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Cloning, Sequencing and Phylogenetic Analysis of *inv* E Gene of *Salmonella typhimurium*

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A B S T R A C T

Salmonella enterica subsp. *enterica* serovar *typhimurium* is a gram-negative serious contagious pathogen having a broad host range. It has been associated with a wide spectrum of infectious diseases, including typhoid fever and nontyphoidal salmonellosis, which cause public health problems worldwide. Several genes contribute in virulence and determine pathogenicity of this isolate. *inv* E is an important invasion gene, member of *Salmonella* pathogenicity island1 (SPI-1) which helps pathogen to invade the host intestinal epithelial cell and also mediates the translocation of several bacterial effector proteins that have the capacity to stimulate cellular responses leading to bacterial internalization and the reprogramming of gene expression in the infected cells. In present study *inv* E gene was cloned, sequenced and phylogenetic analysis was done using bioinformatics approach. Genomic DNA was isolated from field isolate of *Salmonella* Typhimurium (A-201) and *inv* E gene was amplified using gene specific primers and cloned in pJET vector by the positive selection system. Amplification of *inv* E gene yielded a product of approximately 1.1 kb. Subsequently gene was sequenced and a complete ORF of 1119 bp was obtained. The sequence was submitted to NCBI Genbank and Accession No. KC607873 was allocated. The sequence was analysed using bioinformatics tools for the homologous sequences. Sequence homology was searched at nucleotide level and exhibited 99% similarity with *Salmonella enterica* strain FORC030 complete genome and *Salmonella enterica* subsp. *enterica* serovar Indiana strain D90, complete genome.

Introduction

Salmonellosis is one of the most common food-borne diseases of humans and animals. In human beings there are two forms of Salmonellosis. These are Typhoidal and Non-typhoidal Salmonellosis (Gal-Mor *et al.*, 2014). Like other gram-negative bacteria virulence of *Salmonella* is regulated by chromosomal and plasmid mediated genes

(Singh *et al.,* 2018). Multiple drug resistance have been found to be associated with the infections of *Salmonella* (Tamuly *et al.,* 2014; Kalaiyarasu *et al.,* 2008; Verma *et al.,* 2014) which produced difficulties in treatment of disease.

Though by the use of modern biological tools efforts have been made to differentiate organism at molecular level by analysis of plasmid profile (Shivshankara *et al.,* 2000), DNA markers (Verma *et al.,* 2014; Saxena *et al.,* 2012) and protein based markers (Singh *et al.,* 2018).

The studies exhibited high genetic variations among the field isolates. Therefore, there is a need of targeting conserved proteins for development of safe and effective vaccine against *Salmonella*. Efforts have been made by targeting outer membrane proteins (Saxena *et al.,* 2012; Pandey *et al.,* 2015; Jha *et al.,* 2015) along with new generation adjuvant system (Tamuly and Saxena, 2012; Tamuly *et al.,* 2014) and a protection upto a certain level could be achieved in lab animal models. In the continuation of same Inv E protein can be targeted for development of suitable vaccine against *Salmonella*. Inv E is a virulence factor and responsible for invasion of organism in host cell (Shi *et al.,* 2012).

So it can be considered as toxin factor of *Salmonella* which may be proven as an efficient target for vaccine development. But to be proven as vaccine candidate *inv* E should fulfill few pre-requisites. Firstly, it should be a conserved protein among the various serovars and secondly, it must have effective B cell and T cell epitopes.

Therefore, in present study we cloned and sequenced *inv* E gene. The conserved domain and phylogenetic analysis of *inv* E was made to deduce the conservation status of the protein among the various serovars.

Materials and Methods

Bacterial strain

The field isolate of *Salmonella typhimurium* (A-201) used in this study was obtained from Animal Biotechnology Center, G.B. Pant University of Agriculture & Technology, Pantnagar. The culture was revived and the purity of the culture was determined by *Salmonella* specific PCR and biochemical test specific to *Salmonella*. Further, culture was maintained in Hektoen enteric agar slants and glycerol stocks during the study. *E. coli* DH5*á* used in cloning was purchased from Bangalore GeNei, India and grown in Luria Bertani broth while blunt cloning vector pJET 1.2, blunting enzyme, and T4 DNA ligase were procured from Qiagen, USA. Ampicillin (100 µg/ml) used for selection of recombinants were procured from Himedia, India.

Cloning of *inv* E gene

Genomic DNA of *Salmonella typhimurium* was isolated by CTAB method. The sequence information available on NCBI as well as gene tool software was used for designing primers for *inv* E gene of *Salmonella typhimurium*.

inv E (Forward): 5' GGA TCC ATG ATT CCT GGT TCC ACC TCC 3'

inv E (Reverse): 5' AAG GTT TTA AGA CGG CTT TTC AAT AGT ACG A 3'

Amplification of *inv* E gene was carried out with 50 µl of PCR reaction mixture containing 20 ng of genomic DNA (template), 200 µM dNTPs, 20 picomoles of each primer, 5 ul of 10X AccuTaq™ LA PCR buffer with 3 unit of Jumpstart™ AccuTaq™ LA (Sigma). Final volume of PCR reaction mixture was making up to 50 µl by using sterilized water. The gene was amplified by under following cycling conditions, initial denaturation at 94°C,

followed by denaturation at 94°C, annealing at 54°C, and elongation at 68°C. PCR product was analyzed in 1.5% agarose gel and the size of the amplicon was measured by comparing with standard molecular weight marker. The elution of PCR product was carried out from agarose gel using Qiagen mini elute gel extraction kit. Further, 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. The ligated product (5 ul) was checked on 1.5 % agarose gel and transformed into *E.coli* DH5α cells by Calcium chloride method (Sambrook *et al.,* 1989). Recombinants were screened by selection on the basis of antibiotic resistance and analyzed for the presence of insert by colony PCR. The insert was released by double digestion with *Hind* III and *Bam* H1 and the size of insert was measured by comparing with standard molecular weight marker. Plasmid DNA was isolated from clones and the selected clones were sent to University of Delhi, South Campus for sequencing. The deduced sequences were submitted to NCBI.

Open reading frame

The Open reading frame (ORF) of *inv* E gene of *Salmonella typhimurium* was deduced by using Gene tool software.

GC content

The percentage of GC content in *inv* E gene of *Salmonella typhimurium* was calculated by using DNA/RNA GC content calculator. (http://www.endmemo.com/bio/gc.php).

Conserved domain search

Conserved domain analysis of the *inv* E protein sequence was performed by using CD Search tool of NCBI.

Sequence similarity and phylogenetic analysis

The deduced sequence was analyzed for homology using BLASTn (http://www.ncbi. nlm.nih.gov/). The sequences showing maximum similarity with *inv* E gene were 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.

The 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.

Results and Discussion

The purity of the procured *Salmonella typhimurium* culture was confirmed using biochemical characterization and PCR with *Salmonella typhimurium* specific primers. The culture was found to be MR+, VP- and Urease- biochemically, which represents characteristic of *Salmonella typhimurium*.

PCR Amplification and Cloning

The PCR amplification of *inv* E gene of *Salmonella typhimurium* was conducted with isolated genomic DNA and gene specific primers resulting in a product of approximate size 1119 bp (Fig 1). The PCR product was purified using QIAGEN quick gel extraction kit, cloned in pJET 1.2 cloning vector (Fermentas, USA) and transformed into *E.coli* DH5*á* cells. Further, recombinant clones were selected by colony PCR (Fig. 2). Plasmid with *inv* E gene insert was isolated and sequenced at University of Delhi, South Campus. The sequence with complete cds of *inv* E gene was submitted in NCBI Gene Bank (Accession number KC607873).

Open reading frame analysis

The sequence showed a complete ORF of 1119 bp using Gene tool software. (Fig 3)

GC content analysis

GC content varies with different genes. The length of the coding sequence is directly proportional to higher G+C content as the stop codon has a bias towards A and T nucleotides. Therefore, shorter the sequence the higher will be the AT bias. The total GC content in the *inv* E gene sequence of *Salmonella typhimurium* was found to be 45.5%.

Conserved Domain Search

Nucleotide sequence (1119 bp) was used to predict the domain region in the sequence. A single domain, type III secretion system regulator *inv* E was identified in the *inv* E gene sequence of *Salmonella typhimurium*. HrpJ-like domain family represents a conserved region approximately 200 residues long within a number of bacterial hypersensitivity response secretion protein HrpJ and similar proteins. HrpJ forms part of a type III secretion system through which, in phytopathogenic bacterial species, virulence factors are thought to be delivered to plant cells. This family also includes the inv E invasion protein from *Salmonella*. This protein is involved in host parasite interactions and mutations in the InvE gene render *Salmonella typhimurium* non-invasive. *Inv* E *S. typhimurium* mutants fail to elicit a rapid Ca^{2+} increase in cultured cells, an important event in the infection procedure and internalisation of *S. typhimurium* into epithelial cells. This family includes bacterial SepL and SsaL proteins. SepL plays an essential role in the infection process of enterohemorrhagic *Escherichia coli* and is thought to be responsible for the secretion of EspA, EspD and EspB while SsaL of *Salmonella typhimurium* is thought to be a component of the type III secretion system.

Fig.1 1.5% Agarose gel showing 1.1 kb PCR product of *inv* E gene. **Fig.2** The cloned gene was confirmed by release of insert by double digestion with *Bam* H I *and Hind III, M*: 1kb ladder *(*GeneRuler™, Fermentas)

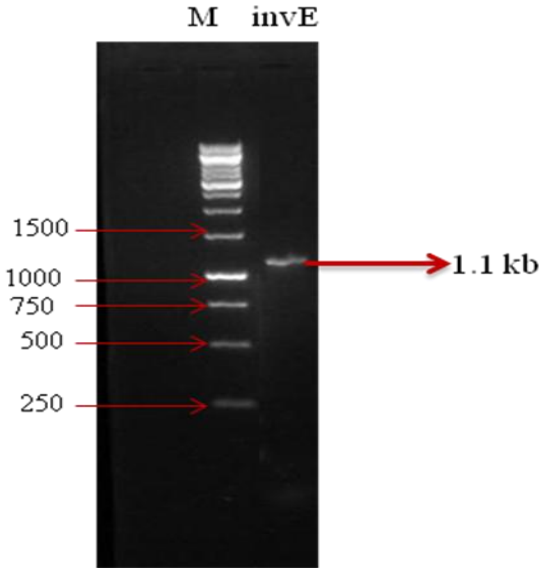

Fig.1

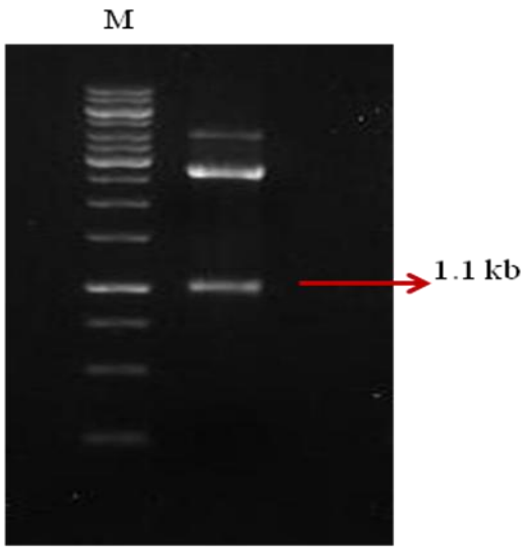

Fig.2

Fig.3 Open reading frame (ORF) analysis of *inv* E gene sequence of *Salmonella typhimurium* (ORF 1: 1-1119)

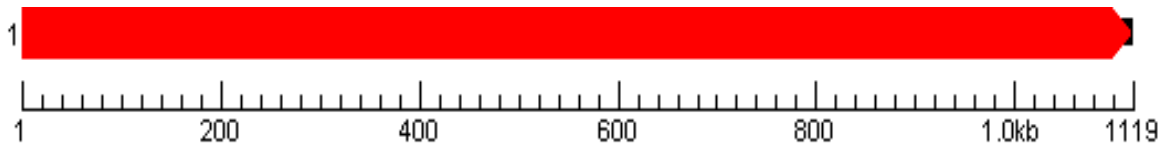

Fig.4 Total GC content in the *inv E* gene sequence of *Salmonella typhimurium*

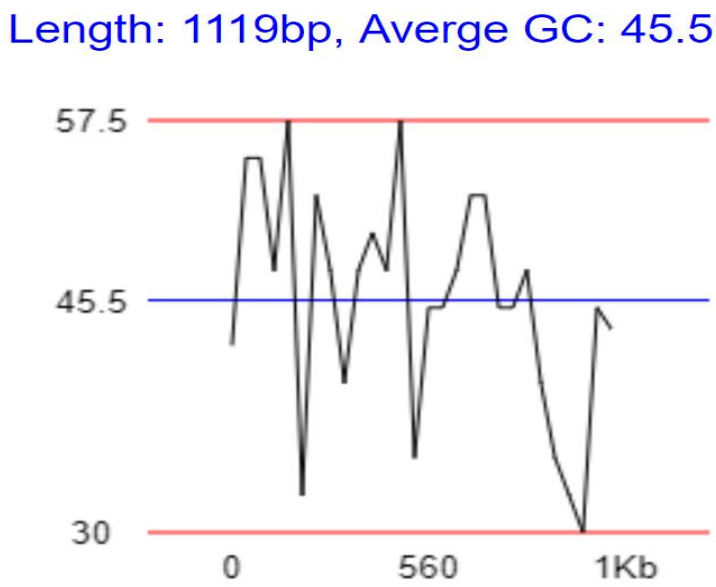

Fig.5 Conserved Domain of Inv E protein of *Salmonella typhimurium*

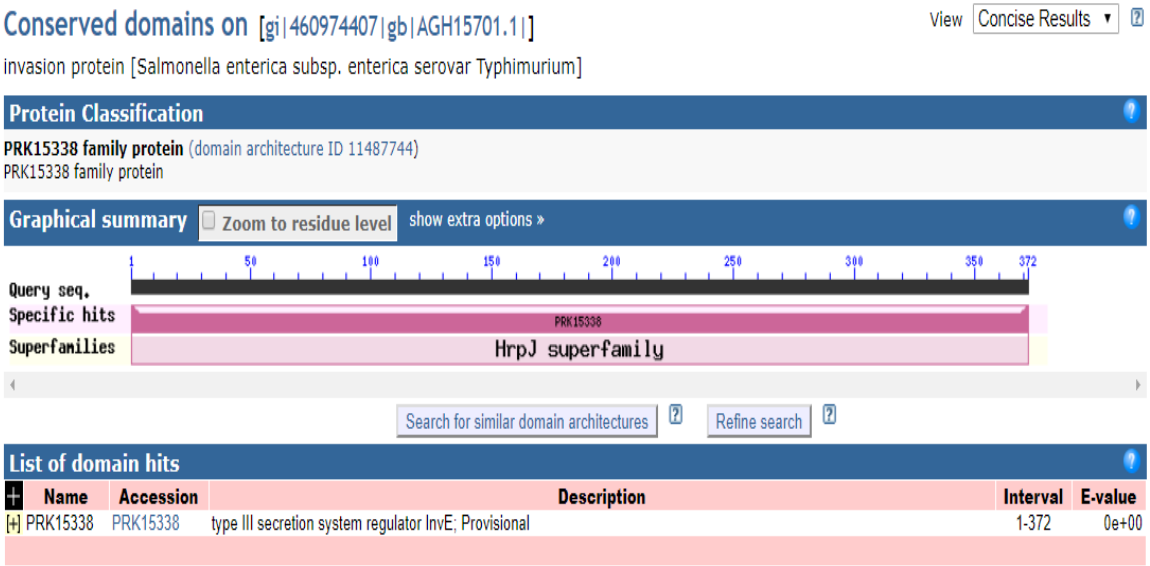

Fig.6 Dendrogram for phylogenetic analysis at nucleotide level of *inv* E gene marked with red square box with other closely related sequences

Phylogenetic analysis of *inv* E gene of *S. typhimurium* at nucleotide level

The NCBI BLASTn search of the *inv* E gene showed maximum homology (99%) with *Salmonella enterica* subsp. *enterica* strain LSP 389/97 complete genome and *Salmonella enterica* strain DA34833 chromosome complete genome at nucleotide level (Fig. 6). Though the sequence was shared by other serovar of *Salmonella* like *S. enteritidis*.

Salmonella is an important contagious gram-negative pathogen which affects both humans and poultry. It is mainly causative agent of typhoid fever caused by *S. typhi* in humans and non-typhoidal salmonellosis caused by *S.typhimurium*. Both of the forms of disease result in high mortality as well as severe economic loss (Kemal, 2014). In past few years the emergence of multi-drug resistant strains of *Salmonella* in many countries of the world has become a major concern (Chandane *et al.*, 2017). Vaccination is considered as the prominent option but effective vaccines are not available as currently available vaccines have certain limitations such as it cannot be used in pregnant women, debatable immune response in children and high cost of production (Ochiai *et al.*, 2014). Although to

overcome these problems several workers have made efforts to develop vaccine targeting recombinant as well as immunogenic outer membrane proteins of *Salmonella* (Bhat and Jain, 2010; Jha *et al.*, 2015). Strategies involving new adjuvant system have also been developed (Tamuly and Saxena 2012, Tamuly *et al.*, 2014) which produces better immune response. The invasion protein Inv E is an important virulence factor located on SPI-1 which is responsible for spread of *Salmonella* in host cells. In order to explore the relationship between pathogenicity of *Salmonella* and virulence factor genes (*inv* E), i*nv* E gene of *Salmonella typhimurium* was isolated, amplified, sequenced and homology was searched for similarity with related sequences of different strains. Sequence was further used for bioinformatics analysis of Inv E protein, which exhibited a single domain. On phylogenetic analysis, sequence exhibited 99% similarity with *Salmonella enterica* strain FORC030 complete genome and *Salmonella enterica* subsp. *enterica* serovar Indiana strain D90, complete genome. In our study we could observe that Inv E is a conserved protein in genus *Salmonella* as it has exhibited homology with different serovars of *Salmonella* like *S. indiana*, *S. stanley*, *S. newport*, *S. enteritidis* etc.

Simultaneously it has exhibited a complete functional conserved domain. Therefore, it fulfills the first pre-requisite as a candidate for vaccine development. In the further studies the epitopes, secondary and tertiary structure of the protein is to be deduced to explore the possibility of development of subunit vaccine.

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References

Bhat, N.H. and Jain, S.K., 2010. Immunogenic evaluation of a recombinant 49-kilodalton outer membrane protein of *Salmonella typhi* as a candidate for a subunit vaccine against typhoid. *Journal of Infectious Diseases and Immunity*, 2(2): 30-40.

Chandane, P., Gandhi, A. and Bowalekar, S. 2017. Study of antibiotic susceptibility pattern of *Salmonella typhi* in children suffering from enteric fever. *Annals of Tropical Medicine and Public Health*, 10(2): 440-443.

Gal-Mor, O., Boyle, E.C. and. Grassl, G.A. 2014. Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Frontiers in Microbiology*, 5: doi: 10.3389/fmicb.2014.00391.

Jha, R., Kumar, A., Saxena, A., Pandey, M., Kumar, R. and Saxena, M.K. 2015. Heterogeneous expression and functional evaluation of *in silico* characterized recombinant OmpC of *Salmonella typhimurium* as a functional poultry vaccine to eradicate zoonotic transmission. *African Journal of Biotechnology*, 14(41): 2862-2870.

Kalaiyarasu, S., Saxena, M.K., Gupta, R.S. 2013. Emerging of multiple drug resistance in *Salmonella typhimurium* serovars isolated from Indian Ganga River. *Advances in Animal and Veterinary sciences*, 1:1-3.

Kemal, J. 2014. A Review on the Public Health Importance of Bovine Salmonellosis. *Journal of Veterinary Science & Technology*, 5: 2: doi: 10.4172/2157-7579.1000175.

Ochiai R.L., Khan M.I., Soofi S.B., Sur. D., Kanungo, S., You, Y.A., Habib, M.A., Sahito, S.M., Manna, B., Dutta, S., Acosta, C.J., Ali, M., Bhattacharya SK., Bhutta, Z.A. and John, D.C.2014. Immune Response to Vi Capsular Polysaccharide typhoid vaccine in Children 2 to 16 years old in Karachi, Pakistan and Kolkata, India. *Clin and Vaccine* Immunol, 5:661-666.

Pandey, M. and Saxena, M.K. 2015. Cloning and immunopotential analysis of Omp 28 of *Salmonella typhimurium* for the development of subunit vaccine for poultry salmonellosis. *Indian Journal of Poultry Science*, 50(2):138-142.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning. *A Laboratory Manual 198*(9) edition *2nd Cold Spring Harbour Laboratory Press Cold Spring Harbour NY*.

Saxena, A., Tamuly, S. and Saxena, M.K. 2012. Cloning, sequencing, and *in silico* characterization of Omp 28 of *Salmonella typhi* (strain MTCC 733) to develop r-DNA vaccine for typhoid fever. *Journal of Natural Science Biological Medicine*, 3(2): 133-138.

Shi, Q., Zhang, Y., Wang, Q.Y., Gao, G., Fang, H., Miao, F., Yuan, Z. and He, H. 2012. Phylogenetic analysis of virulence factor gene of *Salmonella* isolated from clinically symptomatic Chickens. *African Journal of Microbiology Research*, 6(8): 1718-1722.

Shivshankara, N., Singh, V.P., Saxena, M.K. and Kumar, A.A. 2000. Plasmid profile analysis of *Pasteurella multocida*. *Indian journal of Comparative Microbiology, Immunology and Infectious diseases*, 21(2): 115-7.

Singh, Y., Saxena, A., Kumar, R. and Saxena, M.K. 2018. Isolation and Protein Profiling of Outer Membrane Proteins (Omps) of *Salmonella* Typhi. *International Journal of Current Microbiology and Applied Sciences*, *7*(8): 2851-2855.

Singh, Y., Saxena, A., Kumar, R. and Saxena, M.K. 2018. Virulence System of *Salmonella* with Special reference to *Salmonella enterica* http://dx.doi.org/10.5772/intechopen.77210.

Tamuly, S. and Saxena, M.K. 2012. Preparation of Calcium phosphate nanoparticles and evaluation of their effects on muscle cells of rat. *Current Science*, 102(4): 610-612.

Tamuly, S., Saxena, M.K., Saxena, A., Mishra, R.K. and Jha, R. 2014. Comparative Evaluation of Humoral Immune Response Generated by Calcium Phosphate Nanoparticle Adjuvanted and Saponin-Adjuvanted Recombinant Outermembrane Protein 87 (Omp87) of *Pasteurella multocida* (Serotype B: 2) in Mice. *Journal of Nanopharmaceutics Drug Delivery*, 2(1): doi:10.1166/jnd.2014.1045.

Verma, J.K., Pilkhwal, D., Tamuly, S., Kumar, R., Kumar, A. and Saxena, M.K. 2014. Molecular characterization of Ampicillin resistant poultry isolates of *Salmonella typhimurium*. *Journal of Cell and Tissue Research*, 14:4019-4026.

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