Distribution of *Salmonella* pathogenicity island (SPI)-8 and SPI-10 among different serotypes of *Salmonella*

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Many virulence phenotypes of *Salmonella enterica* are encoded by genes located on pathogenicity islands. Based on genome analysis, it is predicted that *Salmonella* pathogenicity island (SPI)-8 is restricted to *Salmonella* serovars Typhi and Paratyphi A, and SPI-10 to *Salmonella* serovars Typhi, Paratyphi, Enteritidis, Dublin and Gallinarum. This study was conducted to investigate the distribution of SPI-8 and SPI-10 among *Salmonella* isolates from sprouts, fish, water and blood. A total of 110 *Salmonella* isolates and 6 *Salmonella* serovars from the Microbial Type Culture Collection, Chandigarh, India, were screened. All isolates belonging to *Salmonella* serovars Washington, Enteritidis and Paratyphi A had both SPI-8 and SPI-10. All *Salmonella* serovar Typhi isolates from water and blood had both SPI-8 and SPI-10, whereas isolates from fish contained only SPI-8. SPI-8 and SPI-10 were also detected in only 3 out of 42 isolates belonging to *Salmonella* serovar Typhimurium. Both SPI-8 and SPI-10 were absent in *Salmonella* serovars Worthington, Dublin, Paratyphi B and Paratyphi C. These results contradict the predictions from *Salmonella* genome sequences available in GenBank and indicate that SPI-8 and SPI-10 are widely distributed among *Salmonella* serovars and that virulence factors other than those on SPI-8 and SPI-10 may be responsible for host specificity. This is the first report on the distribution of SPIs in *Salmonella* isolates from India.

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INTRODUCTION

The genus *Salmonella* contains pathogens that are closely related genetically but differ in their host range (Baumler *et al.*, 1998). *Salmonella enterica* contains more than 2500 serovars. Serovar Typhimurium causes systemic disease in mice, but may colonize other species such as pigs, poultry, horses, cattle, and sheep, and can cause gastroenteritis in humans, whereas serovar Typhi is a prototypical host-restricted serovar that causes typhoid fever in humans and higher primates, but is unable to produce illness in other vertebrate species (Townsend *et al.*, 2001). Large numbers of *Salmonella* outbreaks have been reported as a result of the consumption of fresh, minimally processed foods, poultry meat, contaminated water and undercooked eggs (Baird-Parker, 1990; Lynch *et al.*, 2006; Plym-Forshell & Wierup, 2006). *Salmonella* contamination of different food products

such as fish, vegetables, fruit and sprouts has been reported in India (Bandekar *et al.*, 2004; Dhokane *et al.*, 2006; Saroj *et al.*, 2006).

Many of the virulence phenotypes of *S. enterica* are encoded by genes on pathogenicity-associated islands (PAIs). PAIs consist of large regions of genomic DNA (approx. 10–200 kb) that are present in pathogenic bacterial strains but absent from the genomes of non-pathogenic members of the same or related species (Wilson *et al.*, 2002). PAIs are genetic elements on the chromosomes of a large number of pathogens and are considered to produce quantum leaps in bacterial evolution (Groisman & Ochman, 1996; Schmidt & Hensel, 2004). The acquisition of PAIs by horizontal gene transfer enables bacteria to gain complex virulence functions rapidly from other species. PAIs on *Salmonella* are referred to as *Salmonella* pathogenicity islands (SPIs). At present, 17 different SPIs have been described that encode the most prominent virulence phenotypes, i.e. host-cell invasion and intracellular pathogenesis (Hensel, 2004; Chiu *et al.*, 2005; Vernikos & Parkhill, 2006).

Based on the genomic data available, Hensel (2004) predicted that SPI-8 would be restricted to *Salmonella* serovar Typhi and SPI-10 to serovars Typhi and Enteritidis. Whole-genome analysis has shown the presence of SPI-8 in serovars Choleraesuis and Paratyphi A as well as in serovar

Abbreviations: PAI, pathogenicity-associated island; SPI, *Salmonella* pathogenicity island.

The GenBank/EMBL/DDBJ accession numbers for the SPI-8 and SPI-10 sequences of the *Salmonella* isolates determined in this study are EF033079–EF033108.

A table of sequences of the primers used in this study and figures showing DNA dot blots for SPI-8 and SPI-10 and the molecular typing of *Salmonella* serovars are available as supplementary material with the online version of this paper.

Typhi. In addition, a BLAST search across the current GenBank database has revealed that SPI-10 is present in *Salmonella* serovars Typhi, Paratyphi A, Enteritidis, Dublin and Gallinarum. The identification and distribution of PAIs in different *Salmonella* serovars is essential in understanding the development of disease and the evolution of bacterial pathogenesis. Although *Salmonella* infections are endemic in India, no database on genotypic characteristics of *Salmonella* isolates from India exists. Therefore, detailed characterization of these food isolates with respect to their pathogenicity genes is vital from a public health point of view.

The purpose of this study was to characterize *Salmonella* isolates from sprouts, fish, water and blood with respect to pathogenicity-related genes on SPI-8 and SPI-10.

METHODS

Bacterial strains. All standard cultures were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. *Salmonella* serovar Typhimurium MTCC 98, *Salmonella* serovar Typhi, *Salmonella* serovar Paratyphi A, *Salmonella* serovar Paratyphi B and *Escherichia coli* ATCC 35218 were used to standardize PCR conditions. *E. coli* ATCC 35218, *Salmonella* serovar Bovismorbificans MTCC 1162, *Salmonella* serovar Brunei MTCC 1168, *Salmonella enterica* subsp. *arizonae* MTCC 660, *Salmonella* serovar Virchow MTCC 1163, *Salmonella* serovar Weltevreden MTCC 1169 and 110 *Salmonella* isolates (18 blood, 39 water, 31 fish and 22 sprout) were used for screening of SPI-8 and SPI-10. *Salmonella* isolates from sprouts and fish were from India, whilst water and blood isolates were from Nepal (Bhatta *et al.*, 2007).

Serotyping of the isolates. Antisera (SIFIN) against *Salmonella* serovars Typhi, Paratyphi A, Enteritidis and Typhimurium were used for serotyping as instructed by the manufacturer.

Molecular typing of *Salmonella* isolates. Multiplex PCR was carried out using primers for the *tyv*, *fliC-d* and *viaB* genes as described by Hirose *et al.* (2002) for identification of *Salmonella* serovar Typhi. To identify *Salmonella* serovar Enteritidis, PCR was carried out using *sdfI* primers as described by Agron *et al.* (2001).

DNA amplification. Gene sequences for SPI-8 and SPI-10 were obtained from GenBank. Regions unique to *Salmonella* were determined using a BLAST search. Primers were designed using vector NTI software (Infor Max). The primer sequences are given in Supplementary Table S1 (available in JMM Online). The PCR was carried out in a 25 µl volume containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 1 µl primer pairs (10 pmol each), 0.5 µl (1.5 U) *Taq* polymerase, 0.2 mM dNTPs (Bangalore Genie) and 1 µl genomic DNA (200 pg). The PCR was carried out under the following conditions: 30 cycles with heat denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s and DNA extension at 72 °C for 2 min, with a 10 min final extension at 72 °C, using a programmable thermal controller (Eppendorf AG). The amplified DNA was separated by 1.5 % agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination. Sequencing of amplified products was carried out at Bangalore Genie.

RESULTS AND DISCUSSION

SPI-8 is a 6.8 kb region located adjacent to the *pheV* tRNA gene and SPI-10 is a large insertion of 32.8 kb located at

tRNA *leuX* on the genome of *Salmonella* serovar Typhi (Hensel, 2004). The regions specific for *Salmonella* were determined using BLASTN at the National Center for Biotechnology Information website (www.ncbi.nih.gov/BLAST/). Two different sets of primers for SPI-8 (bacteriocin pseudogenes) and three different sets of primers for SPI-10 (*Salmonella sefC*, *sefR* and *sefB*) were designed using these regions. The primers were specific for SPI-8 and SPI-10; there was no non-specific amplification when tested with *E. coli*. DNA products of 132 and 142 bp were obtained for SPI-8 and of 668, 625 and 535 bp for SPI-10. The possibility of false negatives was ruled out using a DNA dot-blot assay and that of false positives by sequencing of the PCR-amplified products. The results of the DNA dot blot (Supplementary Fig. S1, available in JMM Online) and sequencing of the PCR-amplified products of both SPI-8 and SPI-10 were in accordance with the PCR results. The serotypes of the isolates that showed the presence/absence of SPI-8 and SPI-10 deviating from the BLAST results from GenBank were further confirmed by in-house serotyping using antisera from Germany and by molecular typing (Supplementary Fig. S2 in JMM Online). Based on hybridization studies and comparison of known DNA sequences, the mean DNA sequence similarity among *Salmonella* serovars is between 96 and 99 % (Edwards *et al.*, 2002). One of the prevailing questions in *Salmonella* research today concerns the identification of genetic factors that confer on different *Salmonella* serovars their ability to colonize and in some cases to cause disease in a wide variety of animal hosts (Chan *et al.*, 2003). The pattern of the presence or absence of virulence genes had been used to identify and characterize bacterial isolates using DNA and protein microarrays (Al-Khaldi & Mossoba, 2004). The main difference between non-pathogenic and pathogenic bacteria of the same species or closely related species is the presence of additional genes in the pathogenic bacteria (Oelschlaeger & Hacker, 2004). Virulence functions encoded by certain PAIs are lost with a frequency that is higher than the normal rate of mutation. Genetic analyses have shown that such mutations are caused not by defects in individual virulence genes within the PAI, but rather by loss of large regions of the PAI or even the entire PAI (Schmidt & Hensel, 2004). Therefore, to get information on the complete PAI, two different regions of SPI-8 and three different regions of SPI-10 were analysed by PCR.

SPI-8 was found to be present in all *Salmonella* isolates belonging to serovars Typhi, Enteritidis, Washington and Paratyphi A, and was absent in serovars Paratyphi B, Paratyphi C, Worthington and Dublin. It was absent in *Salmonella* serovar Typhimurium isolated from sprouts and fish, but was present in three out of 14 isolates from water (Table 1). These results contradict the predictions from the available genomic data, which indicated that SPI-8 would be present only in serovars Typhi and Paratyphi A and not in serovars Enteritidis and Typhimurium.

Townsend *et al.* (2001) have shown that the distribution of SPI-10 is restricted to *Salmonella* serovars Typhi, Paratyphi A,

Table 1. Distribution of SPI-8 and SPI-10 among *Salmonella* isolates

Source	<i>Salmonella</i> serovar*	SPI-8 amplicons		SPI-10 amplicons		
		a	b	a	b	c
MTCC	Typhimurium MTCC 98	—	—	—	—	—
MTCC	Paratyphi A MTCC 735	+	+	+	+	+
MTCC	Bovismorbificans MTCC 1162	—	—	—	—	—
MTCC	Brunei MTCC 1168	—	—	—	—	—
MTCC	<i>S. enterica</i> subsp. <i>arizonae</i> MTCC 660	—	—	—	—	—
MTCC	Virchow MTCC 1163	—	—	—	—	—
MTCC	Weltevreden MTCC 1169	—	—	—	—	—
Water/blood	Typhi (15/14)	+	+	+	+	+
Fish	Typhi (12)	+	+	—	—	—
Water/fish/sprouts	Typhimurium (11/6/22)	—	—	—	—	—
Water	Typhimurium (3)	+	+	+	+	+
Water/blood	Paratyphi A (6/2)	+	+	+	+	+
Blood	Paratyphi B (1)	—	—	—	—	—
Blood	Paratyphi C (1)	—	—	—	—	—
Fish	Worthington (12)	—	—	—	—	—
Water	Enteritidis (3)	+	+	+	+	+
Sprouts	Dublin (1)	—	—	—	—	—
Sprouts	Washington (1)	+	+	+	+	+

*The numbers of serovars from the respective sources are shown in parentheses.

Dublin, Enteritidis and Gallinarum. It has been predicted that the fimbrial operon on SPI-10 may play a role in determining host specificity for *Salmonella* serovar Typhi. However, our studies demonstrated that it was also present in *Salmonella* serovars Typhimurium and Washington. It was present in all isolates belonging to *Salmonella* serovars Typhi and Paratyphi A except for serovar Typhi isolated from fish (Table 1), and was absent in *Salmonella* serovars Worthington, Dublin, Paratyphi B and Paratyphi C. Isolates belonging to *Salmonella* serovar Typhimurium obtained from sprouts and fish were negative for SPI-10, but isolates from water contained SPI-10. *Salmonella* isolates belonging to serovar Enteritidis isolated from water were also positive. Both SPI-8 and SPI-10 were absent in *Salmonella* serovars Typhimurium MTCC 98, Bovismorbificans MTCC 1162, Brunei MTCC 1168, Virchow MTCC 1163 and Weltevreden MTCC 1169 and in *S. enterica* subsp. *arizonae* MTCC 660.

Interestingly, *Salmonella* serovar Typhi isolated from fish only possessed SPI-8, whilst all other *Salmonella* serovar Typhi were positive for both of the PAIs as predicted. However, three *Salmonella* serovar Typhimurium, three *Salmonella* serovar Enteritidis and one *Salmonella* serovar Washington were positive for both SPI-8 and SPI-10. A significant observation was that none of the isolates was negative for SPI-8 and positive for SPI-10, giving a strong indication that whenever SPI-10 occurs, SPI-8 is also present. Genotyping data by PFGE analysis has shown that *Salmonella* serovar Typhimurium isolates with differences in PAI distribution are from different clones (Saroj *et al.*, 2008).

SPI-8 and SPI-10 were detected in food (sprouts) and environmental (water) isolates in addition to the clinical (blood) isolates, demonstrating the wide distribution of SPI-8 and SPI-10 among *Salmonella* serovars. This could be due to horizontal gene transfer taking place in *Salmonella*. Acquisition of virulence genes by horizontal gene transfer in *Salmonella* has contributed to the emergence of new pathovars and the acquisition of factors that extend their host range (Rabsch *et al.*, 2002). Serovars that showed the absence of SPI-8 were negative for both of the regions examined, whilst those that showed the absence of SPI-10 were negative for all three regions examined. Serovars showing the presence of SPI-8 and SPI-10 were positive for all of the regions examined. In addition, sequencing of PCR products from *Salmonella* serovars Typhi, Typhimurium, Paratyphi A, Washington and Enteritidis show that these regions are highly conserved (100% identity). This observation is particularly significant as these regions (SPI-8 and SPI-10) were conserved among the *Salmonella* isolates from India, isolates from the distant geographical region of Nepal and also in the sequenced *Salmonella* serovars. This clearly suggests that these PAIs may have been acquired before *Salmonella* species evolved into different serovars. Gain or loss of these islands depends on the environmental niche occupied, as three isolates belonging to *Salmonella* serovar Typhimurium obtained from water showed the presence of both islands, whilst in other isolates these regions were absent. Similarly, *Salmonella* serovar Typhi isolated from fish has lost SPI-10.

The presence and absence of SPI-8 and SPI-10 in *Salmonella* serovar Typhimurium, Washington and Typhi

(from fish) did not correlate with the expected results from the genome sequence data available in GenBank. To check the authenticity of these serovars, serotyping results were confirmed using antisera from Germany and molecular serotyping was carried out as described by Hirose *et al.* (2002) and Agron *et al.* (2001).

The results matched the previous serotyping results. None of the Typhimurium or Washington isolates reacted with antisera against Typhi, and PCR results correlated well with the serology (Supplementary Fig. S2).

Genome-based studies have uncovered a set of *Salmonella* genes that is restricted among these organisms and which may be important in deciding their host specificities. However, their predictions regarding the distribution of SPI-8 and SPI-10 are incorrect. Our results showed that SPI-8 and SPI-10 are widely distributed among *Salmonella* serovars. The exact role of each of these SPIs and their distribution need to be studied further to understand the host specificity and invasiveness of *Salmonella* serovars. Comparative genomics requires input from multiple genomic sequences; however, complete genome sequences of only a few bacteria have been completed to date. Therefore, there is a need to conduct laboratory experiments to support predictions made by genome database analysis and routine characterization of food and environmental isolates for pathogenicity-related genes from a public health point of view.

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