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Research Article

Rpoe Gene Mutant Salmonella Enterica Serovar Typhimurium Protects From Wild Type Typhimurium Infection In Mice Model

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Abstract

Typhoid is a food and water-borne infectious disease transmitted through the faecal-oral route. The causative bacterium Salmonella Typhi has emerged resistant to several drugs and causes thousands of death worldwide. Ironically, the two available vaccines protect for 3 to 7 years only, with protection rates ranging between 50 and 70%. Therefore, the rpoE mutant of Salmonella species was screened for its vaccine potential against typhoid with the expectation of a long-lasting immune response. The immune potential of the ΔrpoE strain (ΔME) of Salmonella Typhimurium was evaluated in the BALB/c mice, a surrogate model. We took four groups with six mice in each. The LD50 (lethal dose) was decided for oral and intraperitoneal routes. The experimental groups were vaccinated with different doses of ΔrpoE strain, including the oral group. Multiple-dose of the mutant was given. Further, the mice with a lethal dose of wild type were challenged to see the protection against S. Typhimurium. In addition, we examined the faecal excretion of wild-type bacteria and humoral immune response. The booster dosage on the 7th and 14th days enhanced antibody titre significantly. In control groups, no mice survived after ten days of challenge, whereas in the vaccinated groups, more than 83% survived.

Keywords: Mutant; rpoE; Typhi; Typhoid; Vaccine

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Introduction

Typhoid fever is caused by *Salmonella enterica* serovar Typhi (henceforth *S.* Typhi), a gram-negative bacteria belonging to Enterobacteriaceae family. It causes 22 million cases, with more than 200,000 deaths yearly [1]. This orofecally transmitted bacteria is highly prevalent in populations with poor sanitation and inadequate hygiene. It is difficult to diagnose enteric fever from other febrile illnesses clinically. Unfortunately, apart from prolonged illnesses, death in typhoid may occur due to intestinal perforation, hemorrhages, and other neurological manifestations if left untreated [2]. Although appropriate antibiotics reduce the duration of fever, bacterial shedding and mortality rate, in the recent past, indiscriminate use of antimicrobials has led to the emergence of multidrug resistance in enteric fever-causing serotypes. Intriguingly, humans are the only reservoir for these serovars. Despite developing the first typhoid vaccine (whole-cell killed), >100 years ago [3] we have only two anti-typhoid licensed vaccines internationally. Live attenuated Ty21a is given orally to a population of more than five years of age. The other vaccine is ViCPS, an injectable polysaccharide vaccine derived from Vi antigen of serovar Typhi that may be given to children >2 years of age. Currently available vaccines provide 65-70% protection for 3-7 years [4-6]. Live attenuated vaccine (LAV) is considered superior to subunit vaccine because LAV induces humoral and cell-mediated immunity. Different live attenuated candidate vaccines targeting mostly metabolic or virulence genes are under different phases of clinical trials. One such candidate vaccine is CVD909, a triple mutant of *aroC, aroD* and *htrA* and is under phase II trial constitutively expressing Vi antigen. The other candidate, Ty800 is a *phoP-phoQ* gene mutant developed by Avant Immunotherapeutics and under phase II trial. Several other mutants developed by targeting *clpX, htrA, ssaV, aro* operon, and *cya/crp* are under clinical trials. Other genes such as *dam, wecA, cdt, recA, recB, relA, spoT*, and *rpoS* mutant have also been checked for their vaccine potential. The search for an ideal vaccine against *S.* Typhi infection is still on (Plotkin and Cam, 1995). *The* lifestyle of this restricted human pathogen is a major hurdle in vaccine development. It survives extra- and intracellularly and tolerates our host defense system well. It has various mechanisms to evade and colonize the host organs and tissues.

Bacteria have a set of two types of genes; one is housekeeping and the luxury gene. A stress regulator is a luxury gene set induced in stress conditions. These stress regulator genes are shut off in normal situations and are induced exclusively to withstand harsh conditions. The *rpoE* is a stress regulator gene that is induced under periplasmic stress. The outer surface of bacteria is the primary site interacting with the host and targets of the defense system [3]. The *SigmaE* gene (*rpoE* in *Salmonella*) is an alternative sigma factor of *Escherichia coli*, which plays a role in maintaining cell envelope integrity in normal growth in stress conditions. It is required for cell viability and maintenance of membrane physiology during stress [7-9] . It is mainly induced either by heat stress or ethanol which disrupts the protein folding in the cell membrane. It may also be induced when overproduction of outer membrane porin or deactivation of periplasmic

chaperones under normal conditions occurs for any reason [10-15]. the 32-kD sigma-factor responsible for the heat-inducible transcription of the heat shock genes. rpoH is transcribed from at least three promoters. Two of these promoters are recognized by RNA polymerase containing sigma 70, the predominant sigma-factor. We purified the factor responsible for recognizing the third rpoH promoter (rpoH P3 The irony is that we do not have an exact animal model for *S.* Typhi infection. However, this is encouraging that *S.* Typhimurium, a murine pathogen mimics a similar infection process [16] It is also interesting to note that serotype Typhimurium encoded *rpoE* gene, controls its survival and proliferation inside macrophages, as is in the case of *S.* Typhi. It regulates Type-III secretion systems (T3SS) encoded by *Salmonella* Pathogenicity Island-1(SPI-1) and SPI-2, stress responses, and several global regulators. In addition, it controls SPI-2 by upregulation of *ssrB* and downregulation of the H-NS gene, which is required for intracellular survival [17] We hypothesize that if *the rpoE* (S*igmaE*) gene is deleted from *S.* Typhimurium, it will succumb to the stressful conditions inside the macrophage and be killed host in the macrophages. These macrophages may present the *Salmonella* antigen on their surface as antigen-presenting cells. This phenomenon may lead to induction of the better humoral and cell-mediated immune responses. Therefore, in the present study, we planned to delete the *rpoE* gene of *S.* Typhimurium and evaluate its efficacy in the mice model (BALB/c).

Results and Discussion

Deletion and characterization of *rpoE* mutant

The lambda red recombination system removed the *rpoE* from *Salmonella enterica* serovar Typhimurium. Mutants were confirmed by colony PCR using the internal sequences of *rpoE* gene (Figure. 1).

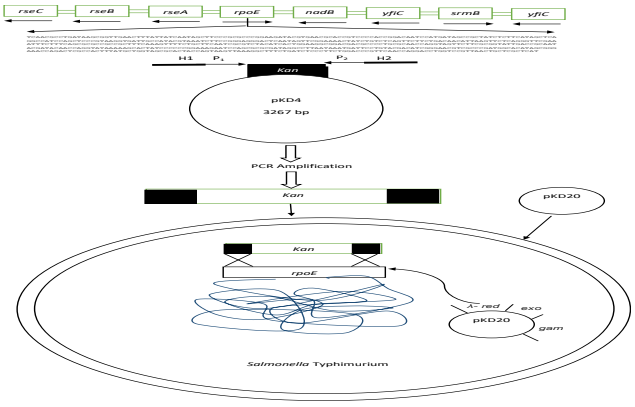

Figure 1: Showing the plan for deletion of *rpoE gene.*

The flanking genes of *rpoE*, left side genes are *rseA, rseB, rseC* and right side are *nadB, yfiC, srmB*. The sequence of *rpoE* gene is available in Pubmed data and shown just below the rpoE gene. The primers, H1 and H2 have been selected from the immediate neighbouring genes of *rpoE, i.e. rseA on the left and nadB on the right side.* The primers P1 and P2 show a priming site for extending the resistance gene (kanamycin) present on the pKD4 plasmid.

The growth curve was identical when the ΔME and WT were incubated at 37°C without acid exposure in the liquid medium (Figure. 2a); however, after giving a 10 min and 20 min exposure to pH 3.5, the WT could grow with a longer log phage of 7.5h at both the temperatures of 37°C and 42°C while ΔME could not enter the log phase despite the viability of the bacterial cells, which was evident by the recovery on inoculating the acid-exposed ΔME in bile broth with 9h of log phase (Figure 2a-c).

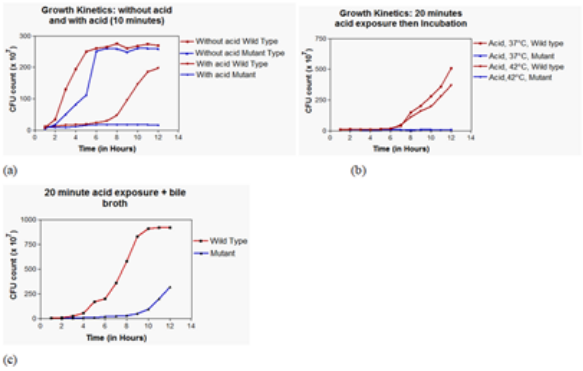

Figure 2: Growth kinetics (a) Without acid exposure and with 10 minutes acid exposure. (b) Acid exposure (20 minutes) and incubation at different temperatures. (c) 20 minutes acid exposure and bile broth enrichment.

Evaluation of single-dose immunization through intraperitoneal route by ΔME

A single immunization via IP route with ΔME at 1.310^4 CFU/mouse gave 55.55%, and 1.310^6 CFU/mouse gave 61.6% protection (Table 1).

Evaluation of triple-dose immunization through intraperitoneal and oral routes by ΔME

The three booster doses of 1.310^4 CFU/mouse and 1.310^6 CFU/mouse given IP provided a protection rate of 61.1 and 83.3%, respectively, while oral doses of 1.310^4 CFU/mouse gave protection of 66.7%, and 1.310^6 CFU/mouse and 1.310^7 CFU/mouse protected 88.8% of the mouse (Table 1).

WT-STM (CFU/mouse), IP	Mortality (%)	Total doses and route	ΔME (CFU/mouse),	Mice survived (total number =18)	Percentage of survival	Antibody titre against the somatic antigen
1.1102	00	Single dose, IP	1.3104	10	55.5	<1:20
1.1103	50		1.3106	11	61.1	<1:20
1.1104	70	Triple dose, IP	1.3104	11	61.1	>1:160
1.1105	100		1.3106	15	83.3	>1:640
		Triple dose, O	1.3104	12	66.7	>1:160
			1.3106	16	88.8	>1:640
			1.3107	16	88.8	>1:320

Table 1: Evaluation of different doses through oral and intraperitoneal routes of ΔME for the protection from the IP challenge of WT of S. Typhimurium at the LD50 dose (1.1×103 CFU/mouse).

IP - Intraperitoneal route and O – oral

Safety of ΔME in mice model through intraperitoneal and oral routes

The table shows that the ΔME at doses 1.310^4, 1.310^5, 1.310^6, 1.310^7, 1.310^8 orally and IP up to the dose of 1.310^7 CFU/mouse did

not cause mortality. All the groups could detect viable ΔME after 7 days of the last vaccination dose in their stool. However, the LD50 for WT was 1.110^3 CFU/mouse (Table 2).

WT-STM (CFU/ mouse), IP	Mortality (%)	ΔME (CFU/ mouse), IP	Mortality (%)	ΔME (CFU/ mouse), O	Mortality (%)
1.1102	00	1.3104	00	1.3104	00
1.1103	50	1.305	00	1.305	00
1.1104	70	1.3106	00	1.3106	00
1.1105	100	1.3107	00	1.3107	00
				1.3108	00

Table 2: Determination of lethal and safety of wild (WT) and mutant (ΔME) S. Typhimurium in BALB/c mice through different routes.

Even after 125 years of the first effective vaccine developed by Almroth Edward Wright, we do not have near-ideal vaccines against typhoid fever. These human-restricted human bacterial *Salmonella* serotypes can be eradicated from the globe if we could develop a satisfactory and acceptable vaccine. A live attenuated oral vaccine is the most desired of many vaccine-development designs since this bacterium may also go intracellularly, inducing a cell-mediated immune response. For the development of live oral vaccines, several approaches, e.g. chemical mutagenesis, genetically engineered vaccines with desired mutations in one or many genes, e.g. *rpoS*, *phoPQ*, ssaV, and htrA intended to block systemic infection have been tried [18]. They are either in the experimental or early stages of clinical trials. In a similar line, we planned to delete the *rpoE* gene. This gene is responsible for controlling 40% of the regulator genes of Salmonella (168 out of 411) [19]. for several functions, e.g. coordinates multiple stress response, intramacrophage survival and proliferation, maintenance of cell envelope integrity, regulates expression of both type 3 secretion system (T3SS) and all aspects of membrane physiology under stress environments. The intracytoplasmic pH is usually lower than 7.0, especially in Salmonella-containing vacuoles (SCV), where the *S.* Typhi resides. The lower pH in the SCV induces the expression of several proteins responsible for inhibition of fusion of SCV with lysosomal vesicles and intramacrophage survival. The genes regulated by this sigma factor *(rpoE)* can inhibit the killing of a bacterium by the host cell. Based on this principle, we deleted the *rpoE* gene expecting the bacterium to be killed in the cytoplasm since this deletion will result in the fusion of SCV with vesicles containing lysosomes. After killing, the phagosomes may present the Salmonella antigen for interaction with immune cells to induce a specific immune response. In the present study, we took *S.* Typhimurium, and its natural host, a rodent (mice), as a surrogate because enteric fever-causing serotypes can infect only human beings and, to some extent, a few higher primates (Chimpanzees). This is an accepted surrogate model for animal experimentation for *S.* Typhi and other serotypes. For creating the *rpoE* mutant, we used the one-step gene inactivation method [20]. After deciding on the lethal dose of the wild-type *S.* Typhimurium through oral and intraperitoneally routes in BALB/c mouse, single and multiple doses of the mutant were given through both routes mentioned above. The mice were observed for eight weeks after the challenge with the wild-type strain.

Interestingly, a 10^6 CFU/mouse IP dose protected more than 88% of the animals under experimentation. Interestingly higher doses (10^7 CFU/mouse) of mutant strain with multiple booster doses were required to achieve a similar level of protection if given orally. When antibody titres were estimated based on Widal's principle four weeks after the mutant strain's last dose, a significant rise in antibody titre could be seen in the mice given 10^6 CFU/mouse IP and 10^7 CFU/mouse orally.

It is worth mentioning that neither oral nor intraperitoneally administered mutant strain at a dose of 10^8 CFU/mouse result in fatality in any experimental mouse. The findings in the present study showed that *rpoE* gene mutant was very safe and able to induce a protective level of immunity in the BALB/c mouse model.

A live attenuated bacterial vaccine to eradicate typhoid fever is essential. The ideal candidate vaccine should induce both humoral and cell-mediated immunity systemically and at mucosal surfaces after a single oral administration. However, a significant rise in antibody titre against *S.* Typhimurium indicates the potential of the *rpoE* gene mutant to induce an immune response.

The *rpoE* mutant of *S.* Typhimurium generated in the present study survived at pH 3.5 for 20 min. This observation indicates that the mutant can pass through the stomach without compromising viability before reaching the small intestine. This is further supported by isolating mutant strains from faeces for 7 days after vaccination. Intriguingly, the faecal excretion was not associated with any visible morbidity.

It is worth mentioning here that despite being safe, the lacunae of the mutant were that a single dose was unable to induce a protective level of immunity. As the bacterium was excreted in the faeces for more than a week, it might have been able to boost the immune response. However, given the lack of data relevant to mechanisms involved in disease clearance, it may not be denied that *rpoE* mutant experimentally proven to be safe and highly immunogenic may nonetheless fail to offer satisfactory protection when administered orally/IP as a single dose. If a schedule of administration of the present mutant is standardized in animals providing satisfactory protection even with multiple doses will negate the need to re-engineer the mutant further to provide significant immunity.

Deleting stress response genes makes the bacteria susceptible to the hostile intracellular environment of reticuloendothelial cells leading to killing and its' antigens being presented to immune responsive cells. We had Δ*rpoE* mutant of *S.* Typhimurium, which was observed to be sufficiently attenuated and gave adequate protection with booster doses.

Materials and methods

The ethical clearance for this work was taken from the Institutional Animal Ethics Committee of the Institute of Medical Science, Banaras Hindu University, Varanasi, 221005 (no. Dean/2015-16/EC/1709 dated 19/03/2016).

Generation of the *rpoE* deletion mutant in *S.* Typhimurium

The Δ*rpoE* (ΔME) was constructed in the wild-type reference strain *of S.* Typhimurium ATCC 14028. The Δ*rpoE* (ΔME) was generated by a one-step gene inactivation strategy described by Wanner et. al., [20]. The ATCC 14028 strain of Typhimurium was transformed with pKD20 carrying lambda red recombinase system under the arabinose inducible promoter. The transformants carrying the helper plasmid pKD20 were grown in LB containing ampicillin (100 mg/ml) and 7 mM L-arabinose (Himedia) till the OD reached 0.5–0.6

at 600 nm. Electro-competent cells were prepared by washing the cells thrice with ice-cold Milli-Q water and 10% glycerol. PCR product containing the kanamycin resistance gene (amplified from pKD4) flanked by sequences upstream and downstream of the *rpoE* gene was amplified with primers (*rpoE* H1 and *rpoE* H2) were electroporated into the *S.* Typhimurium carrying pKD20 as per instructions given by the manufacturer's instructions (Bio-Rad, USA). Mutants were selected for their ability to grow on LB-containing kanamycin (75 mg/ml). The deletion was confirmed by specific primers targeting the inside sequences of the *rpoE*. In the Δ*rpoE* strain of *S.* Typhimurium, no amplification could be detected, while in the wild-type, 124 bp segment was amplified. Thus, the deletion mutant was designated as Δ *rpoE* (ΔME) in Typhimurium. The primers used in the study are shown in (Table 3).

	Sequences	Annealing temperature	Amplicon Size
Deletion primers			
rpoE:H1 F	5'GGTCCTGGTTGAACGGGTCCAGAAGGGAGATCAGAAAGCCGTGTAGGCTGGAGCTGCTTC3'	58°C	1.5 kbp
rpoE:H2 R	5'GAACTTTATTATCAATAGCTTCCCCGCGCCCGGAAGATACGCATATGAATATCCTCCTTAG3'		
Confirmation primers for deletion			
rpoE: F	5'CATGATAGCCGCTATCTCTTC3'	59°C	124 bp
rpoE:R	5'GTTGTCAGAAGAACTGAGACAG3'		

Table 3: In-house designed primers for deletion of rpoE and confirmation of mutant with a deletion in *Salmonella Typhimurium*.

The plan for mutant generation is shown in figure-1

The growth curve at different pH

Ten microliters of overnight broth culture were inoculated in 50 ml LB in triplicate and incubated at 37°C, shaking at 150 rev/min know the *in-vitro* growth kinetics of ΔME and WT strain.

Animal experimentation

Six to eight-week-old BALB/c female mice were procured from Central Drug Research Institute, Lucknow and maintained in Central Animal Facility, Banaras Hindu University, Varanasi, India, under specific pathogen-free conditions. The plan of experimentation has been shown in (Tables 1 & 2).

Determinations of Lethal Dose (LD50) of WT and ΔME

Six to eight-week-old BALB/c female mice were inoculated to determine the LD_{50} value. First, four mice were injected with the wild and mutant serotype intraperitoneally *(IP), and* orally, as shown in (Table 2). Deaths of the animal were recorded for up to 2 weeks.

Fecal shedding of the *Salmonella* Typhimurium (WT) and ΔME

Three groups of mice were inoculated intraperitoneal routes with WT and ΔME through oral and IP at the dose of 10^6 CFU/mouse. Freshly recovered faecal pellets were collected, and the colony count of the bacterium per gram of stool was calculated.

Evaluation of vaccine potential of mutant strain (ΔME)

Single-dose immunization through oral and IP routes and multiple booster doses through oral and IP routes, as shown in Table 3, was evaluated after giving the challenge of WT S. Typhimurium at the LD_{50} dose of 1.110^3 CFU/mouse.

Estimation of humoral immune response

One millilitre of blood was collected and preserved for control. Antigen was prepared from overnight growth WT. It was washed thrice and fixed with formalin. The concentration of bacteria was brought to $1.5\ 10^8$ CFU/ml. The agglutination was done with the pre and post-vaccination serum collected from the experimental mice following the tube method of agglutination.

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Conflicts of interest

No conflict of interest declared.

Significance and impact of the study

The *SigmaE* gene (*rpoE* in *Salmonella*) is a luxury gene induced in stress conditions which play a role in maintaining cell envelope integrity in normal growth in stress conditions *rpoE.* Lower pH in the intracellular niche leads to the killing of the mutated bacteria and antigens are expressed on the surface of macrophages inducing a better immune response. In the present study, we have observed a significant rise in antibody titre against *S.* Typhimurium indicates the potential of the *rpoE* gene mutant to induce an immune response. Thus, *rpoE* mutants may be a potential candidate vaccine for typhoid.

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