Journal of Animal Research: v.6 n.1, p. 21-26. February 2016

DOI: 10.5958/2277-940X.2016.00004.8

Cloning and Sequencing of Thioredoxin Reductase (*trxB*) Gene of *Salmonella enterica* serovar Typhimurium Isolated from Poultry

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Received: 10 November, 2015 **Accepted:** 08 January, 2016

ABSTRACT

Thioredoxin reductase (TrxB) is a flavoprotein which acts as an integral part of Thioredoxin (Trx) system. This Trx system produces reducing equivalent in various oxidation-reduction reaction which ultimately targets in various metabolic processes inside cell. TrxB must be playing role in combating various stress conditions confronted by *Salmonella* Typhimurium (ST) inside its host. The aim was to clone and sequence Thioredoxin reductase *(trxB*) gene of ST strain E2375 and to construct a phylogenetic tree based on the information obtained after aligning the *trxB* sequence with other serovars of ST. *Salmonella* Typhimurium (ST) strain E2375 was procured from National *Salmonella* Centre, Indian Veterinary Research institute, Bareilly, India. Genomic DNA was isolated from the bacteria followed by amplification of Thioredoxin reductase(*trxB*) gene by PCR. The gene was restriction digested and cloned into a vector pET28c(+). The cloned *trxB* plasmid was transformed into NEB 5-alpha cells. The gene was sequenced and submitted in the gene bank. After multiple alignment analysis of that sequence by BLAST, a phylogenetic tree was constructed with the help of MEGA4.0 software. The sequence of *trxB* was found to be highly conserved among the *salmonella* serovars.

Keywords: Salmonella Typhimurium, cloning, sequencing, thioredoxin, phylogenetic tree

Food borne zoonosis has been a major concern for human health in less developed countries. 1.9 million People lost their lives annually across the glove due to many food borne diseases like caused by *Salmonella*, *Cryptosporidium* and *Toxoplasma* etc. (Schlundt *et al.* 2004). Out of these pathogens only *Salmonella* is responsible for 1.3 billion cases of gastroenteritis worldwide (San Román *et al.* 2013). Non typhoidal *Salmonellosis* (NTS) is one of the important food borne disease in the form of gastroenteritis, bacteraemia leading to focal infections. The causative agents for this NTS are many but for humans, most important is ST (Hoelzer *et al.* 2011). This is a Gram-ve, rod shaped bacteria possessing flagella (Behera *et al.* 2015). Poultry meat and eggs are important carrier for this organism (Sarvanan *et al.* 2015). *Salmonellae* grow over a wide range of temperature and pH, e.g. minimum temperature for growth in chicken is 6.7 to 7.8°C (Malick *et al.* 2015). Vaccination targeting control of this transmission is not much successful (McGregor *et al.* 2013). Resistance towards Ampicillin and Sulphafurazole is becoming a matter of concern in therapeutic management of salmonellosis (Kumar and Lakhera, 2013). Therapeutic management of infection is difficult due to the intra-phagocytic life cycle of *Salmonella*. The pathogen get internalize inside macrophages with the help of type-III secretory system which may be the basis of its chronic course of infection (Kato *et al.* 2015). Survival strategies of ST, to combat oxidative stress encountered inside macrophage is least studied. There might be some kind of reducing system which is playing role in survivability of *Salmonella* under these oxidative conditions. Trx complex is an electrochemical circuit which provides reducing equivalents to various enzymes which can help the bacteria to survive under oxidativestress conditions. Thioredoxin

with low redox potential contributes maximum towards reduction of dithiols formed inside bacteria (Nordberg and Arner 2001). Trx system donates electron to various enzymes like Ribonucleotide reductase, Thiredoxin peroxidase and Methionine sulfoxide reductase (Gromer *et al.* 2004). They do also play an important role in signaling, mediated via thiol redox control. Thioredoxin reductase is a dimeric (70 kDa) flavoprotein with a redox active site for dithiols. It comprises up of a protein thioredoxin which provides electron to subsequent enzymes and a flavo protein thioredoxin reductase which keeps these protein in their reduced state in a NADPH dependent manner (Arnér and Holmgren, 2000). *msrA* mutant of *Salmonella* has shown compromised growth against oxidative attacks of neutrophils (Trivedi *et al.* 2015). This MsrA (Reduces the oxidised methionine i.e. Met-SO to normal methionine) was found to be associated with virulence of *Salmonella* (Denkel *et al.* 2011), maintains its reduced state by receiving electrons from Trx system. In *Salmonella,* thioredoxin system contains two different thioredoxins i.e. TrxA and TrxC along with a reductase (TrxB) (Peters *et al.* 2010). Trx A was found to be associated with type-III secretory system, so must be contributing towards survivability of bacteria inside macrophages (Minamino 2015). In the current study we have cloned and sequenced the *trxB* gene of *Salmonella* Typhimurium Strain E2375, a poultry isolate and have constructed a phylogenetic tree showing evolutionary stability of this gene across various serovars of *Salmonella*.

MATERIALS AND METHODS

Ethical approval

The study was done after the approval of institutional animal ethical committee.

Bacterial culture

Salmonella Typhimurium Strain E2375 was a poultry isolate obtained from National *Salmonella* center, IVRI, India. The initial procurement was maintained in our laboratory at -80 ºC. The culture was revived by thawing and was streaked on a Hektoen Enteric Agar (HIMEDIA, M-467) plate and left for overnight incubation. One single isolated colony was inoculated into 10 ml of Luria Bertani (LB) broth and was incubated over night at 37 ºC. The culture was later used for isolation of genomic DNA.

Amplification of *trxB* gene

Isolation of genomic DNA was done from the overnight grown culture by following the standard protocol given by Purelink™ Genomic DNA mini kit. The purity and concentration of the isolated DNA was checked by using NanoDrop spectrophotometer (ThermoScientific) which gave the absorbance and ratio of A_{260}/A_{280} to scrutinize any impurities present. Primers for the *trxB* were designed manually by calculating the Tm by considering the attribute of each base pair and compatibility was checked by using Oligo Analyzer 1.0.2 software. The primers were flanked by restriction enzyme site for NheI and BamHI which were incorporated at the 5' end of forward and reverse primers respectively. The sequences of primers are given in table 1. PCR was performed using the isolated genomic DNA as template. 100 µL of reaction mixture was prepared containing 10X Dream Taq buffer-10 µL,10 nm dNTP-10 µL,1 µL of each primer, 4 µL of isolated genomic DNA, 1µL of Taq DNA Polymerase (Thermo scientific) adjusting the final volume by nuclease free water. The reaction was performed upto 32 cycles in a thermo cycler (Applied Biosystem) as per the following reaction conditions, initial denaturation at 95ºC for 5 min, denaturation at 95 ºC for 30 sec, annealing at 62 ºC for 30 sec,extension at 72 ºC for 30 sec and final extension at 72 ºC for 10 min. PCR product was checked by gel electrophoresis on 1 % agarose gel containing Ethidium Bromide (0.5 µg/ ml) under UV illumination.

Table 1: Sequence of primers used for the amplification of *trxB* gene of *Salmonella* Typhimurium Strain E2375

Target gene	Primer sequence (5`- 3`)	Product size
trxB	For: ATATATGCTAGCATGGGCACGACCAAACACAGT	993bp
	Rev: ATATATGGATCCCTATTTGCTCGCGTCGGCCAG	

Cloning and sequencing of *trxB* gene

The PCR product was gel eluted by following the standard protocol of QIAquick® Gel extraction kit (Cat. No.-28704). The purity and concentration of the eluate was checked by NanoDrop spectrophotometer. Both the PCR product and vector pET28c(+) was restriction digested initially with NheI for 3 hours followed by Bam HI for 1 hour at 37ºC. The RE enzymes were inactivated by incubating them at 80ºC for 10 min.

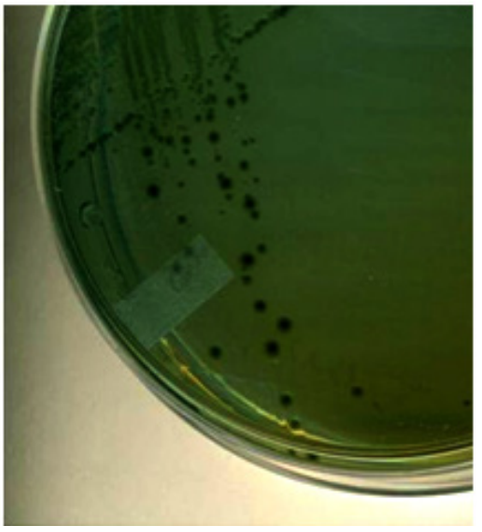

Fig. 1: *Salmonella* Typhimurium strain E2375 colonies grown on Hektoen Enteric Agar

To avoid the self-ligation of digested pET28c(+), it was dephosphorylated by using Shrimp alkaline phosphatase, (1U/ µL) Promega, USA. The digested PCR products and pET28c(+) were gel purified by QIAquick® Gel extraction kit (Cat. No.-28704). The ligation reaction was carried out by using T_4 DNA ligase (Thermo Scientific). A 3:1 molar ratio of insert to vector was taken and incubated for 1hr at room temperature followed by overnight incubation at 4 ºC. 5 µL of recombinant plasmid having *trxB* insert was transformed into chemically competent NEB5-alpha (C2987H/C2987I) cells following 5 minutes transformation protocol provided by the manufacturer. The transformed cells were plated on Kan^+ (30µg/ mL) LB plates and was incubated overnight at 37 ºC. Screening of the positive colonies was done by colony PCR. The recombinant plasmid having *trxB* insert was isolated by Gene Jet Plasmid mini prep kit following manufacturers instruction and was confirmed by PCR amplification, agarose gel electrophoresis, insert release following RE digestion and sequencing with T_7 promoter/terminator sequencing primers. The data obtained from custom sequencing of *trxB* gene was analyzed by BLAST and aligned by Gene Tool™. After proper annotation it was submitted to gene bank by using the Bankit portal. MEGA4.0 software was used to construct the phylogenetic tree by taking 41 similar sequences of different *Salmonella* serovar as an output to the query against *trxB* gene from BLAST analysis (Felsenstein,1985; Tamura *et al.* 2004; Tamura *et al*. 2007).

RESULTS

Bacterial culture

ST strain E2375 produced typical smooth, transparent, black centered colonies with greenish surrounding when grown on HEA plates (Figure 1).

Amplification of *trxB* gene

The concentration and A_{260}/A_{280} ratio was found to be 151 ng/mL and 2 respectively. The amplified PCR product with *trxB* gene specific primers was detected just below 1000bp region (Figure 2) (Amplicon size: 969bp +Additional sequences of primers: 24bp = 993bp).

Cloning and sequencing of *TrxB* gene

The gel purified *trxB* and pET28c(+) was ligated after RE digestion. Transformation into NEB5-alpha cells and seeding those transformed cells onto Kan^+(30µg/ mL) plates, produced a few white transparent, recombinant colonies (Figure 3) after overnight incubation.

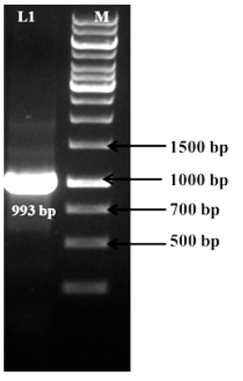

Fig. 2: 1% Agarose gel electrophoresis of PCR product of *trxB* amplified from genomic DNA of *Salmonella* Typhimurium strain E2375. M: 1 kb Plus ladder; L1: PCR product

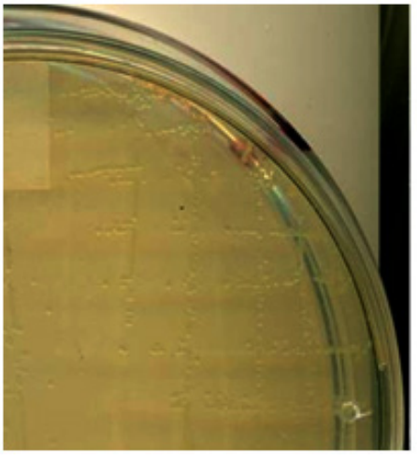

Fig. 3: Transformed NEB5-alpha colonies grown on Kan+ Luria-Bertani agar plates

Colony PCR with T_7 Test primers gave an amplicon of desired size i.e. above 1000 bp ladder (Figure 4). The recombinant plasmid having *trxB* insert was found to be little retarded on gel electrophoresisas compare to the non-recombinant pET28c(+) (Figure 5). PCR with *trxB* gene specific primers, amplified a product size of 993 bp by using the recombinant pET28c(+) as a template (Figure 6). Again an insert of desired size (969 bp) was released after RE digestion of recombinant pET28c(+) (Figure 7). After submission of the sequence in Genebank an accession no as KT153578 was provided by the same. A scaled phylogenetic tree was constructed by using Neighbor – Joining method based on distance matrix (Figure 8). The bootstrap value was found to be 99.

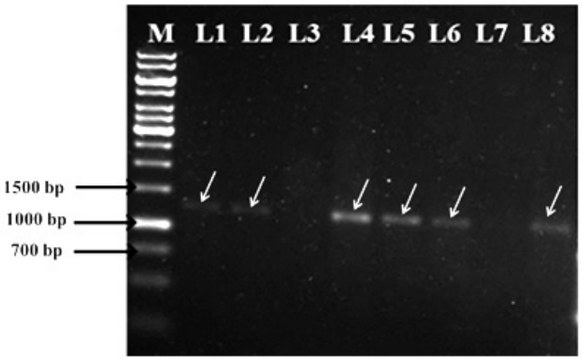

Fig. 4. 1% Agarose gel electrophoresis of Colony PCR products of *trxB* cloned NEB5-alpha cells. M: 1 kb Plus ladder; L1-L8: Colony PCR product

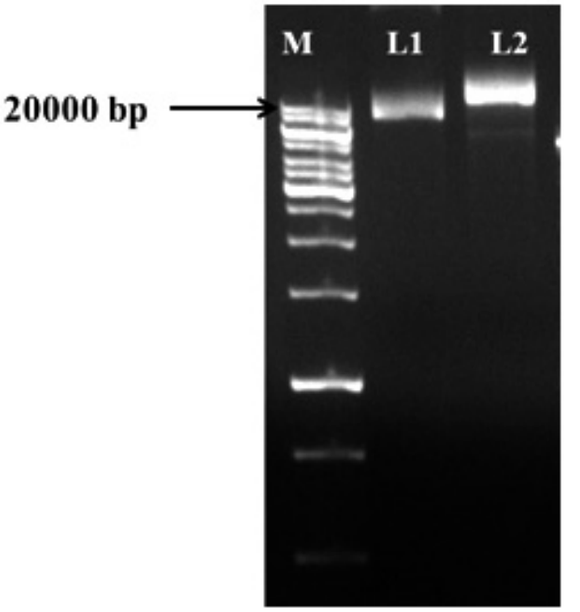

Fig. 5. 0.8% Agarose gel electrophoresis of un-cloned pET-28c(+) and *trxB* cloned pET-28c(+) plasmids. M: 1 kb Plus ladder; L1: un-cloned pET-28c(+); L2: *trxB* **c**loned pET-28c(+)

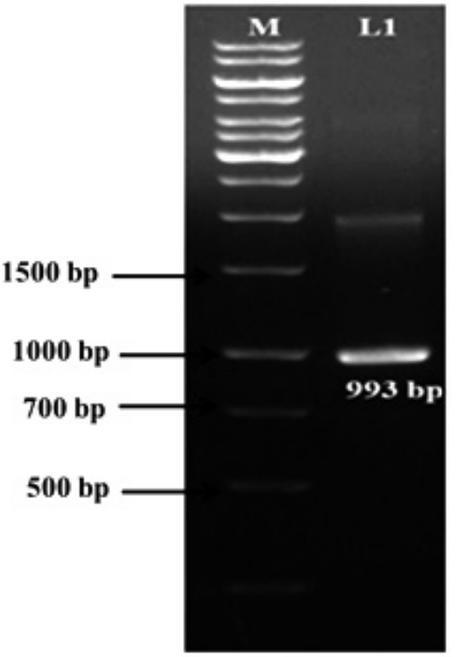

Fig. 6. 1% Agarose gel electrophoresis of PCR product using recombinant pET-28c(+) as template. M: 1 kb plus ladder; L1: PCR amplified product.

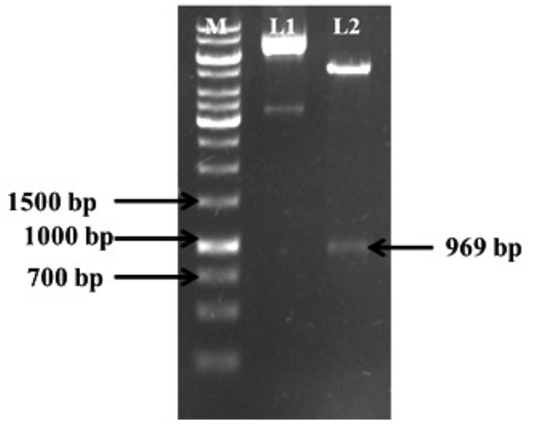

Fig. 7: Confirmation of trxB cloned pET-28c(+) by insert release and running it on 1% Agarose gel electrophoresis. M: 1 kb plus ladder; L1: Digested non-recombinant pET28c(+) ; L2: Digested recombinant pET28c(+)

DISCUSSION

Non typhoidal *Salmonellosis* has been a major food borne zoonotic disease across worldwide. *Salmonella* Typhimurium is one of the crucial causative agent of this disease which gets transmitted mostly via animal products, specifically poultry products like meat and eggs (OIE, 2010).

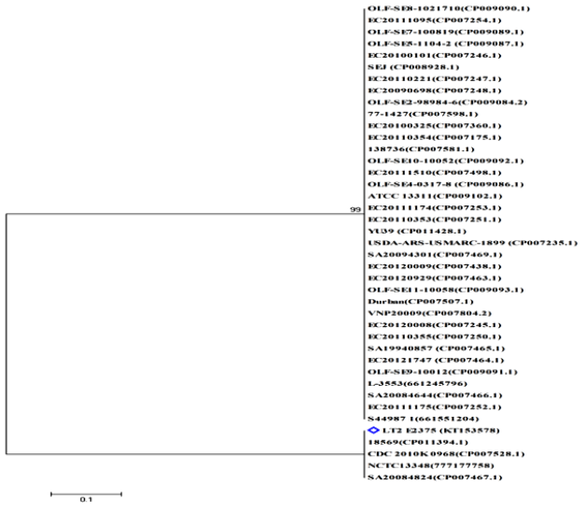

Fig. 8. Phylogenetic analysis of *trxB* **i**n comparison with different serovars of *Salmonella spp.*

Inside poultry, ST experiences various stress conditions during its intraphagocytic life cycle (Trivedi *et al.* 2015). Oxidative stress is one of the important defense strategy executed by host to damage various macromolecules of *Salmonella*. These damages to macromolecule must be repaired and restore in order to survive inside host. Thioredoxin system, being a universal reducing equivalent donor must have some contribution towards this survival procedure(Arnér and Holmgren, 2000). A relation between MsrA (An enzyme, reduces oxidised methionine residues to their normal form) and survival of *Salmonella* inside neutrophils has been recently established (Trivedi *et al.* 2015). MsrA receives reducing equivalent from this Trx system to maintain its active state. Contribution of TrxA has been studied in *Salmonella* virulence (Bjur *et al.* 2006). Though much work has not been done on this component of Trx system i.e. Thioredoxin reductase (TrxB), still it can be an effective target for current generation prevention strategy for *Salmonella*. In the current study we have shown that *trxB* is undergoing high selection pressure and is highly conserved across various serovars of *Salmonella*. Mutant construct of *trxB* may be helpful for further elucidation of its importance in *Salmonella* life cycle.

CONCLUSION

Salmonella Typhimurium is causing major losses to poultry industry due to its zoonotic importance since time. Effective vaccination strategies can help to prevent transmission of this disease. The intra-phagocytic lifecycle of *Salmonella* exposes it to various oxidative stress conditions. Thioredoxin reducatse (TrxB) is an essential component of thioredoxin sytem. It must be playing role in combating these oxidative stress. In the present study we have found that the *trxB* sequence is highly conserved across various serovars of *Salmonella.*

ACKNOWLEDGMENTS

The authors are thankful to the (1) Director, IVRI, (2) Dr Manish Mahawar (Division of Biochemistry) and NFBSFARA for providing necessary facilities and funds for the current study.

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