatggtcagggcgttat	97–117	275
		cttaaagaacgggtgccatc	352–371	
sopB	Effector/SPI-5	cccagtgcttatgagggaaa	610–636	569
		gggcgtaaatcattgcctaa	1159–1179	
		gcatcagaaggcgtctaacc	192–211	225
		gtgcgtgctgcaataagttc	397–416	
pipA	SPI-5	ctcatgacagccttgccata	128–147	283
		tcctgagccacggtagtttc	391–410	
pagN	SPI-6	ttggatgcagcttttgtgtc	296–315	390
		aaggcgggcaggatattact	667–686	
		catcgcttcccttctggtaa	24–43	205
		aaagtcataaccgatagcaactcc	205–228	
viaB	Vi antigen/SPI-7	ttggctccggcttattagaa	80–99	460
		tgcaaacacatcagcgtaca	521–540	
		caacaagcagggatttttgc	689–708	238
		gcctggctatgcttaacgtc	907–926	
pefC	Plasmid-encoded fimbriae/Plasmid	acagtaaacgcccgtacctg	1577–1596	266
		gcatcatgttggtctggatg	1824–1843	
spvC	Plasmid-encoded virulence	aatttgccggtgacaagttc	296–316	235
		cgtgtcttgtggagaaacga	511–531	
spvR	Plasmid-encoded virulence regulator	gcagtgcgtgatctgttgat	573–593	202
		tttcatgaggggggctaaaaa	756–775	
shdA	Fibronectin-binding/Plasmid	cgtaccggcagaggtaatgt	637–656	291
		caacaaggcagtacgctgaa	908–927	
fliC	Flagellin/chromosomal	attgtccttatcggcaccag	448–467	492
		gccaacgacggtgaaactat	920–940	

presented in Table 2. In our study, analysis of clinical isolates of *S.* Typhi for genes located on seven different SPIs, plasmid-borne and chromosomally-encoded virulence genes revealed that the clinical isolates, by far, are homogenous. The virulence genes *invA*, *hilA* located on SPI-1 and other virulence genes not present on SPIs such as *stn*, *fliC*, *agfA* and *iroB* were detectable in all the isolates in concurrence with previous studies suggesting universal presence of these genes in *S. enterica* subspecies I [25].

spiC is one of the genes in *spiCAB* structural component of a type III secretion system encoded on SPI-2 that contributes for intracellular survival and replication of *Salmonella* [9], while the genes responsible for Mg^{2+} uptake designated as *mgt* are present on SPI-3 [4]. All *S.* Typhi isolates of our study harbored *spiC* and *mgtB* as evidenced by PCR results. *spi4R*, *sopB* and *pagN* represent SPIs IV, V and VI, respectively. Except one (NIO-C5), all isolates yielded positive PCR amplicons for *spi4R*, *sopB* and *pagN*.

Table 2 Distribution of various virulence genes in *Salmonella* Typhi (clinical isolates) and *Salmonella* Newport (backwater isolates) determined by PCR

Gene	Number (%) of isolates positive	
	Clinical ($n = 30$)	Back water ($n = 60$)
mgtB	30 (100)	60 (100)
viaB	22 (73.3)	40 (66.3)
pagN	29 (96.60)	59 (98.3)
spi4R	29 (96.6)	59 (98.3)
Spi4D	29 (96.6)	59 (98.3)
stn	30 (100)	60 (100)
sopB	29 (96.6)	59 (98.3)
pipA	29 (96.6)	59 (98.3)
iroB	30 (100)	59 (98.3)
fliC	30 (100)	60 (100)
agfA	30 (100)	60 (100)
spiC	30 (100)	59 (98.3)
ttrC	30 (100)	59 (98.3)
invA	30 (100)	60 (100)
hilA	30 (100)	60 (100)
pefA	0	0
pefC	0	0
sopE	12 (40)	30 (50%)
spvR	0	0
spvC	0	0
shdA	30 (100)	33(55)

In order to rule out the possibility of sequence variations leading to a negative PCR result or the isolate lacking a single gene within a pathogenicity island, we designed additional primers for *spi4R*, *sopB* and *pagN* (Table 1). We also tested this isolate for other genes associated with SPI-IV (*spi4D*) and SPI-V (*pipA*), and the isolate was found to be negative in all these PCR assays. Thus, the isolate (NIO-C5) appears to be deficient in genes of multiple SPIs and can make a good candidate for comparative studies involving genotyping and cells invasion assays. The target gene for SPI-7 was *viaB*, a component of cluster of genes encoding capsular polysaccharide. We targeted *viaB* because this locus is present in all Vi antigen-producing *S.* Typhi unlike the other genes such as *viaA* present in many serotypes of *Salmonella* and also in *E. coli* [32]. *viaB* was detectable in 22 (73.3%) of all the isolates tested using two sets of primers. SPI-7 is the largest of all the known SPIs and has been associated with serotypes causing systemic infections, though serotype Paratyphi lacks SPI-7 while still being able to cause systemic infection, thus casting doubt on the role of Vi antigen in typhoid fever [23].

The PCR results with environmental isolates, all belonging to *Salmonella* serotype Newport, was similar and comparable with clinical isolates except for *shdA*. As in clinical isolates, *mgtB*, *stn*, *fliC*, *agfA*, *invA* and *hilA* were detected in all of the 60 isolates. All isolates lacked plasmid-borne *pefA*, *pefC*, *spvC* and *spvR* genes. Human-adapted serotypes *S.* Typhi, Paratyphi A & B, and *Salmi-nella* Sendai are known to lack virulence plasmids and consequently the *spv* operon [5]. Our results demonstrate that *S.* Newport lack *spv* and *pef* genes. We analyzed all the isolates of this study for the presence of large plasmids by extracting plasmids from them. While the control strain *S.* Typhimurium showed the presence of a large plasmid ($\sim$90 kb), none of the *S.* Newport or *S.* Typhi showed the presence of plasmids. Thus, the absence of *spv* and *pef* genes may be correlated with the absence of large plasmids in these isolates.

Among the SPI-associated genes, variations were observed in the incidence of *viaB*, *sopE* and *shdA* (Table 2). *viaB* and *sopE* were detected in 66% and 50% of the isolates respectively. These results clearly indicate that irrespective of the source and serotypes, the occurrence of *sopE* and *viaB* in *Salmonella* is variable. However, a clear difference was noticed in the occurrence of *shdA* between *S.* Typhi and *S.* Newport. *shdA* was detected in all isolates of *S.* Typhi, but only in 50% of *S.* Newport isolates. One isolate (BW8) was negative for *spi4R*, *spi4D*, *sopB, pipA* and *pagN*, apart from being negative for *pefA*, *pefC*, *spvC* and *spvR*. Two other isolates that showed variable genotypes were BW20 ($iroB^{-}$, $spiC^{-}$, $ttrC^{-}$) and BW22 ($spiC^{-}$, $ttrC^{-}$) (data not shown). As in the case of *S.* Typhi, the absence of a particular gene was confirmed by using two separate primer pairs. In addition, the negative isolates were tested for additional genes of the respective pathogenicity island.

Eighteen isolates were positive for all virulence genes tested barring the plasmid-borne genes. In our study, the variations in genotypes were limited to, mainly, *viaB*, *sopE*, and *shdA*, all known to be highly unstable loci in the genome of *Salmonella*. Apart from these and the plasmid-borne genes, the isolates were positive for genes associated with PIs I-VI. *shdA* encodes a thin aggregative fimbrae required for prolonged fecal shedding of *Salmonella* [18, 19]. The ubiquitous presence of *shdA* in *S.* Typhi but not in *S.* Newport warrants further investigations on whether this gene contributes to any variation in the virulence between these two serotypes.

Antimicrobial Susceptibility

The clinical isolates of *S.* Typhi were sensitive to the commonly used drugs in the treatment of typhoid fever (data not shown). All clinical isolates were sensitive to tetracycline, quinolone and fluoroquinolone, β-lactam and the cephalosporin antibiotics. However, the antimicrobial resistance phenotypes of *S.* Newport were very different

Table 3 Antibiotic susceptibility data on *Salmonella* Newport isolated from the backwater environment

Antibiotic	No. (%) of resistant *Salmonella* isolates ($n = 60$)
Amoxicillin	15 (25)
Ampicillin	15 (25)
Bacitracin	60 (100)
Cephalexin	9 (15)
Cephalothin	9 (15)
Chloramphenicol	9 (15)
Ciprofloxacin	9 (15)
Erythromycin	30 (50%)
Gentamicin	0
Kanamycin	12 (20)
Nalidixic acid	14 (23.3)
Nitrofurantoin	11 (18.3)
Norfloxacin	3 (5)
Penicillin-G	60 (100)
Streptomycin	0
Tetracycline	14 (23.3)
Tobramycin	60 (100)
Trimethoprim	19 (31.6)

from *S.* Typhi (Table 3). The antibiotic susceptibility test revealed the presence of multi-drug resistant *S.* Newport in the backwater (Table 3). Fifteen isolates (25%) were resistant to ampicillin, 14 (23.3%) to tetracycline, 19 (31.6%) to trimethoprim/sulfamethoxazole, 9 (15%) to ciprofloxacin, 9 each (15%) to cephalothin and cephalexin, 14 (23.3%) to nalidixic acid and 9 (15%) were resistant to chloramphenicol. A study by the Centre for Disease Control and Prevention reported emergence of Newport-MDRAmpC strains resistant ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline [8]. Seven isolates in our study were resistant to ampicillin, chloramphenicol, sulfamethoxazole and tetracycline, but not to streptomycin. Of these, one each was resistant to additional antibiotics kanamycin or norfloxacin. Interestingly, similar antibiotic resistance phenotype was not found among clinical *S.* Typhi that were resistant only to erythromycin and bacitracin.

Our study is limited to detecting few genes associated with a specific SPI and thus does not explore the possibility of variations within the SPIs. Such variations in SPIs caused by insertions or deletions of specific genes may be important determinants of virulence and host ranges of *Salmonella* serotypes. Our future study will focus on elucidating such variations within individual SPIs in *S.* Newport and *S.* Typhi. Put together, the results of our study show that *S.* Newport distributed in the environment have similar and comparable virulence genotypes to clinical *S.* Typhi. However, this observation is based on the distribution of virulence genes known to contribute to the pathogenicity of *S. enterica*. Comparison of whole genome sequences has identified 11% of the *S.* Typhimurium LT2 genes absent from the whole genome of *S.* Typhi [24]. In addition, more than 300 genes confined to the *S. enterica* subspecies I are predicted to have roles in the host specificity and pathogenicity of *Salmonella* [24]. Thus, the whole genome comparison using microarray technique is necessary to fully identify variations in gene contents, followed by animal studies to correlate these genetic variations with the virulence phenotype.

In developed countries where scientific monitoring and record keeping is followed, the morbidity due to emerging antibiotic resistant strains and the virulent types is well understood. According to one estimate, Newport MDR-AmpC strains were responsible for >2% of the 1 million cases of salmonellosis in 2001 in the U.S [15]. Studies from India have reported the isolation of multidrug-resistant *S.* Newport from water, animals, fish and vegetables [20, 33, 34]. Though epidemiological data on *S.* Newport infections in India are lacking, cases of neonatal meningitis and nursery outbreaks with *S.* Newport have been documented [21, 30]. The high prevalence of multiple drug resistant *Salmonella* in the environment as revealed by this study definitely represents significant disease burden in the region under study. Thus, future epidemiological studies in this region need to focus on the contribution of non-typhoidal *Salmonella* to the morbidity and mortality, sources of contamination and the transmission cycle to contain the endemicity of *Salmonella* infections.

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