Cloning and sequencing of protein \( L \)-isoaspartyl \( O \)-methyl transferase of \textit{Salmonella} Typhimurium isolated from poultry

S. K. Dixit\textsuperscript{1}, D. P. Hota\textsuperscript{2}, M. Kumawat\textsuperscript{2}, T. K. Goswami\textsuperscript{1} and M. Mahawar\textsuperscript{2}

1. Immunology Section, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India; 2. Division of Biochemistry, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.

Corresponding author: S. K. Dixit, e-mail: sunildixit1987@gmail.com, DPH: durgaprasad.hota04@gmail.com, MK: 0711mworld@gmail.com, TKG: goswami.tapas@gmail.com, MM: manishbiochemistry@gmail.com

Received: 16-05-2014, Revised: 28-07-2014, Accepted: 04-08-2014, Published online: 25-09-2014


Abstract

Aim: To clone the \textit{Salmonella} Typhimurium protein \( L \)-isoaspartyl \( O \)-methyl transferase (\textit{PIMT}) enzyme and to analyze the sequence with \textit{PIMT} gene of other pathogenic serovars of \textit{Salmonella}.

Materials and Methods: \textit{Salmonella} Typhimurium strain E-2375 was procured from the National Salmonella Center, IVRI. The genomic DNA was isolated from \textit{Salmonella} Typhimurium. Polymerase chain reaction (PCR) was carried out to amplify \textit{PIMT} gene using the designed primers. The PCR product was cloned into pET28c plasmid vector and transformed into \textit{Escherichia coli} DH5\textalpha cells. The plasmid was isolated from \textit{E. coli} and was sequenced. The sequence was analyzed and submitted in Genbank.

Results: The PCR product revealed a distinct amplicon of 627 bp. The clone was confirmed by PCR. Sequencing data revealed 100% homology between \textit{PIMT} sequences from \textit{Salmonella} Typhimurium strain E-2375 (used in the current study) and \textit{PIMT} sequences of standard reported strain (\textit{Salmonella} Typhimurium str. LT2) in NCBI data base. This submitted sequence in Genbank having accession no. KJ575536.

Conclusions: \textit{PIMT} gene of \textit{Salmonella} is highly conserved in most of the pathogenic \textit{Salmonella} serovars. The \textit{PIMT} clone can be used to isolate \textit{PIMT} protein. This \textit{PIMT} protein will be helpful to identify isoaspartate containing proteins thus can help in study \textit{Salmonella} virulence.

Keywords: cloning, sequencing, \textit{Salmonella} Typhimurium protein \( L \)-isoaspartyl \( O \)-methyl transferase, virulence.

Introduction

\textit{Salmonella enterica} serovar Typhimurium (shortly \textit{Salmonella} Typhimurium) is a Gram-negative, flagellated and facultative intracellular anaerobe grouped under the family \textit{enterobactericeae} is widely known for its highly zoonotic importance [1]. This pathogenic bacterium infects wide range of animal host causing severe morbidity and mortality in animals and human [2,3]. Poultry birds mainly act as an asymptomatic reservoir host and its contaminated meat and eggs are the source of infection causing severe gastroenteritis in human and typhoid like disease in rodents [4]. Growing resistance to antibiotics [5], endemicity of infection and their career state encourages understanding the several aspects of its physiology and metabolism for novel vaccine development and its effective control. There are several virulence factor associated with the severity of infection [6]. The virulence associated enzyme mediated protein repairing modification mechanism associated with \textit{Salmonella} for the confrontation of a hostile environment inside the host immune system. The phagocytic cells damage various macromolecules such as DNA, RNA and proteins by producing a battery of antimicrobials such as reactive oxygen species and reactive nitrogen species [7]. The proteins are the major target for this oxidative damage due to their abundance and oxidation leads to covalent modification of the amino acid residues and conformational changes in protein structure [8]. This protein modification brought about by various protein repairing enzymes may be present in \textit{Salmonella} Typhimurium rescue from the oxidative damage and assist for intraphagosomal survival and its replication.

Out of the several protein repairing enzymes reported, protein \( L \)-isoaspartyl \( O \)-methyl transferase (\textit{PIMT}) have been found to be effective for all the domains of the life, and this enzymes extensively studied in \textit{Escherichia coli} [9] and \textit{Pyrococcus} [10] and \textit{Vibrio cholera} [11]. The way \textit{PIMT} works are by repairing the damaged proteins like \( L \)-isoaspartyl residues to normal \( L \)-aspartyl in proteins by methyl transferase activities [12]. The housekeeping role of this enzyme catalyzes the methyl esterification by transfer of methyl group from the \( S \)-adenosylmethionine to the \( \alpha \)-carboxyl group of \( L \)-isoaspartyl and \( \beta \)-carboxyl group of \( D \)-aspartyl residues via succinimide intermediate formation, which is hydrolyzed to a mixture of \( L \)-aspartyl and \( L \)-isoaspartyl residues in the ratio of 1:3 and subsequently restore their biological functions [13].
As per our knowledge, no work has been reported until date regarding the importance of PIMT in Salmonella Typhimurium. Keeping this in view, the present study was carried out to clone and sequence the PIMT gene of S. Typhimurium and to analyze the sequence relationship with other pathogenic Salmonella.

Materials and Methods

The poultry isolate of Salmonella Typhimurium strain E-2375 was procured from National Salmonella Centre (Veterinary), Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izamagor, India. The cultures were revived by growing in LB broth at 37°C overnight, and the isolated colonies were obtained by streaking on Hektoen Enteric Agar (HEA) plate. The cultures were confirmed by testing the purity, morphology and biochemical characteristics as per standard protocol [14]. Serotype confirmation was done by slide agglutination test using polyvalent antisera. Further, the recombinant clone was confirmed by colony PCR and RE digestion. The PIMT gene cloned in pET28c plasmid was confirmed by sequencing by using plasmid as template and T7 terminator and promoter as sequencing primers. Further, the recombinant clone was confirmed by SDS-PAGE after addition of isopropyl β-D-thiogalactopyranoside in the bacterial culture at the final concentration of 0.5 mM for 3 h at 25°C. The sequencing results were assembled and analyzed using DNASTAR package analysis software. The final sequence was aligned with the available PIMT gene of S. enterica serovar Typhimurium str. LT2. The sequence was submitted to NCBI Genbank. Further, the nucleotide sequence was subjected to nucleotide BLAST to compare for sequence similarities with other sequences available in the NCBI database (http://blast.ncbi.nlm.nih.gov).

Results

Salmonella Typhimurium strain of poultry origin was used in the current study. The strain showed typical morphological, cultural and serological characteristics of the Salmonella. On HEA plate, the isolate produced smooth, transparent, black centered colonies with greenish periphery (Figure-1). In slide agglutination test, the organism reacted with the standard positive serum.

The genomic DNA from Salmonella Typhimurium strain E-2375 was extracted as per the standard protocol (purelink™ genomic DNA isolation kit, Cat. no. K-1820-01, Invitrogen™, USA). To check the integrity of the DNA, about 0.5 μg sample was run on 1% w/v agarose gel (UltraPure™ agarose, Invitrogen, USA) prepared in 1X TAE [15]. The PIMT of Salmonella Typhimurium was amplified by using designed oligonucleotides primers (Table-1). The restriction enzymes NheI and BamHI sites were incorporated on 5’ of forward and reverse primers, respectively.

PCR reaction was carried out using following constituents, 7.5 μl of 2 mM dNTPs mix, 1.5 μl of PIMT FP and PIMT RP (10 pM/μl each), 1 μl of 50 mM MgCl2, 5 μl ×10 PCR reaction buffer, 5 μl Enhancer, 2 μl (96 ng/μl) of Salmonella Typhimurium genomic DNA as template and 0.5 μl (2.5 U/μl) of Pfx polymerase and the final reaction volume made up to 50 μl with Nuclease free water. The PCR program was standardized with one cycle of the initial denaturation at 95°C for 5 min, followed by 30 cycles each of denaturation (95°C for 30 s), annealing (60°C for 30 s) and extension (72°C for 45 s) and final extension (72°C for 10 min). The PCR products were checked by sub-maringe gel electrophoresis on 1% agarose gel.

The amplified PCR product digested with NheI (5 h at 37°C) and then, BamHI was added to the reaction, and the mix was incubated for an additional 1 h. Finally, heat inactivation was carried out at 80°C for 10 min. The RE digested PIMT PCR product was gel purified using QIA quick gel extraction kit (Qiagen™, Germany) using manufacturers protocol. The pET28c vector was digested in a similar manner with NheI and BamHI restriction enzymes to generate complementary overhangs. Following RE digestion the vector was dephosphorylated at 37°C for 30 min using shrimp alkaline phosphatase. The enzymes were heat inactivated at 80°C for 10 min. The digested and dephosphorylated pET28c was gel purified. NheI-BamHI digested, gel purified PIMT gene was ligated into the digested pET28c expression vector. A 3:1 molar ratio of insert (PIMT) to vector (pET28c) was used. The resultant recombinant plasmid (pET28c-PIMT Plasmid) was initially transformed into chemically competent E. coli DH5α cells with 5 μl of ligated reaction mix as per standard protocol. Screening of recombinant clones was done by colony PCR and RE digestion. The PIMT gene cloned in pET28c plasmid was confirmed by sequencing by using plasmid as template and T7 terminator and promoter as sequencing primers. Further, the recombinant clone was confirmed by SDS-PAGE after addition of isopropyl β-D-thiogalactopyranoside in the bacterial culture at the final concentration of 0.5 mM for 3 h at 25°C. The sequencing results were assembled and analyzed using DNASTAR package analysis software. The final sequence was aligned with the available PIMT gene of S. enterica serovar Typhimurium str. LT2. The sequence was submitted to NCBI Genbank. Further, the nucleotide sequence was subjected to nucleotide BLAST to compare for sequence similarities with other sequences available in the NCBI database (http://blast.ncbi.nlm.nih.gov).

Table-1: Designed primers of PIMT of S. enterica subsp. enterica serovar Typhimurium

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5'-ATATAT GCTAGC ATG GTA</td>
</tr>
<tr>
<td>PIMT F (NheI)</td>
<td>AGT GGA CGT GAT CAG-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'-ATATAT GGATCC GGC CAG</td>
</tr>
<tr>
<td>PIMT R (BamHI)</td>
<td>CTG TCC TCT GAC-3'</td>
</tr>
</tbody>
</table>

PIMT: Protein L-isospartyl O-methyl transferase, S. enterica=Salmoneilla enterica
analyzed using DNA star software and NCBI home page and 100% homology between PIMT sequences from Salmonella Typhimurium strain E-2375 (used in the current study) and retrieved S. Typhimurium LT2 PIMT (Figure-3). After SDS-PAGE following induction a band migrating ~28 kDa was observed in induced lane that was absent in the corresponding uninduced control lane (Figure-4).

The sequence when subjected to nucleotide BLAST search revealed a high degree of similarity with other Salmonella serovar (Table-2). The PIMT gene is highly conserved in most of the pathogenic serovars of Salmonella as evidenced from the sequence analysis. The obtained sequences shown 100% similarity with PIMT gene sequences of Salmonella Typhimurium DT104 (HF937208), Salmonella Typhimurium str. 08-1736 (CP006602), Salmonella Typhimurium str. D23580 (FN424405) and Salmonella Typhimurium SL1344 (FQ312003) and 99% similarity with str. DT2 (HG326213), S. Newport str. SL254 (CP001113), Salmonella Bareilly str. CFSAN000189 (CP006053), Salmonella Enteritidis str. EC20100325 (CP007360), Salmonella Anatum str. ATCC BAA-1592 (CP007531), Salmonella Heidelberg str. SL476 (CP001120) and S. Enteritidis str. EC20100130 (CP007358). The expressed protein was isolated from

**Table-2**: Results of nucleotide blast showing similarity of PIMT gene of S. enterica serovar Typhimurium strain E-2375 with other Salmonella serovars.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Description</th>
<th>Maximum score</th>
<th>Total score</th>
<th>Query coverage %</th>
<th>Maximum indent %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF937208</td>
<td>S. enterica subsp. enterica serovar Typhimurium DT104 main chromosome, complete genome</td>
<td>1153</td>
<td>1153</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CP006602</td>
<td>S. enterica subsp. enterica serovar Typhimurium str. 08-1736, complete genome</td>
<td>1153</td>
<td>1153</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>FN424405</td>
<td>S. enterica subsp. enterica serovar Typhimurium str. D23580 complete genome</td>
<td>1153</td>
<td>1153</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>FQ312003</td>
<td>S. enterica subsp. enterica serovar Typhimurium SL1344 complete genome</td>
<td>1153</td>
<td>1153</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>HG326213</td>
<td>S. enterica subsp. enterica serovar Typhimurium str. DT2, complete genome</td>
<td>1147</td>
<td>1147</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>CP001113</td>
<td>S. enterica subsp. enterica serovar Newport str. SL254, complete genome</td>
<td>1136</td>
<td>1136</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>CP006053</td>
<td>S. enterica subsp. enterica serovar Bareilly str. CFSAN000189, complete genome</td>
<td>1120</td>
<td>1120</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>CP007360</td>
<td>S. enterica subsp. enterica serovar Enteritidis str. EC20100325 genome</td>
<td>1109</td>
<td>1109</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>CP007531</td>
<td>S. enterica subsp. enterica serovar Anatum str. ATCC BAA-1592, complete genome</td>
<td>1131</td>
<td>1131</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>CP001120</td>
<td>S. enterica subsp. enterica serovar Heidelberg str. SL476, complete genome</td>
<td>1125</td>
<td>1125</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>CP007358</td>
<td>S. enterica subsp. enterica serovar Enteritidis str. EC20100130, complete genome</td>
<td>1109</td>
<td>1109</td>
<td>100</td>
<td>99</td>
</tr>
</tbody>
</table>

PIMT: Protein L-isoaspartyl O-methyl transferase, S. enterica=Salmonella enterica
the soluble fraction and migrated as around 28 kDa band on SDS gel as histidine was fused with PIMT.

**Discussion**

The importance of PIMT has been demonstrated in *E. coli*. The *E. coli* Δpimt (mutant PIMT) showed compromised survival in stationary phase of growth when subjected to oxidative stress [9]. This protein repairing enzyme is highly conserved across the phylogenetic domain including eubacteria, archaeabacteria, protozoa, fungi, nematodes, mammals and plants [16]. The structural sequence similarities reveal the same functional characterization of almost all *Salmonella* serovars. To know more about PIMT role and establishment of the importance of this enzyme, cloning and sequencing is primary attempt that can be further helpful to produce the PIMT protein for its functional characterization by identifying its target to establish its virulence properties.

**Conclusion**

Salmonellosis caused by *Salmonella* Typhimurium is an important food borne intracellular pathogen and disease spreads mainly due to consumption of contaminated poultry products. Intra phagocytic survival of *Salmonella* Typhimurium depends upon its ability to quench phagocyte generated oxidants and repair of macromolecules. Although this bacterium harbors several primary antioxidant enzymes, its survival inside the macrophages at least in part depends upon repair of damaged proteins. The repair of the isoaspartyl proteins under oxidative stress is crucial to reactivate their

---

**Figure-3:** Sequence analysis of protein L-isoaspartyl O-methyl transferase (PIMT) from positive clones resembling 100% homology: Query-PIMT sequence obtained by sequencing, subject-retrieved PIMT sequence of *S. Typhimurium* LT2 strain from NCBI.
functions. This PIMT enzyme is responsible for the repair of the same to rescue its functions. This PIMT enzyme of Salmonella is highly conserved in the nucleotide sequence among the pathogenic Salmonella serovars. The similarity in the sequences of the cloned product with other Salmonella serovars suggests its importance in the utility of PIMT enzyme for its restoration of protein function as a mean of intraphagosomal survival. It is not yet cleared whether PIMT can be used as detective anti-sera for confirmation of clinical cases of Salmonella, which needs further experimental study.

Authors’ Contributions

SKD, TKG and MM designed the experiments. SKD and DPH carried out the experimental work. MM, TKG and SKD were involved in scientific discussion and analysis of the data. SKD and MK drafted and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Authors are thankful to the Director, IVRI and National Fund for Basic, Strategic and Frontier Application Research in Agriculture (NFBSFARA), the Indian Council of Agricultural Research (ICAR), New Delhi for providing the necessary funds (in part) and facilities for the current study. Authors are also thankful to the Head, Bacteriology and Mycology, IVRI, Izatnagar for providing Salmonella Strain.

Competing Interests

The authors declare that they have no competing interests.

**********

References