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# Induction of deletion mutation on *ompR* gene of *Salmonella enterica serovar Typhi* isolates from asymptomatic typhoid carriers to evolve attenuated strains for vaccine development

Senthilkumar B[1*], Anbarasu K[2], Senbagam D[3], Rajasekarapandian M[4]

[1]*Centre for Biotechnology, Muthayammal College of Arts & Science, Rasipuram-637 408, Tamil Nadu, India*
[2]*Department of Marine Biotechnology, Bharathidasan University, Tiruchirappalli-620 024, Tamil Nadu, India*
[3]*Department of Biotechnology, Vivekanandha College of Engineering for Women, Tiruchengode-637 205. Tamil Nadu, India*
[4]*Department of Zoology, Arignar Anna Government Arts College, Namakkal-637 001, Tamil Nadu, India*

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ABSTRACT

**Objective:** To develop attenuated strains of *Salmonella enterica serovar Typhi* (*S. typhi*) for the candidate vaccine by osmolar stress. **Methods:** *S. typhi* SS3 and SS5 strains were isolated from asymptomatic typhoid carriers in Namakkal, Tamil Nadu, India. Both strains were grown in LB (Luria Bertani) medium supplemented with various concentration of NaCl (0.1- 0.7M) respectively. The effect of osmolar stress was determined at molecular level by PCR using MGR 06 and MGR 07 primers corresponding to *ompR* with chromosomal DNA of *S. typhi* SS3 and SS5 strains. Attenuation by osmolar stress results in deletion mutation of the *S. typhi* strains was determined by agglutination assays, precipitation method, SDS PAGE analysis and by animal models. **Results:** The 799 bp amplified *ompR* gene product from wild type *S. typhi* SS3 and SS5 illustrate the presence of virulent gene. Interestingly, there was only a 282 bp amplified product from *S. typhi* SS3 and SS5 grown in the presence of 0.5, 0.6 and 0.7 M NaCl. This illustrates the occurrence of deletion mutation in *ompR* gene at high concentration of NaCl. Furthermore, both the wild-type and mutant *S. typhi* outer membrane SDS-PAGE profile reveals the differences in the expression of *ompF*, *ompC* and *ompA* proteins. In mice, wild type and mutant strains lethal dose ($LD_{50}$) were determined. The mice died within 72 h when both the wild type strains were injected intraperitoneally with 3 log $CFU \cdot mL^{-1}$. When the mice were injected with the mutants in same dosage, no clinical symptoms were observed; whereas the serum antibody titre was elicited within two weeks indicated that the mutants have the ability to induce protective humoral immune response. These results suggest that *S. typhi* SS3 and SS5 may be used as good candidate strains for the development of live attenuated vaccine against salmonellosis. **Conclusions:** This study demonstrates that the *S. typhi* strains were attenuated and could be good vaccine candidates in future.

## 1. Introduction

*Salmonella enterica serovar Typhi* (*S. typhi*) are Gram-negative, motile, non-lactose fermenting, facultative, intracellular pathogenic bacteria causing typhoid fever exclusively in humans, responsible for both morbidity and mortality in worldwide. It has been well documented that typhoid fever is a systemic infection characterized by the presence of *S. typhi* in the liver, spleen, and bone marrow, invades and survives within macrophages and tissues of the reticuloendothelial system[1]. *S. typhi* colonizes gall bladder and survive in the infected individual after the symptoms disappeared, serving as a reservoir for the spreading of the typhoid fever[2]. In developing countries, *Salmonellae* infection poses a greatest health concern in the water, food

*Corresponding author: Balakrishnan Senthilkumar, Centre for Biotechnology, Muthayammal College of Arts & Science, Rasipuram-637 408, Tamil Nadu, India.
Tel: +91-9443286292
Fax: +91+91-4287 220227
E-mail: senthilmicro@yahoo.co.in

industry[3] and continues to be a worldwide health problem. It has been reported that the morbidity of typhoid fever is highest in Asia with 93% of the global level (WHO). Number of studies has been describing the emergence of multidrug resistant *S. typhi* which renders concern on use of antibiotics in the treatment process[4-8]. Given the fact, currently use of vaccines to prevent the Salmonellae infections holds great promise in clinical research as an alternative approach to antibiotics as well to reduce the disease burden. It has been reported that *S. typhi* like other enteric pathogens has to respond quickly to the changing host environments encountered *in vivo*, which will exert different demands and stresses *ie*. osmolarity, pH, oxygen tension and nutrient starvation on the bacterial cell[1]. Bacteria possess systems for sensing these external environments, responding by co-ordinately controlling the expression of genes whose products are employed to assist survival under different condition[9].

Traditional approaches to the development of vaccines for bacterial diseases include parenteral injection of purified components of live vaccines or killed whole cell organisms. In contrast live oral vaccines have several advantages over parenteral vaccine such as low cost, easy to preparation and administration, safe to the organism as well as to the administrator. But the development of live vaccines has limitations such as understanding of the pathogenesis of the disease at molecular level. It is prerequisite that the candidate live vaccine strains must be non- revertable genetic alterations that affect the virulence of the organism, but not its induction of an immune response. Attenuation of virulent *Salmonella* strains has been demonstrated by evaluating *Salmonella typhimurium mutants* in the murine typhoid model[10].

Several classes and combinations of mutant of *Salmonella typhimurium* attenuation have already been reported, including those with mutations in genes encoding key enzymes in the aromatic biosynthetic pathway (*aro* mutants) [11] and those with mutations in controlling the expression of genes in response to environmental stimuli (*ompR* mutants) [12]. It has been well documented that attenuated strains of *S. typhi* in humans can stimulate both humoral and cell-mediated immune responses. It is proven that cell-mediated immune responses are very important mediators of the protection conferred by *Ty21a* and other attenuated *S. typhi* live oral vaccine strains[13].

CVD 909 live attenuated *S. typhi* induces cell mediated immune response, however second dose affects ability due to constitutive expression of Vi Ag[14]. *S. typhi Ty2* derivative TSB7 harbouring deletion mutation in ssaVand *aroC* demonstrated to elicite immune response[15].

The importance of *ompR-envZ* genes is well characterized in *S. typhi* in response to osmolar stress and its virulence in human beings[10]. Moreover, the mutation in *ompR* can attenuate the virulence characteristics also reported[1]. Considering the importance of NaCl concentration in the expression of *ompR* gene in the present study, we propose that a high osmolar concentration can be a strong stress and used for induction of deletion mutations in *ompR* gene to attain attenuated strains which is not revert in animal models to develop candidate vaccines.

## 2. Materials and methods

### 2.1. Sample collection

Early morning stool samples were collected from the asymptomatic typhoid carriers in Namakkal District and transported to the laboratory using screw-capped tubes with Cary-Blair medium.

### 2.2. Identification of S. typhi strains

The preliminary morphological and biochemical tests[16] were performed for the identification of *S. typhi* including Gram's staining, motility test, catalase test, oxidase test, sugar fermentation, indole test, methyl red test, voges-proskauer test, citrate utilization test, triple sugar iron test and urease test. *S. typhi* was also identified based on the growth pattern on enrichment medium (Selenite-F broth), Differential medium (Mac Conkey Agar), and Selective medium (Bismuth Sulphite Agar, Xylose, Lysine and Deoxycholate Agar).

### 2.3. Confirmatory test for identification of S. typhi strains

The bacterial agglutination test (high titre serum agglutination test) was adopted to identify *S. typhi*[17]. A drop of saline was placed in clean microscopic slide and a small amount of culture of *S. typhi* from the solid medium was emulsified using inoculation loop followed by a drop of antiserum (SPAN) of *S. typhi* was added and mixed with sterile stick. The slide was observed for agglutination within 2 min.

### 2.4. Induction of mutation on SS3 and SS5 strains

LB broth was prepared and various concentrations of sodium chloride ranging from 0.1 to 0.7 M were supplemented. *S. typhi* SS3 and SS5 strains were inoculated into the LB broth and incubated for two weeks of period with repeated subculture. From these cultures, DNA was isolated[18] and subjected to amplification of *ompR* gene using MGR 06 and MGR 07 primers[1].

### 2.5. Polymerase chain reaction (PCR)

Total genomic DNA of *S. typhi* isolates grown on LB broth was prepared by phenol/chloroform/isoamyl alcohol extraction (25:24:1) and spooling from ethanol, as described by Sambrooke *et al*[19]. RNA contamination in DNA was eliminated using RNase A (BioBasic INC, Canada) PCR was performed in a final volume of 50 μL containing 1 μL of genomic DNA (100 μg) as template, 0.5 μL of each primer, (*ompR* gene forward primer 5′AGG GGC GTT TTC ATC TCG-3′ (MGR 06) and reverse primer 5′-ACC AGG CTG ACG AAC AG-3′ (MGR 07) (First Base, Singapore) (20 μm), 48 μL of Master Mix (Promega) to make a final concentration of 15 mM $MgCl_2$, 100 mM Tris-Hydrochloride, 4 μL of 2.5 mM dNTPs mix (dATP, dCTP, dGTP, dTTP) and 2.5 Units of Thermo stable DNA polymerase (Promega). Subsequent amplifications were then performed in a Thermal Cycler (Techgene, UK), according to the following profile: 35 cycles of 2 min at 94 ℃, 30 sec at 94 ℃, 1 min at 49 ℃, 1 min at 72 ℃ for extension, and 7 min at 72 ℃ for the final extension. Amplified products were purified using Eppendrof perfectprep gel cleanup kit and analyzed by electrophoresis in 1% (w/v) agarose (Sigma) gel. DNA sequence was done by automated sequencer at MWG Biotech Private Limited, Bangalore, India. DNA sequence data reported here was deposited in GenBank under the accession number of EU834745 and EU849617.

### 2.6. Detection of Vi antigen in SS3 and SS5 strains

*S. typhi* strains were placed on a clean glass slide, heated at 60 ℃ for 30 min and cool. Equal amount of Vi antiserum (Acme Progen Biotech India Pvt Ltd, India) was placed and mixed gently for agglutination test[1].

### 2.7. Detection of intra cellular accumulation of Vi polysaccharide

*S. typhi* strains were grown overnight on LB agar (Hi Media) plates containing aromatic compounds at 37 ℃. Cells were harvested, suspended in PBS and the optical density was adjusted to 0.8-1.0 at 650 nm. Then the cells were disrupted by sonication in 30 sec bursts for a total of 2.5 min with an interval of 30 sec on ice. Counter current immunoelctrophoresis was done[20] to detect intracellular accumulation of Vi polysaccharide.

### 2.8. Outer membrane protein isolation from SS3 and SS5 wild and mutant strains

To begin with total cell, envelopes were removed by sonication of aerobically grown LB broth cultures. The sonication was carried out with 5 mL aliquots of cells (OD at 650 nm of 0.8-1.0) suspended in 10 mM sodium phosphate buffer (pH 7.2). The resulting sonicated material was subjected to centrifugation to remove the cell debris at 100 000 *g* for 10 min. Eventually, the inner membrane was solubilized by 1% sodium lauryl sarcosinate treatment, and outer membrane enriched fraction was collected by a final centrifugation at 100 000 *g* for 30 min. The pellets were resuspended in phosphate buffer and then analyzed in 10% SDS-PAGE[21].

### 2.9. Assessment of virulence in mice

Six mice per group for SS3 and SS5 wild type strains and mutants were used. Female mice aged 6-8 weeks were used to determine $LD_{50}$ of the wild strains with 0.5 mL doses of log phase culture of attenuated SS3 and SS5 strains of having 3 log CFU·$mL^{-1}$. Second injection was being given on 8th day with the same dose. After the 14th day mice was bleed by retero orbital plexus. The antiserum was separated and stored in aliquots with 0.1% sodium azide (Merck) at -20 ℃[22]. Mice were injected intraperitoneally with various log phase culture (12 h of LB broth culture again inoculated in the LB broth and incubated for 3 h) of 10 fold dilutions of SS3 and SS5 wild isolates were used. The animals were monitored for morbidity and mortality[23,24].

## 3. Results

### 3.1. Identification of S. typhi strains

Based on biochemical tests and specific morphological growth characteristics in the respective medium such as the production of red colour deposits in the Selenite F broth, jet-black colour colonies on BSA, red smooth colonies on XLD and colourless colonies on Mac Conkey agar, the isolates were identified as *S. typhi*. A clear agglutination of emulsified colony confirms the isolates were *S. typhi*, with antiserum on the glass slide (bacterial agglutination test).

### 3.2. Amplification of ompR gene encoding the virulence region SS3 and SS5 strains

PCR primers MGR 06 (F) and MGR 07(R) corresponding to the region of *ompR* used to amplify the chromosomal DNA from *S. typhi* isolates. The PCR products of *ompR* genes were analyzed by agarose gel electrophoresis. The amplified product was also compared with standard DNA ladder (Sigma). *S. typhi* SS3 and SS5 strains were give rise to 799 bp for *ompR* (Figure 1). In case of the mutants, 282 bp *ompR* gene products (Figure 2) were observed using same MGR 06 (F) and MGR 07 (R) primers, and 517 bp was found to be deleted due to osmolar stress at 0.5, 0.6 and 0.7 M NaCl

(Table 1).

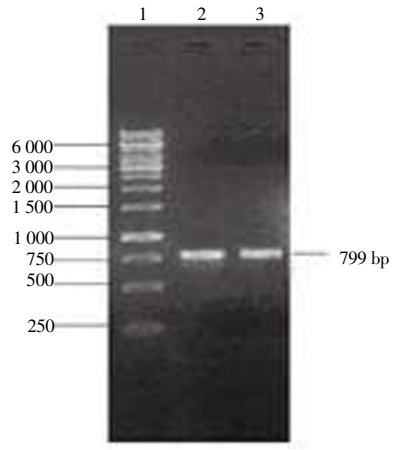


**Figure 1.** Amplified *ompR* gene of *S. typhi* wild strains.
Lane1-1 kb DNA ladder (Sigma), Lane 2- *S. typhi* SS3, Lane 3- *S. typhi* SS5.

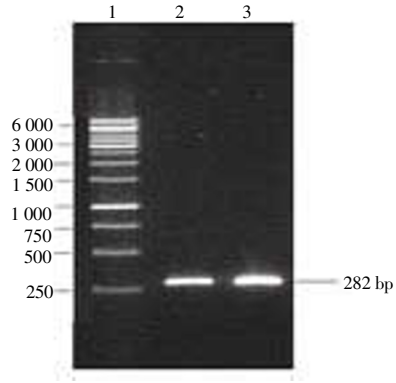


**Figure 2.** Amplified *ompR* gene of *S. typhi* mutant strains.
Lane1: 1 kb DNA ladder (Sigma), Lane 2: *S. typhi* SS3, Lane 3: *S. typhi* SS5.

**Table 1**
NaCl concentration to induce mutation on *S. typhi* SS 3 and SS 5 strains.

| *S. typhi* strains | | Osmolar concentration (M) | *OmpR* PCR product (bp) | Remarks |
|---|---|---|---|---|
| SS3 | SS5 | 0.1 | 799 | Wild |
| SS3 | SS5 | 0.2 | 799 | Wild |
| SS3 | SS5 | 0.3 | 799 | Wild |
| SS3 | SS5 | 0.4 | 799 | Wild |
| SS3 | SS5 | 0.5 | 282 | Mutant |
| SS3 | SS5 | 0.6 | 282 | Mutant |
| SS3 | SS5 | 0.7 | 282 | Mutant |

### *3.3. Intra cellular accumulation of Vi polysaccharide*

The lack of agglutination of *ompR* mutants with antiserum was due to a defect in the expression of Vi polysaccharide. Counter current immunoelctreophoresis was performed on the bacterial sonicates with Vi antiserum to test the presence of Vi polysaccharide. The sonicates of mutant strains failed to produce any line of precipitation indicated that Vi polysaccharide was not being accumulated intracellularly in *ompR* mutants, whereas wild strains were produced the precipitation band (Figure 3).

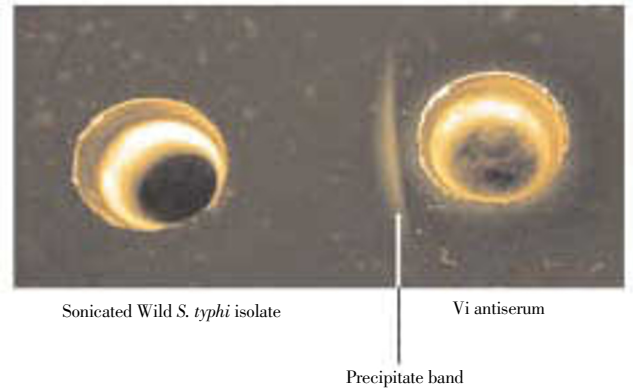


**Figure 3.** Immunodiffusion pattern for sonicated *S. typhi* strain reacted with Vi antiserum.

### *3.4. Expression and regulation of the porins ompC and ompF in the SS3 and SS5 strains*

Because *ompR* is known to regulate expression of porin channel, in the present study we probe into the effect of the *ompR* mutation on the expression of *ompC* and *ompF* by comparing with outer membrane proteins of the mutant and wild type strains of *S. typhi*. The expression of *ompC*, *ompF*, and *ompA* in the mutants as well as in the wild type strains were analyzed by SDS-PAGE. We observed *ompF*, *ompC* and *ompA* were found to be 36 kDa, 35 kDa and 33 kDa respectively in the wild type strain, whereas the mutant isolates showing only *ompA* which do not have porin forming properties. Thus, it was found that expression of *ompC* and *ompF* was down regulated in mutants (Figure 4).

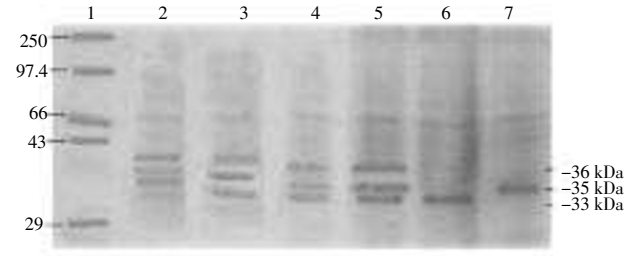


**Figure 4.** SDS-PAGE (10%) analysis of OMPs from *S. typhi* upon treatment with various concentrations of NaCl.
Lane 1-Protein molecular weight marker (GeNei, India), Lane 2-0.1 M NaCl osmolar induced *S. typhi*, Lane 3- 0.2 M NaCl osmolar induced *S. typhi*, Lane 4- 0.3 M NaCl osmolar induced *S. typhi*, Lane 5- 0.4 M NaCl osmolar induced *S. typhi*, Lane 6-0.5 M NaCl osmolar induced *S. typhi*, Lane 7- 0.6 M NaCl osmolar induced *S. typhi*.

### *3.5. Phenotypic characterization of SS3 and SS5 mutant strains*

Phenotypic test was carried out to identify the mutant

strains which were no longer agglutinated with Vi antiserum (Table 2). These strains were also screened for the presence of *ompR* deletions. From these results, it is suggested that the *ompR* component systems were involved in the regulation of Vi synthesis in *S. typhi*.

**Table 2**

Agglutination test of *S. typhi* mutants grown on LB agar with different osmolarity.

| NaCl Con (M) in LB agar | Slide agglutination of *S. typhi* with Vi antiserum | |
|---|---|---|
| | SS3 | SS5 |
| 0.1 | + | + |
| 0.2 | + | + |
| 0.3 | + | + |
| 0.4 | + | + |
| 0.5 | − | − |
| 0.6 | − | − |
| 0.7 | − | − |

### 3.6. Assessment of virulence in mice

$LD_{50}$ was found to be 3 log CFU·$mL^{-1}$ for SS3 and SS5 strains in this study. The experimental animals died within 72 h after inoculation intraperitoneally indicating these strains were virulent. However, the mutant strain injected mice group showed no mortality or any clinical symptoms. Serum was collected from the mutant mice injected with the attenuated strains of SS3 and SS5. The collected serum was added with broth culture of SS3 and SS5 wild-type strains on a glass slide. Agglutination within 2 min was observed and the results indicated antibody was raised against the mutant isolates without any symptoms. The immunized animal's serum antibody titre was found to be 1:60. This study obviously revealed that the mice immunized with attenuated strains were found to be protected with elicited humoral immune response.

## 4. Discussion

Typhoid fever continues to be an important infection in endemic countries and among travellers to these areas. Typhoid fever has been declining in many middle-income countries, whereas the bulk of cases occur in the Indian subcontinent and in south-east Asia[25].

A total of 138 enteric fever cases were found in United Kingdom during 2005-2010. Of these 65% and 35% cases were caused by *S. typhi* and *Salmonella paratyphi* respectively. These individuals were acquired the infection form abroad in the age group 16-40 years and under 16 years. Indian, Pakisthani, Banhgladesh patients have been made the majority (87%) of the typhoid cases. New entrant and foreign visitors accounted for 10% of the cases, 92% cases had a history of recent foreign travel; 57%patients had travelled to visit friends and relatives and 23% patients were new entrants / foreign visitors from the Indian subcontinent[26].

Typhoid bacilli has become a major threat to the public due to the severity of the disease , recurrence of disease through asymptomatic carrier state[27] emergence of multi drug resistance[4,28] and its use as a potential candidate in bioterrorism[29], thereby it is necessary to develop potential candidate vaccine for typhoid fever[30].

Attenuated strain development for bacterial vaccines by virulent gene knock out is one of the key research areas at present against infectious bacterial diseases prevention. In this study, we described a modified approach for the generation of mutant *Salmonella* strains to be attenuated against Salmonellosis in mice model study. Development of attenuated *S. typhi* strains and their ability to elicit protective immunity in animal models have already been reported[31,32]. It is proved that live attenuated vaccines elicit potent cell mediated responses. It has also been proved that the family of two component regulatory system genes responses to various environmental stimuli in bacteria and its involvement in bacterial virulence. Towards that end it has been studied that alterations in porin channel by demanding bacterial growth with the presence of sugars[33]. On such alteration in the *ompC* porin, R74C substitution (*ompC* R74C or *ompC*1 cys) has been reported. The *ompC* and *ompF* porin genes were transcriptionally regulated by a classical two component signal transduction regulatory system consistent of the *ompR-envZ* proteins[34,35]. Furthermore, *Salmonella* strains harbouring mutations in two component regulatory system of *phoP-phoQ*, could be attenuated to develop *Salmonella* vaccines[36].

*In vivo* down regulation of Vi Ag provides proof of principle that it is possible to generate a live attenuated vaccine that induces specific antibodies after single oral administration[37].

The WHO has been recommended for the programmatic use of new-generation typhoid vaccines in high-risk areas of countries where typhoid fever is still endemic. Past and current typhoid vaccination programs that have taken place in Thailand (using the old whole-cell vaccine) and in China, Vietnam and India (using the new-generation injectable Vi polysaccharide vaccine)[38]. Vi polysaccharide vaccine will not protect the patients if *S. typhi* strains are negative for Vi polysaccharide. The recent data of the community vaccination in high incidence areas of Kolkata, Karachi, and north Jakarta showed the cost effectives of Vi polysaccharide vaccine in children[39].

It is well documented that *ompR* gene of *S. typhi* is involved in the regulation of the synthesis of Vi capsular

polysaccharide in response to osmolority. Considering all these facts we used high osmolar conditions to study the effect of *ompR* gene regulation in the *Salmonella* isolates from asymptomatic typhoid carriers. Remarkably, in agreement with, Pickard *et al*[1], we found that Vi expression is very sensitive with osmolarity of the growth medium. Interestingly, our results showed that 0.5, 0.6 and 0.7 M of NaCl yielded mutant attenuated strains which leads to 282 bp PCR amplified products. We speculate that resulting mutant strains due to deletion of 517 bp in *ompR* gene. Moreover, as expected[22,40] in this study, the SDS-PAGE results showed that in the wild type isolates the outer membrane proteins *viz*. *ompC*, *ompF* and *ompA* were found to be 36, 35 and 33 kDa respectively. In contrast, the mutant strains only expressed *omp A* at 33 kDa, other two proteins of *ompC* and *F* were found to be down regulated.

In mice challenging study, the animals were died within 72 h of intraperitoneal injection of wild type strains SS3 and SS5 indicating that the strains were virulent, whereas, no clinical symptoms were observed when the mice were intraperitoneally injected with the high concentration NaCl osmolar stress induced *ompR* mutant strains alternatively, they produce antibody titre after 15 days.

In 2007, a live attenuated vaccine against typhoid fever DV-STM-07 has been shown to be potent in murine model of salmonellosis[41]. The vaccine developed through independent chemical conjugation of Vi polysaccharide of *S. typhi* and o polysaccharide of S. paratyphi A to CRM197, a non toxic mutant of diphtheria toxin. The vaccine was found to be inducing humoral response than unconjugated Vi polysaccharide[42].

With these findings, we believe that the NaCl osmolar stress induced mutants may be used for vaccine development. In conclusion, this is the first time, we report that *S. typhi* isolated from asymptomatic typhoid carriers with its down regulated expression of Vi polysaccharide upon NaCl osmolar stress induced deletion mutation in *ompR* gene would be a rational object against which to elicit antibody for a candidate of typhoid vaccine.

## Conflict of interest statement

We declare that we have no conflict of interest.

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