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Cloning, Sequencing and Phylogenetic Analysis of *stn* gene of *Salmonella* Typhimurium

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Salmonella* Typhimurium is an important facultative bacillus pathogen having broad host range and high physiological adoptability. Several genes contribute in virulence and determine pathogenicity of this isolate. *Stn* is an important virulent gene which code for *Salmonella* toxin, increases the level of c-AMP in the host, and ultimately results into diarrhoea and vomiting. In present study *stn* gene was cloned, sequenced and on basis of sequence information of stn gene, phylogenetic relation was deduced between different serovars of *Salmonella* Typhimurium. Genomic DNA was isolated from field isolate of *Salmonella* Typhimurium (isolate No-A201) and *stn* gene was amplified using gene specific primer and cloned in pJET vector by the positive selection system. Amplification of *stn* gene yielded a product of approximately 750 bp. Subsequently gene was sequenced and a complete ORF of 750 bp was obtained. The sequence was submitted to NCBI Genbank and allotted the Accession No KF032246 was allocated. Sequence was further used for bioinformatics analysis of Stn protein, which exhibited two major domains and one amino acid substitution at 609 residue. On phylogenetic analysis, *S.*Typhimurium exhibited 99% similarity with *Salmonella* enterica subsp. enterica serovar Newport. Our findings indicate that *stn* is an important toxin gene, which is conserved among many serovars of *Salmonella.

Keywords: *Salmonella*, gene, protein, vaccine.

Gastrointestinal diseases of infectious origin usually arise upon ingestion of contaminated foods or water and can have a wide number of etiological agents, known as enteric pathogens. Among

them, the genus *Salmonella* is of particular clinical relevance in both developed and developing countries, where this pathogen is one of the most common causes of food-borne illness and is a major cause of diarrheal diseases, respectively (Kozak *et al*.,2013; Vojdani *et al*.,2008; Ansari *et al*., 2012 ; Kabir *et al*.,2012; Kariuki *et al,*. 2006). Several outbreaks attributed to different *Salmonella* serovars are reported each year, highlighting the frequency of *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis among the most common causal agents (Kozak *et al*.,2013; Vojdani *et al*.,2008; Ansari *et al*., 2012 ; Kabir *et al*.,2012; Kariuki *et al,*. 2006) *Salmonella enterica* serovar Typhimurium is a Gram negative,

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facultative bacillus and a leading cause of human gastroenteritis, which is often associated with non typhoid symptoms such as diarrhoea and abdominal pain (McClelland *et al*.,2001; Everest *et al*.,1999). In poultry, *S*. Typhimurium has been isolated from broilers, breeders, and commercial egg-laying flocks (Chima *et al*., 1998; Jamshidi *et al*., 2009). The backyard poultry practices are very common in India so there is always a possibility of transferring of *Salmonella* Typhimurium from poultry to human populations where it can cause serious health problems (Moreno *et al*., 2000; Swe *et al*., 2008; Saxena *et al*., 2006).Therefore, *Salmonella* control in poultry would contribute a significant advantage in improving public health. Hence, there is an emergent need for devising various strategies to develop vaccine against poultry salmonellosis for reducing the infection caused by this organism.

Salmonella enterotoxin (Stn) is a putative virulence factor and causative agent of diarrhoea (Chopra *et al.,* 1994;.Chopra *et al.,*1999). It has been shown that the *stn* gene is specifically distributed only in *Salmonella* spp. irrespective of their serotypes (Dinjus *et al*., 1997; Makino *et al.,* 1999; Moore *et al.,* 2007). Biological activities of Stn are important to *Salmonella* virulence, especially acute gastroenteritis. It has shown an enterotoxic activity in a murine ileal loop model (Chopra *et al*., 1999). Therefore, Stn is a *Salmonella* virulence factor and is responsible for the enterotoxicity of *Salmonella*. It is possible that Stn will play a pivotal role in special functions of *Salmonella*. To explore more information about Stn protein in the present study *stn* gene of *Salmonella* Typhimurium was cloned, sequenced and phylogenetic analysis was done by using bioinformatics approach.

MATERIALS AND METHODS

Bacterial Strains. *Salmonella* Typhimurium field isolate (A201) was used in this study which had been characterized by *Salmonella* specific PCR, biochemical characterization and serotyped at National *Salmonella* Centre, IVRI Izzatnagar as *Salmonella* Typhimurium. *Escherichia coli* DH5*á* used in cloning experiment was purchased from Bangalore Genei, India and grown in LB broth. Blunt cloning vector pJET 1.2, blunting enzyme, and T4 DNA ligase were procured from Qiagen, USA. The antibiotics (Ampicillin (100 *ì*g/mL) and Kanamycin (50 *ì*g/mL)) used for selection of recombinants were procured from Himedia, India. The cultures were maintained in LB agar slants and their purity was tested by biochemical tests and *Salmonella* specific PCR.

Cloning of *stn* gene. Genomic DNA was isolated by CTAB method. Primers were designed for Stn gene of *Salmonella* Typhimurium using sequence information available on NCBI and using gene tool software.

Stn 1 (Forward) : 5' GGATCC TTG TTA ATC CTG TTG TCT CGC TAT 3'
Stn 2 (Reverse) : 5' AAGGTT TTA CTG GCG TTT TTT TGG CAT 3'

50 µl of PCR reaction mixture was set up containing 20 ng of genomic DNA (template), 200 µM dNTPs, 20 picomoles of each primer, 3 unit of Jumpstart™ AccuTaq™ LA (SIGMA) with 5 ul of 10X AccuTaq™ LA PCR buffer. Final volume to 50 ul was made up by using sterilised water. The gene was amplified by PCR using the following program, that is, initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C, annealing at 51°C, and elongation at 68°C.

PCR product was loaded on 1.5% Agarose gel and the size of the amplicon was measured by comparing with standard molecular weight marker. To remove PCR dimer, PCR product was eluted from agarose gel using Qiagen Mini Elute Gel extraction kit. PCR product was cloned in pJET vector by blunt end cloning. After blunting of PCR product, 1 µl of pJET vector and 1 µl of T4 DNA ligase were added and ligation was carried out at 22ºC for 4 hours. 5 ul of ligated product was checked on 1.5 % Agarose gel. Ligated product was transformed into DH5á cells using Calcium chloride method (Sambrook *et al*., 1989). Recombinants were screened by selecting Ampicillin resistant colonies and analyzing the presence of insert by colony PCR. Plasmids were isolated from selected clones and insert was released by double digestion with *Bam* H1 and *Hind* III. The size of insert was measured by comparing with standard molecular weight marker. Selected clones were sent to University of Delhi South Campus for sequencing and obtained sequence was analysed for presence of open reading frame. The sequence was submitted to NCBI.

GC Content analysis*:* DNA/RNA GC Content Calculator was used to calculate the percentage of GC content in stn gene. (http://www.endmemo.com/bio/gc.php).

Open reading frame analysis: ORF analysis of stn gene of *Salmonella* Typhimurium (750 bp) was performed by GENE TOOL software.

Conserved Domain Search*:* Conserved domain analysis of the protein sequence was performed using CD Search tool of NCBI.

Sequence Similarity and Phylogenetic Analysis: The sequence obtained was subjected to homology search using BLASTn (http://www.ncbi.nlm.nih.gov/). The sequences showing maximum similarity with stn gene was subjected to multiple sequence alignment and a phylogenetic tree was constructed based on the comparative analysis of related sequences using MEGA (Molecular Evolution Genetics Analysis) tool at nucleotide level. Analysis was performed on the default values of the MEGA software and Neighbour-joining statistical method at 1000 bootstrap replication was used for tree construction.

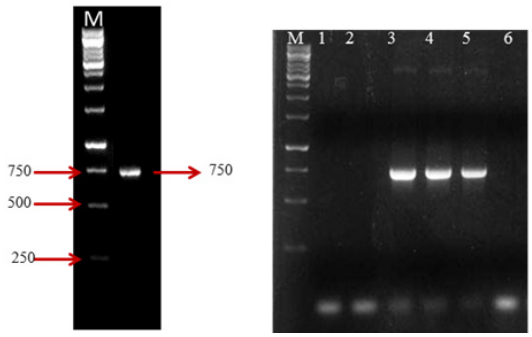

Fig. 1. (1a).The cloned gene was confirmed by colony PCR .1.5% Agarose gel showing 750 bp PCR product of *stn* gene (1b).Lane (1,2): PCR of false recombinants, Lane (3) and (4) corresponds to positive clones of *stn*. A corresponding band of 750 bp was seen for *Salmonella* isolate A201 that acted as a positive control (lane 5), DH5-á as negative control (lane 6), M: 1kb ladder

RESULTS AND DISCUSSION

The purity of the culture was checked by biochemical characterization and *Salmonella*-specific PCR. The culture was found to be MR+, VP-, Urease- biochemically, which is characteristic of *Salmonella* Typhimurium.

PCR Amplification and Cloning*:* The PCR amplification with stn specific primers was conducted with genomic DNA, which resulted in a product of approximate size 750 bp (Figure 1a). The desired product was successfully purified using QIA quick gel extraction kit and cloned in pJET 1.2 blunt cloning vector (Fermentas, USA) and transformed into chemically competent *E.coli* DH5*á* cells. Recombinant clones were selected by colony PCR (Figure 1b). The insert was sequenced and complete cds of 750 bp was obtained. The sequence was submitted to NCBI Gene bank and accession no. KF032246 was allocated by NCBI.

GC Content analysis: GC content is found to be variable with different genes but evidence of GC ratio with that of length of the coding region of a gene has shown that the length of the coding sequence is directly proportional to higher G+C content. This has been pointed to the fact that the stop codon has a bias towards A and T nucleotides, and, thus, the shorter the sequence the higher the AT bias and in this case as shown in the graph (Fig

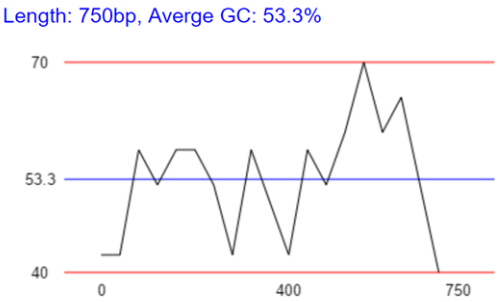

Fig. 2. Total GC content in the stn gene sequence of *Salmonella* Typhimurium

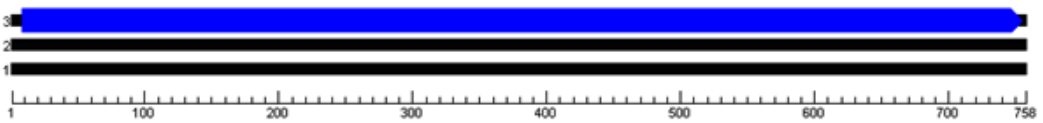

Fig. 3. Open reading frame analysis of stn gene

2) the average GC content is 53.3 % i.e. a good prediction.

Open reading frame analysis

The sequence when subjected to ORF analysis using GENE TOOL software showed a complete ORF of 750 bp. (Fig 3)

Conserved Domain Search

Nucleotide sequence (750bp) was used to predict the domain region in the sequence. Histidine kinase-like ATPases (25-342bp) and Histidine Kinase A (478-672bp), two domains were found in the stn gene sequence of *Salmonella*.

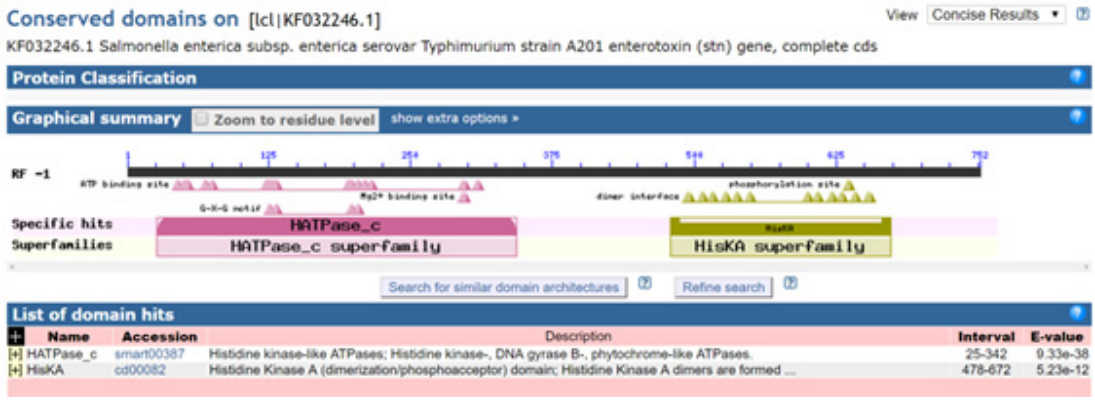

Fig. 4. The sequence was screened for the presence of conserved regions that may exist within a family of gene

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Query  1        TTGTTAATCCTGTTGTCTCGCTATCACTGGCAACCAGATAGTAAAGACCGCGCCTTTACC  60
Sbjct  4459255  TTGTTAATCCTGTTGTCTCGCTATCACTGGCAACCAGATAGTAAAGACCGCGCCTTTACC  4459314
Query  61       CTCAATACTTTTCACCTTAATCGCGCCGCCATGCTGTTCGATGATATTTTGCACCACCGC  120
Sbjct  4459315  CTCAATACTTTTCACCTTAATCGCGCCGCCATGCTGTTCGATGATATTTTGCACCACCGC  4459374
Query  121      CAGCCCCAGACCTGTCCCGTCAGCTTTGGTCGTAAAATAAGGCGTAAAAATCGCCTCCAA  180
Sbjct  4459375  CAGCCCCAGACCTGTCCCGTCAGCTTTGGTCGTAAAATAAGGCGTAAAAATCGCCTCCAA  4459434
Query  181      CTGATCCGGGGCGATCCCTTTCCCGCTATCGGTAACAGTGATGATAACGCGGTCGGTCCC  240
Sbjct  4459435  CTGATCCGGGGCGATCCCTTTCCCGCTATCGGTAACAGTGATGATAACGCGGTCGGTCCC  4459494
Query  241      ACTTTCTTTTGCCTCTACGCTAATCGTTCCCTGGCGGCCAATCGCATGAATAGCGTTCAG  300
Sbjct  4459495  ACTTTCTTTTGCCTCTACGCTAATCGTTCCCTGGCGGCCAATCGCATGAATAGCGTTCAG  4459554
Query  301      GTACAAATTCAACAGCACCTGAGTCAGCCTGTCCGGGTCAGCCTGAATACGCTTAAGCGT  360
Sbjct  4459555  GTACAAATTCAACAGCACCTGAGTCAGCCTGTCCGGGTCAGCCTGAATACGCTTAAGCGT  4459614
Query  361      CTCATTCGCCGTGAACCTCAACTGAATCTCTCTGCTTTGGGCATCCTGACTGACCAGATT  420
Sbjct  4459615  CTCATTCGCCGTGAACCTCAACTGAATCTCTCTGCTTTGGGCATCCTGACTGACCAGATT  4459674
Query  421      CAGGGAGTGAGTAATAATATCATTGAGGTTAACCGTCTGGAGCGTCAGATGCGCGGGCTT  480
Sbjct  4459675  CAGGGAGTGAGTAATAATATCATTGAGGTTAACCGTCTGGAGCGTCAGATGCGCGGGCTT  4459734
Query  481      TACCAGTTCGAGCAATTCGCTTACCACCCGGTTCAAACGGTCGGCCTCTTTGGCCATCAC  540
Sbjct  4459735  TACCAGTTCGAGCAATTCGCTTACCACCCGGTTCAAACGGTCGGCCTCTTTGGCCATCAC  4459794
Query  541      CTGCGCCAGTTCATGCGACTCGCCGCCGGCAGGCGTGCGCTCGGCAAAGTATTTCGCCAG  600
Sbjct  4459795  CTGCGCCAGTTCATGCGACTCGCCGCCGGCAGGCGTGCGCTCGGCAAAGTATTTCGCCAG  4459854
Query  601      CCCTTTGACGGACGAGAGCGGGTTACGAATTTCGTGCGCGACGCCCGCCGCCAGATGCCC  660
Sbjct  4459855  CCCTTTGATGGACGAGAGCGGGTTACGAATTTCGTGCGCGACGCCCGCCGCCAGATGCCC  4459914
Query  661      CATCGCCACCAGCTTTTCTTTACGCTTCATTGCATCAAGCAGTTCTCTGTGCGAGCGCTG  720
Sbjct  4459915  CATCGCCACCAGCTTTTCTTTACGCTTCATTGCATCAAGCAGTTCTCTGTGCGAGCGCTG  4459974
Query  721      ATAACGCTGATGCCaaaaaaaCGCCAGTAA  750
Sbjct  4459975  ATAACGCTGATGCCAAAAAAACGCCAGTAA  4460004
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Fig. 5. Substitution of amino acid at nucleotide level in the *stn* gene of *Salmonella* Typhimurium with the homologous sequences

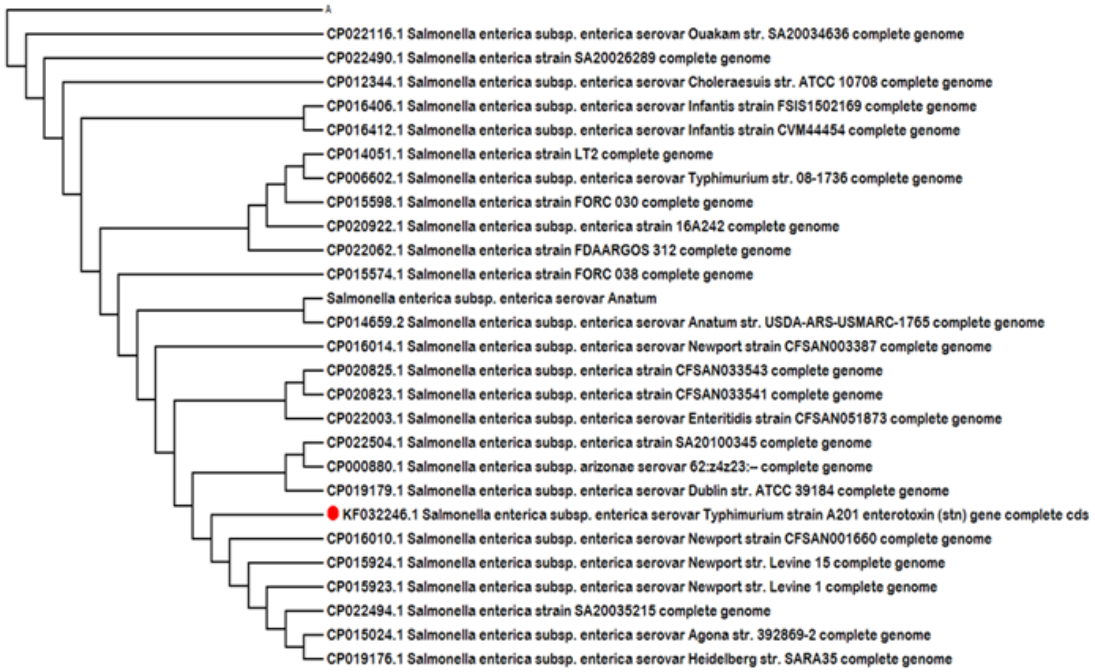

Fig. 6. Dendogram for Phylogenetic analysis at nucleotide level of *stn* gene marked with red square box with other closely related sequences. A is the collapse group of the sequences

Histidine kinase-like ATPases family includes several ATP-binding proteins for example: histidine kinase, DNA gyrase B, topoisomerases, heat shock protein HSP90, phytochrome-like ATPases and DNA mismatch repair proteins.

Histidine Kinase A dimers are formed through parallel association of 2 domains creating 4-helix bundles; usually these domains contain a conserved Histidine residue and are activated via trans-autophosphorylation by the catalytic domain of the histidine kinase. They subsequently transfer the phosphoryl group to the Asp acceptor residue of a response regulator protein. Two-component signalling systems, consisting of a histidine protein kinase that senses a signal input and a response regulator that mediates the output, are ancient and evolutionarily conserved signaling mechanisms in prokaryotes and eukaryotes.

Substitution of amino acids at nucleotide level: Sequence alignment of stn gene of *Salmonella* Typhimurium (nucleotide level) was performed by BLAST tool of NCBI to find the similar homologous sequence. In the Pairwise sequence alignment at nucleotide level the stn gene showed maximum similarity with *Salmonella* enterica subsp. enterica serovar Newport and a substitution was found at position 609 bp with Cysteine to Tyrosine. (Fig 5)

Phylogenetic analysis of *S.* Typhimurium *stn* gene at nucleotide level

The NCBI BLASTn search of the *stn* gene showed maximum homology (99%) with strain *Salmonella* enterica subsp. enterica serovar Newport and *Salmonella* enterica subsp. enterica serovar Dublin complete genome at nucleotide level. (Fig 6).Though the sequence was shared by many serovars of *Salmonella* like *S*.Choleraesuis, *S* infantis, *S* Anatum, *S* Enteritidis, *S* Dublin etc

Salmonella is an important human and animal pathogen. It causes two types of diseases in human being i.e. Typhoid fever caused by S.Typhi and Non-typhoidal salmonellosis caused by *S*.Typhimurium. Both of the forms of disease are dreaded and cause high mortality in human being at global level (Kemal *et al*., 2014). Antibiotic drug resistance is a major concern in case of *Salmonella* as it has been reported in most of the part of the world (Chandane *et al*.,2017) Vaccination is

the only possible viable option but in both the cases (typhoidal & Non-typhoidal salmonellosis) effective vaccines are not available as in case of Typhoid presently available Vi Polysachharide vaccine has serious limitations such as it cannot be used in pregnant ladies, high cost of production and a debatable immune response in children. (Ochiai *et a*l.,2014) Though to overcome from these problems new adjuvant system has been developed (Tamuly and Saxena 2012) which can produce better immune response and by the use of molecular techniques efforts have been made to differentiate the pathogen at molecular level(Shivachandra *et al*.,2006; Zheng *et a*l.,2014) to assess the genetic variation among isolates to resolve the problem of vaccination failure. A conserved and immunogenic protein may be a suitable target for various vaccine development against *Salmonella* (Jha *et al*., 2015). Stn has been considered as putative virulence factor of *Salmonella* and causative agent of diarrhoea (Chopra *et al*., 1999) but in the later stages it was deduced that the mutation in stn gene did not resulted into reduction of virulence of *Salmonella* (Watson *et al.,* 1998) but it is an interested fact that *stn* gene is distributed only in *Salmonella* ssp (Moore *et al*., 2007) and in our study it has exhibited higher degree of homogeneity among the various serovars of *Salmonella*. On amino acid sequencing it has shown some similarity with active site of cholera toxin (Dinjus et al., 1997). Therefore, from the findings of the workers and our study we could conclude that though Stn is not an essential protein for virulence of *Salmonella* but it may be a contributing factor moreover, it seems to be a conserved proteins among the serovars of *Salmonella*. Therefore like other toxoid vaccine like tetanus (Moreira et al., 2016) it may be a suitable target for the development of toxoid vaccine against salmonellosis.

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