Veterinary World, EISSN: 2231-0916
Available at www.veterinaryworld.org/Vol.7/September-2014/17.pdf

RESEARCH ARTICLE
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Cloning and sequencing of protein *L*-isoaspartyl *O*-methyl transferase of *Salmonella* Typhimurium isolated from poultry

S. K. Dixit[1], D. P. Hota[2], M. Kumawat[2], T. K. Goswami[1] and M. Mahawar[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

doi: 10.14202/vetworld.2014.712-716. **How to cite this article:** Dixit SK, Hota DP, Kumawat M, Goswami TK, Mahawar M (2014) Cloning and sequencing of protein *L*-isoaspartyl *O*-methyl transferase of *Salmonella* Typhimurium isolated from poultry, *Veterinary World 7*(9): 712-716.

Abstract

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

Materials and Methods: *Salmonella* Typhimurium strain E-2375 was procured from the National Salmonella Center, IVRI. The genomic DNA was isolated from *Salmonella* Typhimurium. Polymerase chain reaction (PCR) was carried out to amplify *PIMT* gene using the designed primers. The PCR product was cloned into pET28c plasmid vector and transformed into *Escherichia coli* DH5α cells. The plasmid was isolated from *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 *PIMT* sequences from *Salmonella* Typhimurium strain *E*-2375 (used in the current study) and *PIMT* sequences of standard reported strain (*Salmonella* Typhimurium str. LT2) in NCBI data base. This submitted sequence in Genbank having accession no. KJ575536.

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

Keywords: cloning, sequencing, *Salmonella* Typhimurium protein *L*-isoaspartyl *O*-methyl transferase, virulence.

Introduction

Salmonella enterica serovar Typhimurium **(**shortly *Salmonella* Typhimurium**)** is a Gram-negative, flagellated and facultative intracellular anaerobe grouped under the family 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 *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 *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 (*PIMT*) have been found to be effective for all the domains of the life, and this enzymes extensively studied in *Escherichia coli* [9] and *Pyrococcus* [10] and *Vibrio cholera* [11]. The way *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 α-carboxyl group of *L*-isoaspartyl and β-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, Izatnagar, 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 and tube agglutination test using somatic and flagellar group specific and factor antisera.

The genomic DNA was isolated 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 $MgCl_2$, 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 submarine 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.nl.nih.gov).

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

Forward primer, PIMT F (NheI)	5'-ATATAT GCTAGC ATG GTA AGT GGA CGT GTA CAG-3'
Reverse primer, PIMT R (BamHI)	5'-ATATAT GGATCC GGC CAG CTC TCC CTT GAC-3'

PIMT: Protein *L*-isoaspartyl *O*-methyl transferase, *S. enterica*=*Salmonella enterica*

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* was isolated, and the ratio of $OD_{260/280}$ was 1.97, which indicates the DNA was pure. The PCR amplified *PIMT* was purified and the concentration of the PCR product was ~20 ng/µl. This *PIMT* insert was directionally cloned into an expression vector pET28c and was PCR amplified using *PIMT*-pET28c as template. This DNA migrated as a single band of approximately 627bp in 1% agarose gel (Figure-2 and lane 1 and 2) as that of amplified insert DNA (Data not shown). The nucleotide sequence was confirmed by restriction digestion and sequencing and was submitted to NCBI GenBank (accession no. KJ575536). Sequencing data were

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

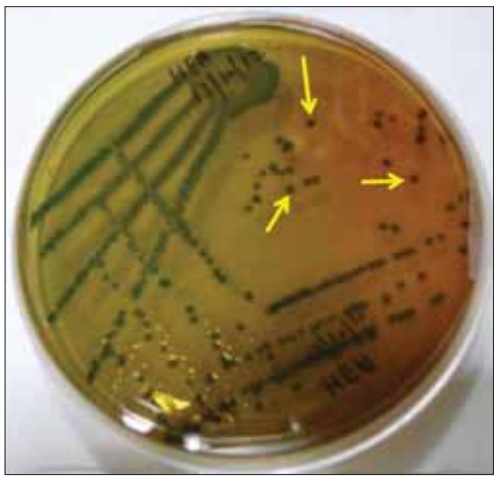

Figure-1: Growth of *Salmonella* Typhimurium E-2375 strain on Hektoen Enteric Agar plate. The cells were streaked and incubated at 37°C for overnight. Isolated colonies are depicted by arrows.

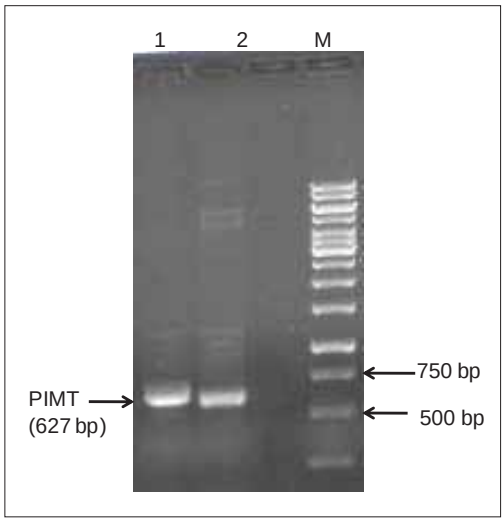

Figure-2: Polymerase chain reaction (PCR) amplification of *Salmonella* Typhimurium protein L-isoaspartyl O-methyl transferase using plasmid DNA template. The amplified products were analysed on 1% agarose gel. Lane M: 1 kb DNA ladder, Lane 1 and 2: PCR amplified products marked by arrow.

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

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

PIMT: Protein *L*-isoaspartyl *O*-methyl transferase, *S. enterica*=*Salmonella enterica*

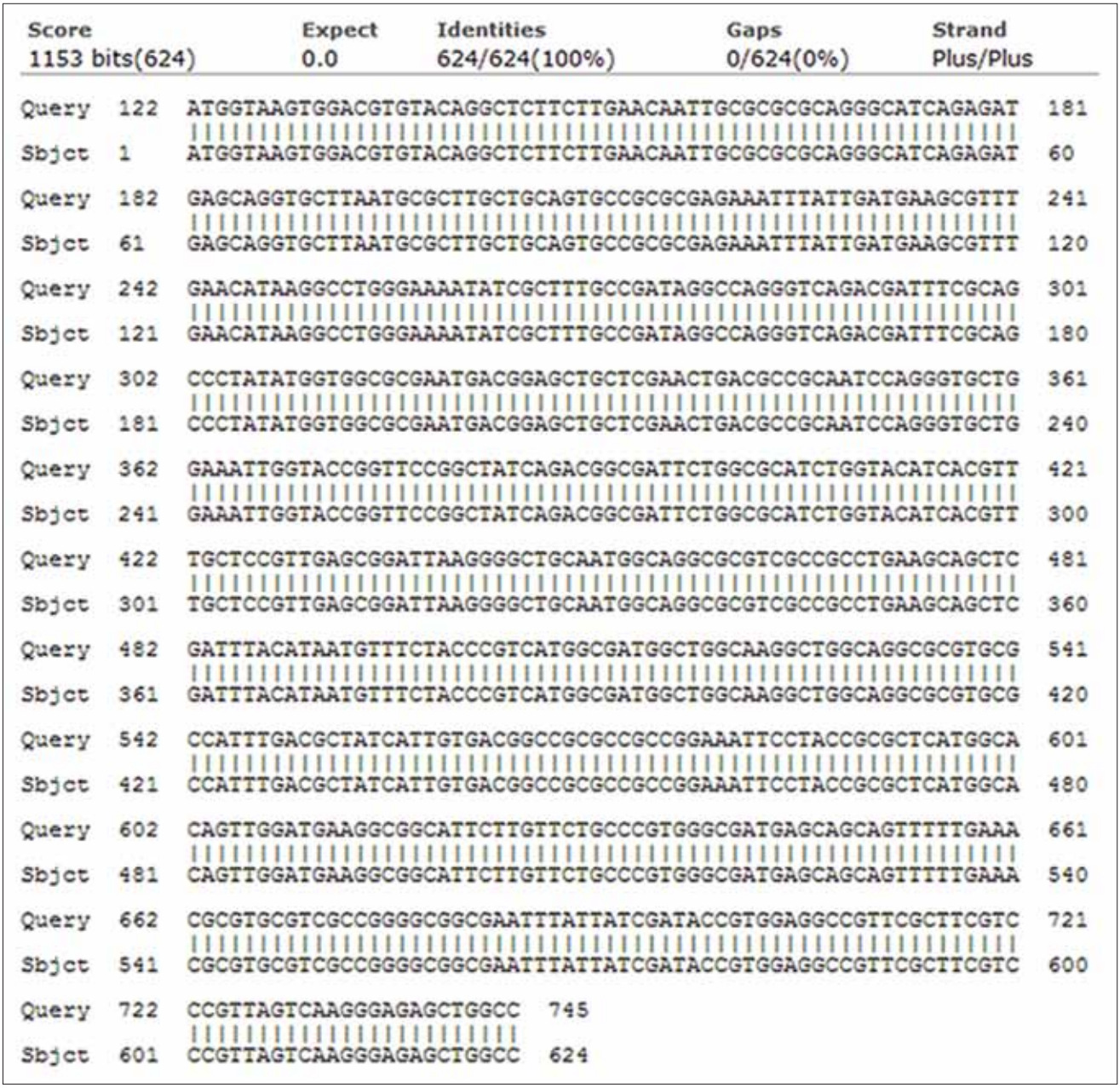

Figure-3: Sequence analysis of protein *L*-isoaspartyl *O*-methyl transferase (PIMT) from positive clones resembling 100% homology: Query-PIMT sequence obtained by sequencing, subject-retrieved PIMT sequence of *S*. Typhimurium LT2 strain from NCBI.

the soluble fraction and migrated as around 28 kDa band on SDS gelas 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, archaebacteria, 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

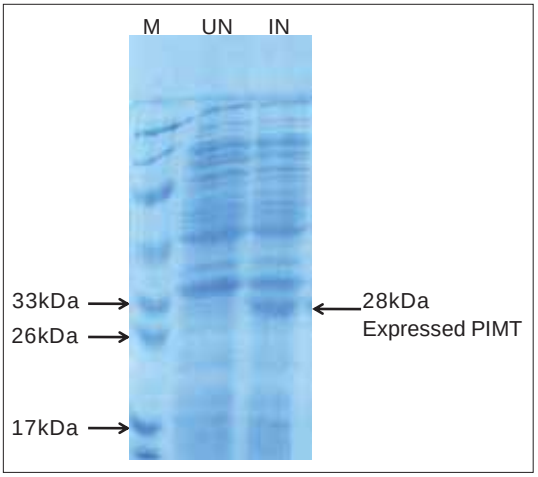

Figure-4: SDS-PAGE analysis of Escherichia coli bacterial cel lysate following induction of IPTG. Lane UN and IN are uninduced and induce cultures. Lane: M is the protein marker.

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 restorement of protein function as a mean of intraphagosomal survival. It is not yet cleared whether *PIMT* can be used as detective antisera 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.

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