STATUS OF Vi GENE, ITS EXPRESSION AND SALMONELLA PATHOGENICITY ISLAND (SPI-7) IN *SALMONELLA* Typhi IN INDIA

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Abstract. *Salmonella enterica* serotype Typhi (*S*. Typhi) is a causative organism of typhoid fever. A number of Salmonella serovars express a capsular polysaccharide antigen known as Vi, the biosynthetic and export proteins of which are encoded within the *viaB* locus of Salmonella Pathogenicity Island -7 (SPI-7). SPI-7 is inserted between two partially duplicated copies of tRNA -*pheU* gene. We have investigated the frequency of *viaB* operon deletion and loss of SPI-7 due to storage of strains collected during the period 1987-2006 by PCR amplification of *fli*C (for confirmation of serotype Typhi), *tviB* (for status of *viaB* operon) and tRNA -*pheU* (for absence of SPI-7). All 111 isolates were observed with positive amplification of 495 bp amplicon for *fliC*. A total of 36 isolates were negative for Vi by agglutination while 39 were negative for *viaB* operon. Interestingly, 106 isolates were found to have SPI-7. The 5 SPI-7 negative isolates were isolated during recent years. Long-term storage and repeated culture had little or no effect on SPI-7, as none of the 18 isolates recovered from blood before 1997 lacked SPI-7. On the other hand, loss of *viaB* operon was directly proportional to duration of storage. Thus, it is proposed that stability of Vi gene is dependent on the presence of selection pressure.

Key words: Salmonella Pathogenicity Island, virulence, *Salmonella* Typhi, *viaB* operon, serotype, *fliC*, Vi agglutination

INTRODUCTION

Salmonella enterica subspecies *enterica* serotype Typhi (*S*. Typhi) is one of the most important causative organisms of enteric fever. Unlike most of the other serovars of *Salmonella*, this serotype is human restricted and expresses a capsular polysaccharide (Vi) antigen. Apart from this, serotypes Paratyphi C and Dublin of *Salmonella enterica* and *Citrobacter freundii* also produce Vi antigen. These Vi- producing bacteria also possess Salmonella Pathogenicity Island-7 (SPI-7), which is possibly an unstable and mobile region in *Salmonella* serovar Typhi genome (Nair *et al*, 2004).

The Vi biosynthetic and export proteins are encoded within the *viaB* locus, a region of DNA located within SPI-7 DNA island. SPI-7 is inserted in between two partially duplicated copies of the tRNA *pheU* gene located at positions 4409511 and 4543074, respectively in the serovar Typhi

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CT18 genome sequence (Pickard *et al*, 2003). Other than *viaB* locus, SPI-7 encodes genes that are responsible for several pathogenic traits, including a gene for type IV pilus implicated in adherence to eukaryotic cells, and *sopE* prophage gene for encoding an effecter protein within its tail fiber, contributing to diversity in *S. enterica* serovars (Thomson *et al*, 2004). The *viaB* region in serovar Typhi consists of 10 genes namely *tviA*, *tviB*, *tviC*, *tviD*, and *tviE* that are involved in synthesis of the capsule and *vexA*, *vexB*, *vexC*, *vexD* and *vexE* encoding proteins for capsule export (Virlogeux *et al*, 1995). In addition, *rcsB* and *rcsC* (*viaA*) and the *ompR-envZ*, a 2 component regulatory system, regulate the production of the Vi polysaccharide (Pickard *et al*, 1994). There are reports that show insertion and deletion mutations in the *tviA* gene, can lead to the loss of Vi expression and an increase in invasive properties (Zhao *et al*, 2001). Furthermore, spontaneous loss of the whole of SPI-7 in Vi-negative mutants has been reported in stored isolates (Nair *et al*, 2004) and precise excision of SPI-7 has also been demonstrated (Bueno *et al*, 2004).

Vi capsule, as such, is not essential for infection, as Vi negative *S*. Typhi mutants are able to establish infection and cause typhoid - like illness in humans (Hornick *et al*, 1970; Hone *et al*, 1988; Saha *et al*, 2000). But *in vitro* studies have shown that Vi is antiopsonic and antiphagocytic, increases the level of resistance of the organism to oxidative killing (Looney and Steigbigel, 1986) and reduces the level of secretion of serovar Typhi induced tumor necrosis factor alpha (TNFα) by human macrophages (Hirose *et al*, 1997). There are reports showing detection of Vi agglutination- negative clinical Typhi isolates from preserved cultures from several countries, including India and Malaysia (Jegathesan, 1983; Mehta and Arya, 2002). A report from Pakistan showed that there is only one out of total 2,221 Typhi isolates stored strains negative for *viaB* operon (Wain *et al*, 2005). Another report from the same country later stated that approximately 10% of the typhoid fever cases were observed due to Vi gene-negative strains of serotype Typhi (Baker *et al*, 2005).

Here, we have determined the effect of storage on frequency of deletion of *viaB* operon alone and of the whole SPI-7 region. Since typhoid fever continues to be a major problem in developing countries, the present findings may provide an insight into the possible role of Vi vaccination in the prevention of typhoid fever.

MATERRIALS AND METHODS

Bacterial strains

Salmonella Typhi strains were isolated from blood during 1987 - 2007 in the Microbiology Laboratory of a tertiary level hospital of eastern part of North India. Individuals yielding positive for the growth of *S.* Typhi were clinically diagnosed with typhoid fever and none of them had prior vaccination for typhoid. Isolates were identified by conventional biochemical and serological test (Ewing, 1986). These strains, positive for Vi agglutination, were stored on peptone agar slopes and kept at room temperature.

DNA extraction and amplification of genes from *Salmonella* Typhi strains

Genomic DNA of the *S*. Typhi isolates was extracted and purified using proteinase K and standard phenol-chloroform method (Ho *et al*, 1995). Table 1 shows the primers used for the detection of *fli*C, *tviA* and SPI-7 including the approximate sizes of the desired amplicons and annealing temperatures for the different primer sets.

Table 1
Primers used for the amplification of *fliC*, *tviA* and SPI-7.

Interpretation if amplicon detected	Target gene/ annealing temperature	Sequence (5′-3′)	Amplicon size (bp)	Reference
Salmonella Typhi DNA present	*fliC*/52ºC	ST15′-TATGCCGCTACATATGATGAG-3′ ST2 5′-TTAACGCAGTAAAGAGAG-3′	495	Song *et al*, 1993
Gene for Vi production present	*tviA*/57ºC	V1 5′-GTTATTTCAGCATAAGGAG-3′ V2 5′-ACTTGTCCGTGTTTTACTC-3′	599	Hashimoto and Khan, 1997
SPI-7 absent	$tRNA^{leu}$/57ºC	DE0032-F-5′-GCTCAGTCGGTAGAGCAGGGGATT-3′ DE0083-R5′-TCATCTTCAGGACGGCAGGTAGAATG-3′	1,275	Pickard *et al*, 1994

PCR amplification was carried out in 50 µl volume using 10 ng of DNA, 1U of Taq DNA polymerase (Banglore, Genei, India), 10 pmol of each primer (Quiagen operon, Cologne, Germany), 0.25 mM of each deoxynucleotide triphosphate (MBI, Fermentas) and 2 mM $MgCl_2$ in standard PCR buffer. The reaction mixture was subjected to 30 cycles of melting at 92ºC for 30 seconds, appropriate annealing temperature for 1 minute and extension at 72ºC for 30 seconds. Initial denaturation was carried out at 92ºC for 5 minutes while final extension at 72ºC for 7 minutes. The PCR products were visualized by ethidium bromide staining after electrophoresis in 1% agarose gel.

RESULTS

Phenotypic identification and genotypic confirmation of serovar Typhi

Phenotypic identification of serovar Typhi was performed by standard laboratory procedures, *viz* fermentation of glucose without gas production, non fermentation of lactose, H_2S production on triple sugar iron (TSI) medium, and non utilization of citrate, and by serological positivity with O9 and Hd antisera. One hundred eleven phenotypically identified *S.* Typhi isolates were confirmed genotypically by PCR using *fli*C gene specific primers. All isolates were observed positive for 495 bp amplicon (see Fig 1 for typical results). The reference strain MTCC 3216 was used as positive control.

Vi agglutination

Of the 111 isolates subjected to Vi agglutination, 36 were found to be negative. Two strains isolated before 1992 were negative for agglutination (Table 2). Of the 16 strains isolated during 1992-1996, 8 (50%) were agglutination negative with

Table 2
Status of agglutinability, *tviA* and SPI-7 in *Salmonella* Typhi isolates.

Year	Number of strains	Vi agglutination		*tviA* PCR		SPI-7	
		+ve	-ve	+ve	-ve	+ve	-ve
1987-1991	2	0 (0)	2 (100)	0 (0)	2 (100)	2 (100)	0 (0)
1992-1996	16	8 (50)	8 (50)	10 (62)	6 (37)	16 (100)	0 (0)
1997-2001	61	40 (66)	21 (34)	38 (62)	23 (38)	58 (95)	3 (5)
2002-2006	32	27 (84)	5 (16)	24 (75)	8 (25)	30 (95)	2 (5)
Total	111	75 (68)	36 (32)	72 (65)	39 (34)	106 (95)	5 (5)

Percent is given in parenthesis.

Vi antisera. The percentage of strains negative for Vi agglutination decreased to 34% (21/61) in those isolated during 1997-2001. Only 5 of the 32 (16%) strains isolated during 2002-2007 were agglutination negative with the Vi antisera.

PCR detection for *viaB* operon

Seventy-two of 111 strains were positive for presence of the *tviA* sequence while 39 were negative (see Fig 2 for typical results). Both strains isolated during 1987-1991 that were negative for agglutination, were also negative for 599 bp amplicon (Table 2). Six of the strains isolated during 1992-1996 and 23 of that isolated during 1997-2001, were observed with no amplification for the *tviA*. During 2002-2006, eight of the isolates did not show amplicon for *tviA.*

Detection of SPI-7

Using tRNA*leu* gene target to amplify 1,276 bp amplicon no band was obtained in amplification of 106 isolates indicating presence of SPI-7 sequence. Only 5 strains were found to give the expected amplicon (see Fig 3 for typical results). These isolates were isolated during recent years (Table 2).

DISCUSSION

We have observed that of the 75 *Salmonella* serovar Typhi strains able to express the Vi capsular antigen, 65 were positive for both *tviA* and SPI-7 while 10 were negative for *tviA* but positive for SPI-7. The properties of the latter 10 strains may be explained on the basis of mutation at the annealing sites of one or both of the primers used in the study. Obviously, these mutations could not change the function of the expressed protein. As Baker *et al* (2005) have shown that strain negative for *tviA*, was positive for the other component genes of *viaB* operon, we did not look for other genes of *viaB* operon.

There were 36 strains negative for Vi agglutination. It is worth mentioning that the longer the duration of storage, the more likely are the strains to loose the agglutination positivity with Vi antisera. It means storage definitely leads to withdrawal of selection pressure for Vi gene which tend to be present *in vivo*. However, it is difficult to explain why in a majority of the strains the *viaB* locus remained intact though stored in similar *in vitro* conditions. It is interesting to note that 7 of

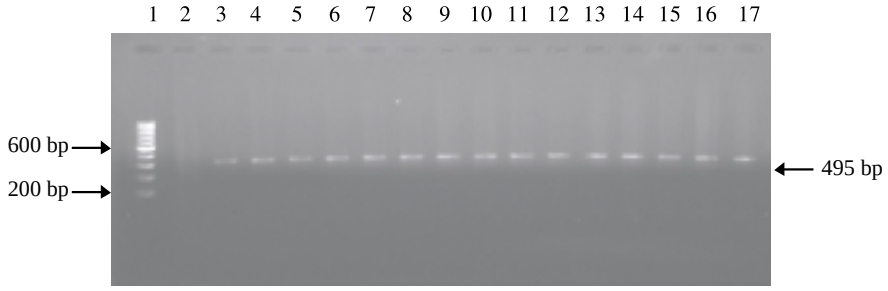

Fig 1–Amplicon of *Salmonella* Typhi flagellin (*fliC)* gene. Lane1, molecular marker; lane 2, -ve control; lane 3, +ve control (*Salmonella* Typhi, MTCC 3216); lanes 4-17: *S.* Typhi showing 495 bp amplicon of *fliC* [primers used for flagellin (*fliC*) gene of *Salmonella* Typhi: ST1 F 5′-TATGCCGCTACATATGATGAG-3′ and ST2 R 5′-TTAACGCAGTAAAGAGAG-3′, annealing temperature 57ºC, cycles 40, expected amplicon size 495 bp].

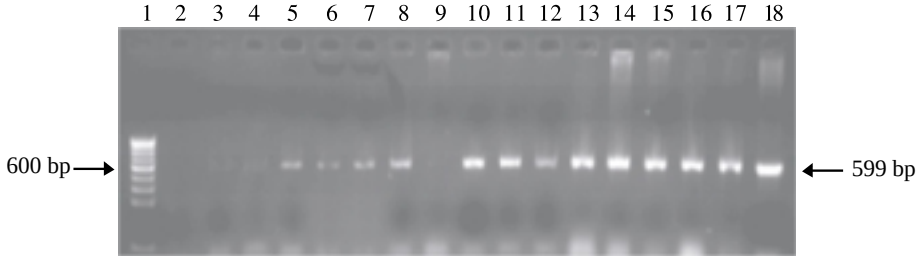

Fig 2–Amplicon of *Salmonella* Typhi *tvi*A gene. Lane 1, molecular marker; lane 2, -ve control; lane 18, + ve control (*Salmonella* Typhi, MTCC 3216); lanes 5,6-8,10-17, *S.*Typhi showing 599bp amplicon *viaB;* lane 3,4,9, -ve for 599bp amplicon (primers used *tvi*A of *Salmonella* Typhi V1 F5′ -GTTATTTCAGCATAAGGAG-3′, V2 R-5′ ACTTGTCCGTGTTTTACTC-3′, annealing temperature 56ºC, cycles 35, expected amplicon size 599 bp).

the 36 agglutination-negative isolates were positive for the presence of both *tviA* gene and SPI-7 DNA segment. Down regulation of *viaB* operon may be a possible explanation for the above observation.

There were 5 isolates negative for both SPI-7 and *tviA*. As, none of the 18 isolates recovered from blood before 1997 was found to be without SPI-7 while the entire 5 isolates negative for SPI-7 were isolated after 1996, this suggested that long term storage and repeated cultures have little or no effect on the maintenance of SPI-7. Baker *et al* (2005) have observed that 10% of blood specimens from typhoid patients do not give positive amplification for *tviA* or *tviB* genes in an under- developed country of Asia , confirming the circulation of Vi negative strains in peripheral blood of the typhoid patients. In India, 10% of freshly isolated strains were also found to be negative for Vi agglutination (Mehta and Arya, 2002). Detailed genetic study is warranted on these 5 strains to determine

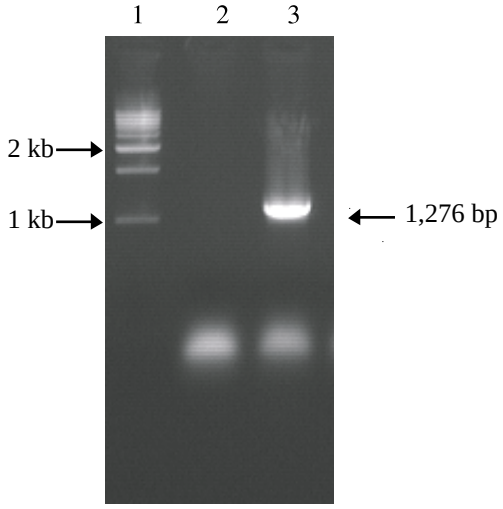

Fig 3–Amplicon of *Salmonella* Typhi tRNA-^{leu} gene. Lane 1, 1kb size marker; lane 2, -ve control; lane 3 showing 1,276 bp amplicon of tRNA^{leu} gene (Amplification : primers used to see the absence of SPI-7 pathogenicity island in *Salmonella* Typhi targeting tRNA-^{leu} DE0032-F 5′ GCTCAGTCGGTAGAGCAGGGG ATT-3′ DE0083-R 5′- TCATCTTCAGG ACGG CAGG TAGAATG-3′, annealing temperature 60°C, cycles 30, expected amplicon size 1,276 bp).

if excision of the whole SPI-7 has definitely occurred. Deletion, insertion or rearrangement of all or part of *viaB* locus may be possible explanations for the 29 strains in which SPI-7 was present but not *tviA*.

It has been suggested by Baker *et al* (2005) that under proper environmental conditions, loss of an operon (*viaB*) of 15kb is much more likely than the loss of whole pathogenicity island (SPI-7) of 134 kb. Our finding of enhanced loss *viaB* operon due to storage appears to be in agreement with this suggestion. Vi-negative serotype Typhi has been reported causing typhoid fever in Kolkata, India (Saha *et al*, 2000). Moreover, the other human restricted serovars, Paratyphi A and Sendai, both inducing a disease clinically indistinguishable from typhoid, are devoid of Vi polysaccharide production. Further, it is worth noting that almost all the chronic typhoid carrier patients have been observed with elevated titer of antibody against Vi antigen, while only 20% of the patient suffering from acute typhoid fever were observed with raised titer of the antibody (Lanata *et al* (1983). We have observed recently that Typhi serotype in chronic typhoid carriers primarily resided in the liver but not in the gallbladder (unpublished data). This intracellular niche of serotype Typhi might be putting selection pressure for the maintenance of *viaB* locus in the bacteria. This hypothesis is further supported by Zhao *et al* (2001) who reported that suppression of Vi expression leads to enhanced invasion in the intestine and destruction in Peyer's patches while enhanced Vi expression leads to decrease invasion by Typhi serotype.

If it is proven that *viaB* locus is essential for the maintenance of the bacterium in a chronic carrier state, then use of Vi vaccine to eradicate Vi positive *S*. Typhi from the community seems logical. However, use of a vaccine giving rise to anti-Vi immunity will not select out Vi negative strains of serotype Typhi. Thus the most important source of typhoid infection may be eliminated. Nevertheless, it seems prudent to know the cause and implications of deletion of *viaB* locus with or without SPI-7.

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REFERENCES

Baker S, Yasra S, Hafsa A, *et al*. Detection of Vi-negative *Salmonella enterica* serovar Typhi in the peripheral blood of patients with typhoid fever in the Faisalabad Region of Pakistan. *J Clin Microbiol* 2005; 43: 4418-25.

Bueno SM, Santiviago CA, Murillo AA, *et al*. Precise excision of the large pathogenicity island, SPI7, in *Salmonella enterica* serovar Typhi. *J Bacteriol* 2004; 186: 3202-13.

Ewing WH. Edwards and Ewing's identification of Enterobacteriaceae. 4th ed. New York: Elsevier Science, 1986: 81-318.

Hashimoto Y, Khan AQ. Comparison of via regions of Vi positive organisms. *FEMS Microbiol Lett* 1997; 157: 55-7.

Hirose K, Ezaki T, Miyake M, *et al*. Survival of Vi-capsulated and Vi-deleted *Salmonella typhus* strains in cultured macrophage expressing different levels of CD14 antigen. *FEMS Microbiol Lett* 1997; 147: 259-65.

Ho CL, Phang, SM, Pang T. Molecular characterization of *Sargassum polycystum* and *S. siliquosum* (Phaeophyta) by polymerase chain reaction (PCR) using random amplified polymorphic DNA (RAPD) primers. *J Appl Phycol* 1995; 7: 33-41.

Hone DM, Attridge SR, Forrest B, *et al*. A *galE via* (Vi antigen-negative) mutant of *Salmonella* typhi Ty2 retains virulence in humans. *Infect Immun* 1988; 56: 1326-33.

Hornick RB, Greisman SE, Woodward TE, DuPont HL. Typhoid fever: pathogenesis and immunologic control. *N Engl J Med* 1970; 283: 686-91.

Jegathesan M. Phage types of *Salmonella* Typhi isolated in Malaysia over the 10-year period 1970-1979. *J Hyg Camb* 1983; 90: 91-7.

Lanata CF, Levine MM, Ristori C, *et al*. Vi serology in the detection of chronic *Salmonella* Typhi carriers in an endemic area. *Lancet* 1983; 2: 441-3.

Looney RJ, Steigbigel RT. Role of the Vi antigen of *Salmonella* Typhi in resistance to host defense in vitro. *J Lab Clin Med* 1986; 108: 506-16.

Mehta G, Arya SC. Capsular Vi polysaccharide antigen in *Salmonella enterica* serovar Typhi isolates. *J Clin Microbiol* 2002; 40: 1127-8.

Nair S, Alokam S, Kothapalli S, *et al*. *Salmonella enterica* serovar Typhi strains from which SPI-7, a 134-kilobase island with genes for Vi exopolysaccharide and other functions, has been deleted. *J Bacteriol* 2004; 186: 3214-23.

Pickard D, Li J, Roberts M, *et al*. Characterization of defined *ompR* mutants of *Salmonella* Typhi: *ompR* is involved in the regulation of Vi polysaccharide expression. *Infect Immun* 1994; 62: 3984-93.

Pickard D, Wain J, Baker S, *et al*. Composition, acquisition, and distribution of the Vi exopolysaccharide-encoding *Salmonella enterica* pathogenicity island SPI-7. *J Bacteriol* 2003; 185: 5055-65.

Saha MR, Ramamurthy T, Dutta P, Mitra PV. Emergence of *Salmonella typhi* Vi-negative strains in an epidemic of multi-drug resistant typhoid fever in Calcutta, Indian. *Nat Med J India* 2000; 13: 164.

Song JH, Cho H, Park MY, Na DS, Moon HB, Pai CH. Detection of *Salmonella* Typhi in the blood of patients with typhoid fever by polymerase chain reaction. *J Clin Microbiol* 1993; 31: 1439-43.

Thomson N, Baker S, Pickard D, *et al*. Prophage-like elements in the genome of *Salmonella enterica* serovar Typhi. Contribution to diversity in *S. enterica* serovars. *J Mol Biol* 2004; 339: 279-300.

Virlogeux I, Waxin H, Ecobichon C, Popoff MY. Role of the *viaB* locus in synthesis, transport and expression of *Salmonella* Typhi Vi antigen. *Microbiology* 1995; 141: 3039-47.

Wain J, Deborah H, Zafar A, *et al*. Vi antigen expression in *Salmonella enterica* serovar Typhi clinical isolates from Pakistan. *J Clin Microbiol* 2005; 43: 1158-65.

Zhao L, Ezak T, Li ZY, Kawamura Y, Hirose K, Watanabe H. Vi suppressed wild strain *Salmonella* Typhi cultured in high osmolarity is hyper invasive toward epithelial cells and destructive of Peyer's patches. *Microbiol Immunol* 2001; 45: 149-58.