

Salmonella enterica serovar Typhimurium strain lacking *pmrG-HM-D* provides excellent protection against salmonellosis in murine typhoid model

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Abstract

The superiority of live attenuated vaccines in systemic salmonellosis has been proven over killed and subunit vaccines, because of its ability to induce protective cell mediated immunity by CD8+ T cells. A live attenuated *Salmonella enterica* serovar Typhimurium vaccine has been developed by systematic site directed deletion of the *pmrG-HM-D* chromosomal genomic loci. This gene confers involved in antimicrobial peptide resistance and is involved in LPS modification, both of which are the major immune evasive mechanisms in *Salmonella*. The efficacy of the newly developed strain in inducing protection against mortality after challenge with the virulent wild type *Salmonella typhimurium* 12023 was evaluated in mice model of typhoid fever. Animals were immunized and then boosted on days 7 and 14. Following challenge with virulent *S. typhimurium* 12023, organ burden and mortality of vaccinated mice were less compared to non-immunized controls. The vaccine strain also induced elevated CD8+ T cells in the vaccinated mice. This multiple mutant vaccine candidate appears to be safe for use in pregnant mice and provides a model for the development of live vaccine candidates against naturally occurring salmonellosis and typhoid fever.

Keywords: *pmrG-HM-D* mutant; *Salmonella typhimurium*; Vaccine

1. Introduction

Salmonella typhi, a causative agent of typhoid fever is raising a major threat due to its potential use in bioterrorism [1] and the non-availability of an efficient candidate vaccine. About 21.6 million people have suffered from typhoid and over 216,500 have succumbed to it, globally, in the year 2000 alone [2]. The incidence of typhoid is high (>100 cases per 100,000 population each year) in south-central Asia, southeast Asia and possibly southern Africa (10–100 cases per 100,000) [2]. The increased appearance of antibiotic resistant strains of *Salmonella* further complicates the situation [3]. The *Salmonella* vaccine strains created so

far had mutations in the metabolic genes or pathogenicity islands. *Salmonella* harboring mutations in SPI2 [4], *aro* A [5], *hrtA* [6] have been tested as a vaccine candidates in both animal models and humans. These vaccine candidates have not been able to fully protect the animals. The only licensed attenuated live oral typhoid vaccine, *S. typhi* strain Ty21a, is well tolerated and immunogenic, but requires three or four spaced doses of $2-6 \times 10^9$ CFU given every other day, an important practical shortcoming [7]. Ty800, a vaccine strain, where *phoP* gene was knocked out, exhibited excellent protection in human volunteers [8]. Creation of vaccine strain which will provide full protection against typhoid fever requires greater understanding of immune evasion strategies used by *Salmonella*. The intricate lifestyle of *Salmonella* inside the host cells, makes this pathogen a better survivor. By evading the immune mechanism, *Salmonella* survives in the host and can cause life-threatening infec-

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tions. Though studies have been done to look at the immune profile of T cells [9], cytokine level [10] and the antibody response [11] in murine model of *Salmonella*, none of these could not rescue the mice from *Salmonella* infection, suggesting that the immunity is not protective. Till date, the candidate antigen of *Salmonella* which can evoke a long lasting protective immunity remains unidentified. As vaccine is the only answer to combat this disease, thought should be given to rationally develop a vaccine with long-term protective immunological memory against *Salmonella* infection.

The key to success for many bacteria in causing infection is colonization of host tissues. An enteric bacterium, such as *Salmonella*, gains entry through the oral route and survives the harsh environment of the intestine. At the intestinal mucosa, these bacteria encounter host defense mechanisms including antimicrobial peptides (AMPs), which are cationic, amphipathic molecules that kill bacteria by membrane permeabilization. Within the intestine, AMPs are secreted into the lumen by Paneth cells located in the base of intestinal crypts. AMPs are also found within phagocytic cells located in the intestinal submucosa. The ability of *Salmonella* to survive within the host intestine and within professional phagocytes is likely to depend, at least in part, on mechanisms of resistance to AMPs. The Pmr systems in *Salmonella* which includes *pmrHIFJKLM*, *pmrD*, *pmrA-B*, *pmrE* and *pmrG* are known to modify LPS, confer resistance to antimicrobial peptides and to regulate other two component regulatory system [12]. Because of the intricate network among *pmr* systems, we have created a multiple mutant where the *Salmonella* is devoid of *pmrD*, *pmrG* and the *pmrHIFJKLM* (*pmrH-M*) operon. This multiple mutant *Salmonella* strain ($\Delta pmrG-HM-D$) is highly attenuated and does not cause disease even at higher doses on oral and intraperitoneal administration. A single dose of this vaccine candidate could protect the mice very efficiently in the challenge experiments, suggesting its ability to evoke a strong protective response in the host.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Salmonella enterica serovar Typhimurium strain NCTC 12023 was used as wild type strain (kindly gifted by Prof. M. Hensel). Bacteria were routinely cultured in LB broth and on LB agar plates. If required to maintain plasmids, carbenicillin (50 $\mu\text{g/ml}$) and/or chloramphenicol (50 $\mu\text{g/ml}$) were added.

2.2. Construction of *pmrG-HM-D* mutant of *Salmonella typhimurium*

$\Delta pmrG-HM-D$ was constructed by one-step deletion strategy of Datsenko and Wanner [13]. *Salmonella* transformants

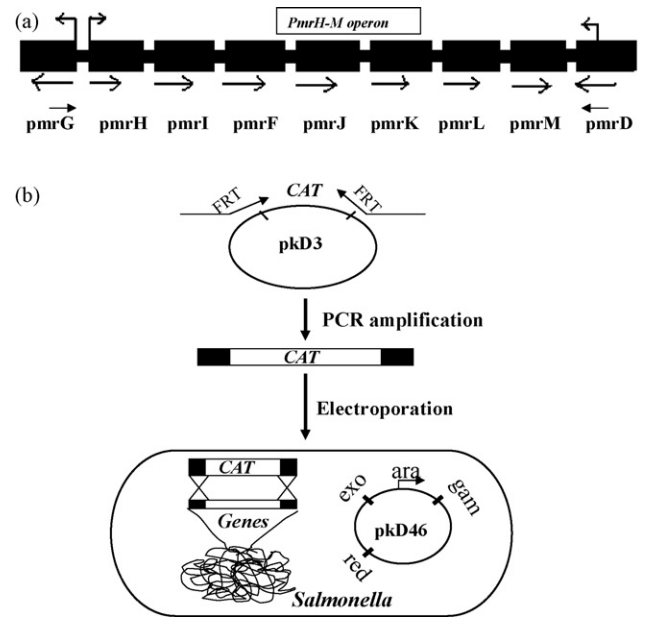


Fig. 1. (a) Genetic make up of *pmrGH-MD* genes in WT *Salmonella*. *pmrG*-putative aluminum inducible gene, *pmrH*-putative LPS modification and polymyxin B resistance gene, *pmrI*-putative sugar transferase, *pmrF*-putative transferase, *pmrJ*-putative cytoplasmic, *pmrK*-effect polymyxinB resistance and LPS modification, *pmrL*-putative mallinin resistant gene, *pmrM*-putative inner membrane protein, *pmrD*-PolymyxinD resistant gene. Thin arrow indicates the region used for designing primers to create the knockout. (b) One step deletion strategy was applied to construct the multiple mutant.

carrying a red helper Plasmid (pKD46) were grown in LB with carbencillin and 10 mM L-arabinose at 30 °C. When the cells reach an OD (600 nm) of 0.4–0.5, electrocompetent cells were made by washing three times with ice-cold autoclaved milli Q water and 10% glycerol. The chloramphenicol cassette (CAT) of pKD3 was amplified with primers having overhangs of flanking regions of *pmrGM-HD* (Fig. 1). This PCR product was electroporated into *Salmonella* having pKD46. Immediately after electroporation 1 ml of SOC was added and incubated at 37 °C for 1 h before plating on to the LB-agar plates containing chloramphenicol (50 $\mu\text{g/ml}$). Mutants were selected by virtue of their ability to grow on the antibiotic plates and confirmed by colony PCR using specific confirmatory primers (Table 1).

Table 1
Showing primer used for construction and confirmation of mutant

Deletion	
Forward primer	5'-ATTTTGTATATCGGCACTGAAGGCTTACCAGCGC-GTGTAGGCTGGAGCTGCTTCG-3'
Reverse primer	5'-AAAATGATCGCCGAGGCGAATTCATGATATTACT-CATATGAATATCCTCCTTA-3'
Confirmation	
Forward primer	5'-GACCTGCCCGAGAAAAC-3'
Reverse primer	5'-AAGAAATCGCATTATGTC-3'

2.3. Eukaryotic cell lines and growth conditions

RAW 264.7 cells (gifted by Prof. Anjali Karande, Dept of Biochem., IISc) and INT407 (NCCS, Pune) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS, Sigma) and 10 mM sodium pyruvate at 37 °C and 5% CO₂. Colo205 (NCCS, Pune) cells were grown in RPMI-1640 supplemented with 10% FCS and 10 mM sodium pyruvate at 37 °C and 5% CO₂.

2.4. Isolation of peritoneal macrophage

Six- to eight-week old female BALB/c mice were intraperitoneally injection of 5 ml Brewer's thioglycolate broth (HiMedia, India). After 4 days, the macrophages were isolated from peritoneal cavity by injecting 5 ml ice-cold RPMI-1640 medium and aspirating after 2 min under sterile conditions.

Peritoneal cells were washed once (300 g, 5 min, 4 °C) and then cultured (1×10^6 cells/ml) in RPMI medium containing 10% FCS in 24-well plates. After 3 h of incubation, non-adherent cells were removed to enrich peritoneal macrophages.

2.5. Growth curve of *S. typhimurium* in LB, minimal medium (MM) and F medium (FM)

Overnight grown cultures of *Salmonella* wild type (WT) and $\Delta pmrG-HM-D$ strains were subcultured (1%) into 25 ml of LB, MM and FM and grown at 37 °C in triplicates with constant shaking at 115 rpm. The optical density of the cultures at 600 nm was measured using spectrophotometer every hour for 24 h. The growth curves were plotted as optical density against time.

2.6. Intracellular survival assay

INT407, RAW 264.7, Colo205 and peritoneal macrophage were seeded at a density of 2×10^5 cells per well in 24-well plates. Stationary phase cultures of wild type and mutant *Salmonella* were added to the macrophages at a multiplicity of infection (MOI) of 10. For epithelial cell line infection, overnight grown culture was diluted at a ratio of 1:33 and incubated for 3 h to activate the SPI1 encoded genes required for invasion in non-phagocytic cells. The cells with bacteria containing media were centrifuged at 1000 rpm for 10 min to facilitate entry of bacteria into cells. Plates were then incubated at 37 °C for 25 min to allow bacterial uptake by cells. The extracellular bacteria were removed by washing thrice with PBS and incubating with 100 µg/ml gentamycin for 1 h. Thereafter, the cells were incubated with 25 µg/ml gentamycin for the rest of the experiment. After 2 and 16 h, the cells were lysed with 0.1% Triton-X 100 and the bacterial load was determined by plating serial dilutions of the lysates on LB agar plates with appropriate antibiotic. Colonies were enumerated after 24 h.

2.7. *Salmonella* burden in various organs

BALB/c mice were bred and housed at the Central Animal facility, Indian Institute of Science. The mice used for the experiments were 6–8 weeks old. All procedures with animals were carried out in accordance with institution approved rules. Mice were starved for 12 h and then infected either orally (10^7 CFU) or intraperitoneally (10^4 CFU) with WT or $\Delta pmrGH-MD$ *Salmonella*. Mice were sacrificed after 4th and 7th day. Liver, mesenteric lymph node (MLN) and Peyer's patch (PP) were aseptically isolated, weighed and homogenized in PBS. The tissue debris were removed by centrifugation at 1500 rpm for 5 min. The bacterial load in the organs were determined by plating serial dilutions of the homogenate on LB-agar and SS agar (*Salmonella*-*Shigella* agar). SS agar was used only for Peyer's patch to select out the commensals present in small intestine. CFU were counted and represented as CFU/g wt. of organ.

2.8. Immunization of mice through oral route

Cohorts of five mice were orally inoculated under aseptic conditions with 1 ml of PBS containing 10^4 CFU of $\Delta pmrGH-MD$. Placebo control mice were given only PBS. Three boosts at same doses were given at 1-week interval followed by final challenge with 10^7 CFU/mouse, WT *Salmonella*. Mice were sacrificed 5th day after challenge. Bacterial load in liver, spleen, MLN and PP were determined as described above. For assessing the safety of the vaccine in pregnant mice, cohorts of four pregnant mice were orally inoculated with $\Delta pmrGH-MD$ or WT *S. typhimurium*. Mice were scored for the delivery of the pups and their survival.

2.9. Immunization of mice through intraperitoneal (i.p.) route

Six- to eight-week-old female BALB/c mice were immunized via i.p. route with 1 ml of PBS containing 10^3 CFU of $\Delta pmrG-HM-D$ strain per mouse in cohort of five mice. Booster doses were given on 7th and 14th day. Placebo control mice were injected with PBS. On 21st day, challenge with either 10^3 CFU/mouse (for organ load) or 10^4 CFU/mouse (for scoring mortality) of WT *Salmonella* was done. Mice were sacrificed after 2 days. Bacterial load in liver, spleen, MLN and PP were determined as described above. Mortality of immunized and non-immunized mice was recorded.

2.10. Immunization with single dose of vaccine and subsequent challenge

Cohorts of five mice were immunized either orally or i.p. with 1 ml of PBS containing 10^3 CFU of vaccine strain ($\Delta pmrG-HM-D$) or PBS alone. The immunization was restricted to single dose only. After 42 days (oral route) and

50 days (IP route), final challenge was performed with WT *Salmonella* at 10^7 and 10^3 CFU/mouse, respectively. Mice were sacrificed after 4 days. Bacterial loads in liver, spleen, MLN and PP were determined as described above.

2.11. CD4 and CD8 T cell population in spleen after immunization

Cohorts of five mice were vaccinated orally and challenged as described before. Splenocytes from the spleen of vaccinated and control mice were isolated by homogenization. Homogenate were treated with RBC lysis buffer to remove RBC. Cells were counted and one million cells were used for FACS analysis (FACS scan analyser Becton, Dickinson and Company, USA). Cells were stained with antibody against CD4 and CD8 using hybridoma supernatants (kind gift from Prof. Dipanker Nandi, Department of Biochemistry, IISc) and Cy2 conjugated anti-rat secondary antibody. FACS analysis was done by using WinMDI software.

2.12. Statistical analysis

The data were subjected to statistical analysis by applying Student's *t*-test and Mann-Whitney test by using Sigma plot and Graph Pad prism 4 software and *p*-value <0.05 was considered as significant. All the experiments were repeated at least thrice to validate the results.

3. Results

3.1. Construction and characterization of mutant

pmrG-HM-D mutant was constructed by gene deletion through homologous recombination using λ -red recombinase system [14] (Fig. 1) and confirmed using colony PCR (data not shown). Long PCR using elongase showed product size of 8 kb for the *pmrG-HM-D*, whereas mutant showed product size of 1.1 kb corresponding to the chloramphenicol cassette. The growth characteristics of mutant with respect to WT remained the same in LB, M and F medium (data not shown).

Δ *pmrG-HM-D* strain was further characterized in terms of intracellular proliferation and survival in macrophage and epithelial cell line. In both the epithelial cell lines, INT407 and Colo205, Δ *pmrG-HM-D* was severely attenuated in terms of intracellular replication (Fig. 2a and b). While peritoneal macrophages showed a decreased survival of the mutant as compared to the WT from 2 to 16h, there was no visible difference in bacterial counts in the RAW264.7 macrophage cells with either strain (Fig. 2c and d).

3.2. Organ load of *pmrG-HM-D* and wild type *Salmonella*

To determine the organ load of wild type and Δ *pmrG-HM-D* in mice, cohort of five mice were inoculated orally or

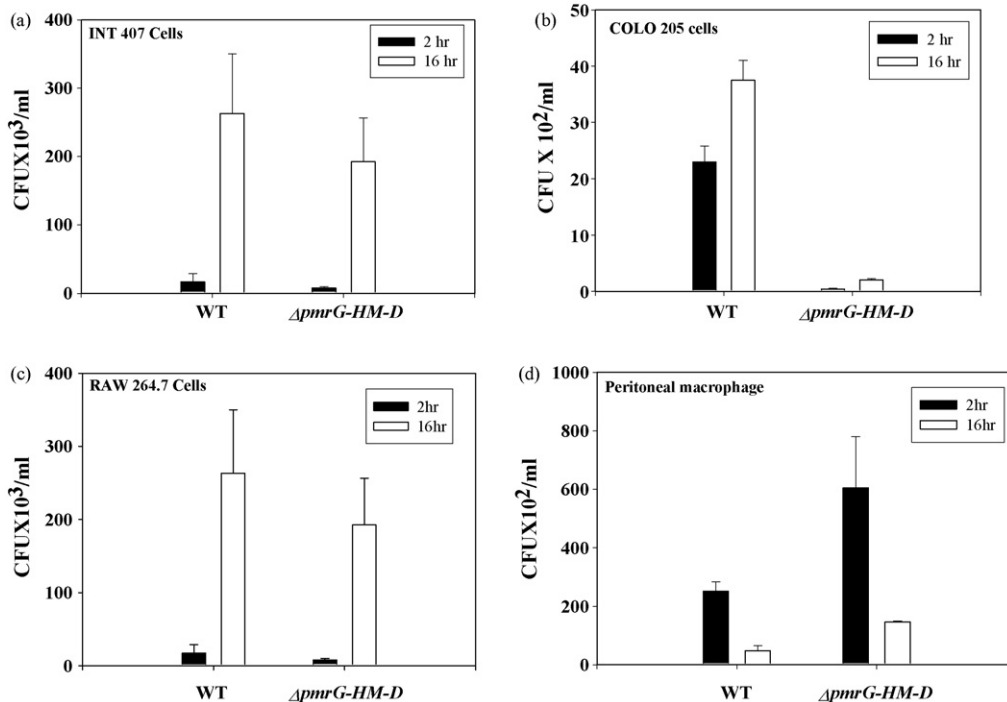


Fig. 2. Intracellular proliferation assays with various cell lines and primary peritoneal macrophage. Intracellular assay was performed with different cell lines. INT 407 (a), Colo205, (b) RAW 264.7 (c) and peritoneal macrophages (d) were infected with Δ *pmrG-HM-D* and wild type *Salmonella*. After 2 and 16h, cells were lysed and serial dilutions of the lysates were plated on LB agar plates. All the experiments were done in triplicate. Data are depicted as mean CFU \pm S.D.

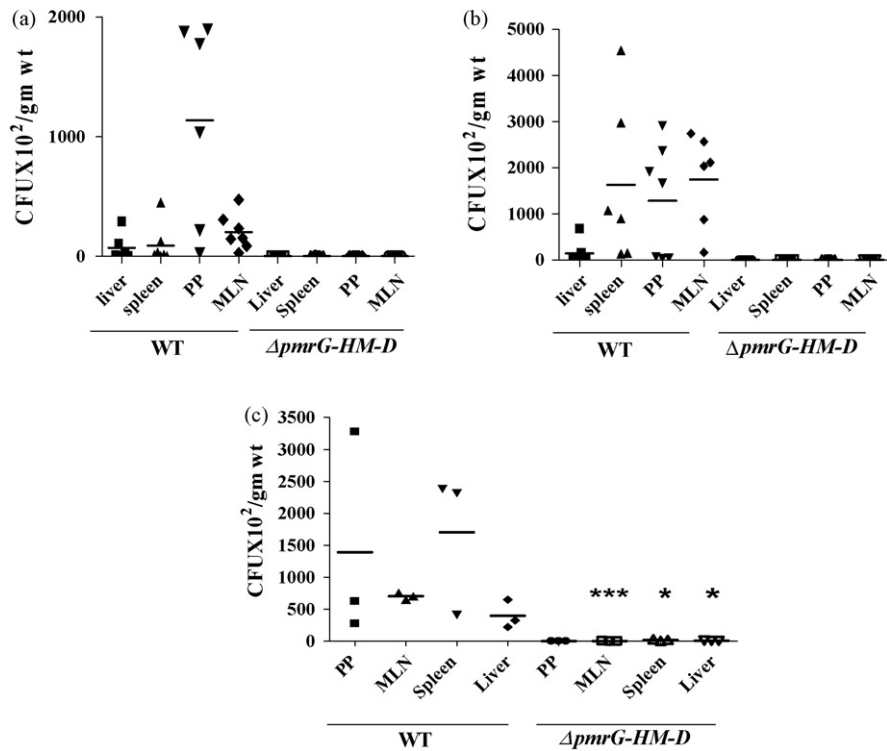


Fig. 3. Organ load of virulent *Salmonella* and the vaccine strain $\Delta pmrG-HM-D$. Two groups of mice were infected with 10^7 CFU/mouse orally or 10^3 CFU/mouse intraperitoneally and mice were sacrificed on day 4 (a) and day 7 (b) after oral infection and on day 3 (c) after i.p. infection. Liver, spleen, PP and MLN were aseptically dissected. Tissues were weighed and homogenized. Serial dilution of homogenized samples were plated on LB agar plates and on SS plates (PP only). The plates were incubated for 24 h and the colonies were counted. The Mann–Whitney U test was used to compare the CFU between vaccinated and non-vaccinated mice. For all statistical analyses, $p < 0.05$.

intraperitoneally with 10^7 and 10^4 CFU, respectively. After 4th and 7th day, *Salmonella* burden was evaluated from various organs. As shown in Fig. 3a and b, WT *Salmonella* was able to survive and proliferate in PP, spleen, MLN and liver after 4th and 7th day of infection. In contrast, $\Delta pmrG-HM-D$ was severely attenuated. Almost 100–1000 fold reduction in the CFU from various organs was observed in mice inoculated with $\Delta pmrG-HM-D$ strain as compared to WT. This suggested that the mutant is efficiently cleared from the PP and the secondary lymphoid organs. As depicted in Fig. 3c, the attenuation of the vaccine strain was also observed during intraperitoneal route of infection after third day of infection. PmrA and PmrHIFJKLM mutant were found to be attenuated only when given through oral route and not through intraperitoneal route [13]. However, our multiple mutant vaccine strain could neither survive the harsh environment of the small intestine nor the peritoneal cavity. We further investigated whether this clearance could generate protective immunity when challenged with WT *Salmonella*.

3.3. Multiple immunizations with *pmrG-HM-D* mutant was able to rescue the mice

BALB/c mice were primed orally with the $\Delta pmrG-HM-D$ followed by two booster doses on 7th and 14th day, all at doses of 10^4 CFU/mouse. After 7th day of the final boost,

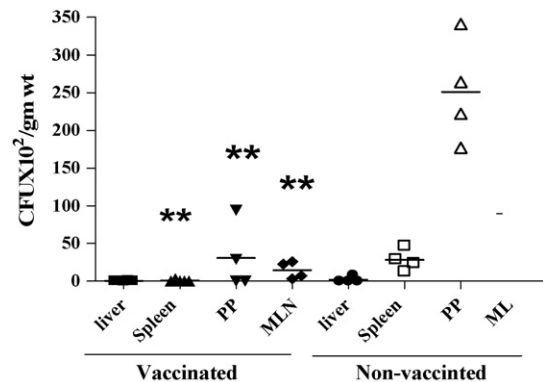


Fig. 4. Oral immunization with $\Delta pmrG-HM-D$ reduced the burden of WT *Salmonella* in liver, spleen MLN and PP. Six- to eight-week-old female BALB/c ($n = 10$) were orally immunized with 10^4 CFU of $\Delta pmrG-HM-D$ or PBS, boosted twice on days 7 and 14 and orally challenged with 10^7 CFU of WT *S. typhimurium*. On day 4 post-challenge, mice were sacrificed and immediately necropsied using standard techniques. The number of *Salmonella* present in the mesenteric lymph nodes (MLN), spleen and PP was enumerated using direct cultures of serially diluted homogenized samples. The Mann–Whitney U-test was used to compare CFU in various organs between vaccinated and non-vaccinated mice. For all statistical analyses, $p < 0.05$.

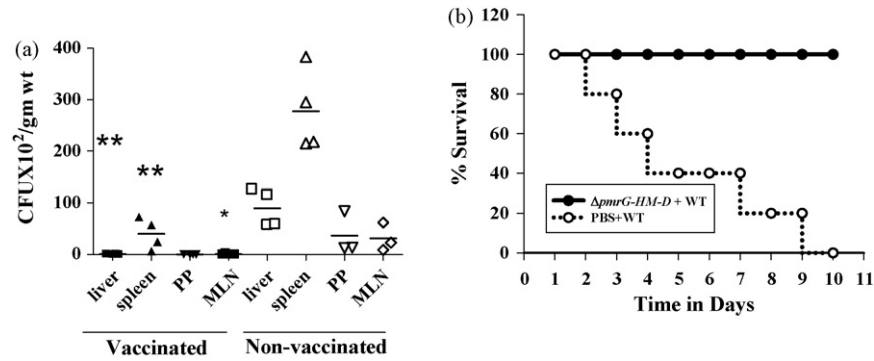


Fig. 5. Intraperitoneal immunization with $\Delta pmrG-HM-D$ conferred protection against WT colonization of liver, spleen MLN and PP in mice. Six- to eight-week-old female BALB/c ($n=10$) were immunized through i.p route with 10^3 CFU of $\Delta pmrG-HM-D$ or PBS, boosted twice with the same dose on days 7 and 14 and challenged with either (a) 10^3 CFU of WT *S. typhimurium* for assessing organ load or (b) 10^4 CFU of WT *S. typhimurium* for assessing mortality. On second day post-challenge, mice were sacrificed and immediately necropsied using standard techniques. The number of *salmonellae* present in tissues mesenteric lymph nodes (MLN), spleen and PP was enumerated using direct cultures of serially diluted homogenized samples. The Mann-Whitney *U*-test was used to compare CFU in various organs between vaccinated and non-vaccinated mice. For statistical analyses, * $p < 0.05$, ** $p < 0.005$.

mice were challenged with 10^7 CFU of wild type *Salmonella*. As shown in Fig. 4, in the immunized mice, there was 100-fold reduction in the bacterial load in spleen, MLN and PP as compared to the organs of placebo control mice.

Intra-peritoneal route of *Salmonella* infection is very lethal, requiring as low as 10^3 CFU for mortality of the mice. BALB/c mice were primed with 10^3 CFU $\Delta pmrG-$

HM-D, followed by two boosters on 7th and 14th day and finally infected with 10^7 CFU of wild type *Salmonella*. There was marked decrease in organ CFU of liver, spleen and MLN in vaccinated mice as compared to control unprimed mice (Fig. 5a). Priming with $\Delta pmrG-HM-D$ was also able to decrease the splenomegaly usually caused by virulent *Salmonella* infection (data not shown). As depicted in Fig. 5b,

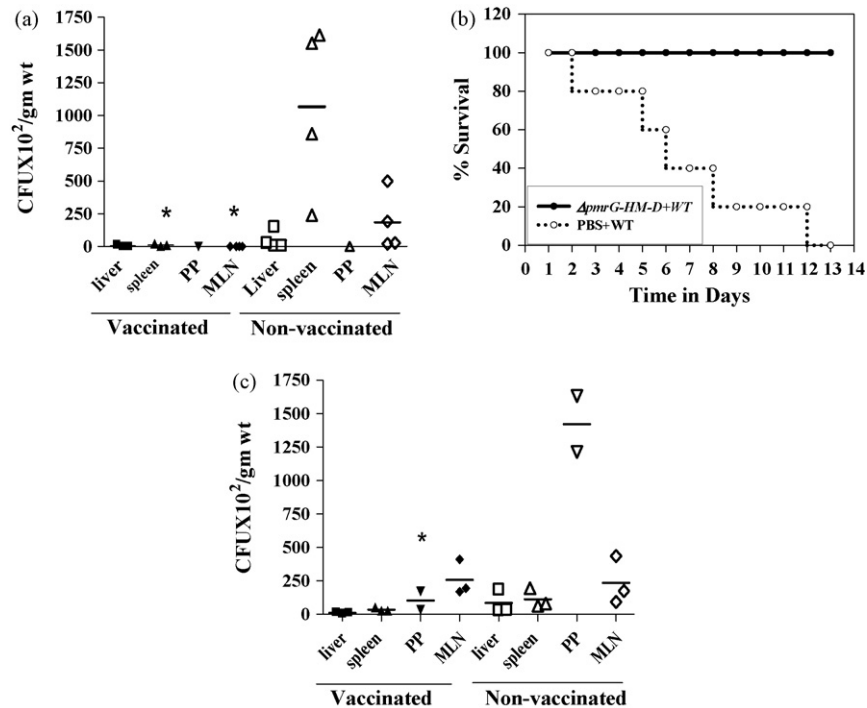


Fig. 6. Single dose of vaccination was able to rescue completely the challenged mice after 50 days of immunization. Each group of five mice were vaccinated via i.p. route and given final lethal challenge of 10^4 CFU/mouse for mortality and 10^3 per mouse for organ load. (a) Organ CFU in mice when challenged with WT CFU of 10^3 per mouse. (b) Mouse viability after lethal challenge of 10^4 CFU/mouse showing complete rescue from infection. (c) Two groups of five mice each were vaccinated orally with either vaccine strain ($\Delta pmrG-HM-D$) or PBS and subsequently challenged with WT *Salmonella* after 42 days. On day 4 after challenge, mice were sacrificed and immediately necropsied using standard techniques. The number of *salmonellae* present in tissues mesenteric lymph nodes (MLN), spleen and PP was enumerated using direct cultures of serially diluted homogenized samples. The Mann-Whitney *U* test was used to compare CFU in various organs between vaccinated and non-vaccinated mice. For statistical analyses, * $p < 0.05$, ** $p < 0.005$.

Table 2

Safety of serovar Typhimurium vaccine strains after oral administration to pregnant mice

No. of pregnant mice	Relevant genotype	No. of abortions	No. of mice (healthy/dead)	Death of pups after delivery (no. of death/total no.)
4	Wild type	1	3/1	4/10
4	$\Delta pmrG-HM-D$	0	4/0	0/20

the vaccinated mice showed 100% survival upon lethal dose challenge with wild type *Salmonella*. On the other hand, all the non-vaccinated mice succumbed to death by 13th day of infection.

The excellent protection conferred by $\Delta pmrG-HM-D$ suggests that the evasion strategy, *Salmonella* uses to escape the defense mechanism of the host is abrogated by this vaccine strain.

3.4. Single dose of *pmrG-HM-D* mutant protected mice from subsequent infection

The number of doses required for vaccination is an important factor for determining the efficiency of the vaccine. Here, we show that vaccination of mice with single dose of $\Delta pmrG-HM-D$, was able to decrease the organ burden when challenged with wild type *Salmonella*, after 50 days (IP vaccination and IP challenge) (Fig. 6a) and 42 days (oral vaccination and oral challenge) (Fig. 6c). Moreover, single dose of vaccination and challenge after 50 days with wild type *Salmonella* showed 100% protection (Fig. 6b).

3.5. Effect of *pmrG-HM-D* mutation on the capacity to prime CD8+ and CD4+ T cell responses

We further evaluated whether the *pmrG-HM-D* mutation affects the modulation of CD4+ and CD8+ T cell response by *Salmonella*. To test this, mice were vaccinated with the $\Delta pmrG-HM-D$ and challenged with WT *Salmonella*. Spleen cells were analyzed for the CD8+ and CD4+ T cell counts. The mice injected with $\Delta pmrG-HM-D$ strain showed an increase in the CD8+ T cell population unlike the WT strain (Fig. 7a). Mice vaccinated with $\Delta pmrG-HM-D$ and then challenged with WT exhibited a marked increase from 0.55% (WT) to 11.44% (vaccinated and challenged) of CD8+ T cells but not CD4+ T cells (Fig. 7b). Taken together these data suggest that probably, *pmr* systems play an important role in immune evasion mechanism by inhibiting CD8+ T cell response.

3.6. Safety of the vaccine strain in pregnant mice model

To assess the possibility of pregnancy failure after challenge with the vaccine strain and WT, groups of four pregnant mice were inoculated orally at 12–15 days of pregnancy. As shown in Table 2, WT virulent *S. typhimurium* strain caused abortion in one mouse and killed one mouse. Among the pups delivered by the remaining two mice, 50% pups died within

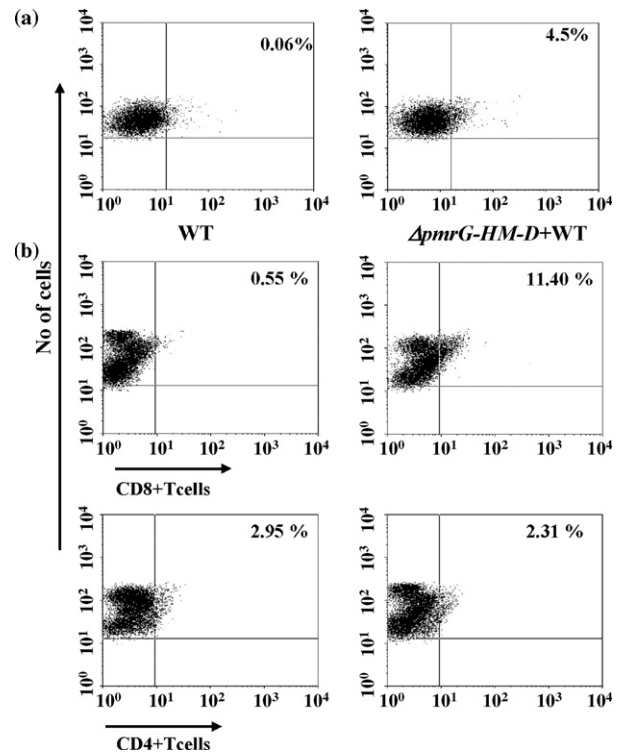


Fig. 7. CD8+ T cell population in vaccinated and non-vaccinated mice. (a) BALB/c mice were given intraperitoneal injection of 10^3 CFU of either WT or vaccine strain ($\Delta pmrG-HM-D$). After third day of injection, mice were sacrificed and spleen cells were processed for presence of CD8+ T cells by FACS analysis. (b) BALB/c mice were vaccinated intraperitoneally with 10^3 CFU of vaccine strain on days 7, 14 and 21. Placebo control mice were injected with PBS alone. Mice were challenged with 10^3 CFU of WT and on third day, spleen were processed for presence of CD8+ and CD4+ T cells by FACS analysis. CD4+ and CD8+ T cells were represented as % positive cells.

a period of 24 h after birth. On the other hand, the pregnant mice inoculated with the vaccine strain $\Delta pmrG-HM-D$ were all healthy and delivered healthy pups. Taken together, these data suggest that the vaccine strain $\Delta pmrG-HM-D$ is safe during pregnancy.

4. Discussion

In the present scenario, *Salmonella* vaccine is known as a fruit not to be eaten. Various research groups are trying to develop a better vaccine which can be well tolerated in children and have better protective immunity. The extra-intestinal infections of *Salmonella*, are very rare but life threatening [14]. With increase in the antibiotic resistant strains, vaccine remains the only choice to curb this disease. Diseases

of the Most Impoverished (DOMI) Program, was launched in 1999 to accelerate the development and use of current and future new-generation vaccines against typhoid fever [15]. This implies the urgent need for a potent vaccine against typhoid fever.

Salmonella is an intracellular pathogen having various strategies to evade the defense mechanism of the host. Murine model is an excellent system available to study the pathogenesis of typhoid fever caused by *S. typhimurium*. Researchers all over the world are trying to create an ideal vaccine strain which could give long lasting protective immunity. The genes of choice to be deleted were either metabolic or pathogenicity island genes like *aroA*, *purD* or SPI2. These vaccine strains were attenuated but were not able to protect the host. We hypothesized that, deleting a set of genes which are thought to play an important role in counteracting the environmental danger signals like AMPs may facilitate the killing of *Salmonella* thereby giving protective immunity. The intricate relation with PmrA-B (two component regulatory system), PmrD and PmrG enable one system to respond to multiple signals, which is important for the bacteria to adapt to the harsh environment of the host. These genes are responsible for the LPS modifications, resistance to AMPs and Fe(III). Deletion of these genes may lead to the susceptibility of the *Salmonella* strains to various AMPs as well as other host environment which may be rich in iron. Hence, in this study we have created a strain which is deficient in the PmrD, PmrH-M and PmrG genes. This multiple mutant strain, $\Delta pmrG-HM-D$, provided excellent protection in the animal model of typhoid fever. Loss of expression of the *pmrHIFJKLM* operon eliminates Ara4A addition to lipid A and polymyxin B resistance [16]. PmrD is hypothesized to interconnect PmrA-B and Pho P-Q system [12]. It was recently demonstrated that dephosphorylation of one of the phosphates in the core region of the LPS by the PmrA-activated PmrG protein confers Fe(III) resistance [17]. Single deletion of *pmrA* and *pmrHIFJKLM* operon, resulted in a attenuated *Salmonella* for the oral route but not intraperitoneal route [16]. All these studies suggest that probably these genes are necessary for the intestinal invasion and survival. The multiple mutant vaccine strain, $\Delta pmrG-HM-D$ was able to disarm the virulence strategies which may be hindering the protective immunity in case of typhoid fever. This multiple vaccine strain may have defective LPS structure with more susceptibility to AMPs and may have dysregulation of the *phoP* regulon. Defect in the *phoP* regulon leads to attenuation of *Salmonella* in the intraperitoneal route of infection. This vaccine strain was not only attenuated when given through oral route, but also through intraperitoneal route.

$\Delta pmrG-HM-D$ strain was not only efficient in conferring excellent protection against *Salmonella* challenge, but also required less dose (10^4 CFU) as compared to other vaccine strains, which were given at doses ranging from 10^5 to 10^8 CFU [18–20]. $\Delta pmrG-HM-D$ strain was markedly attenuated in epithelial cells. The epithelial cells are rich in antimicrobial peptides (AMP), which may probably explain

the drastic decrease in intracellular proliferation rate of this mutant in epithelial cells as observed in our study. However, $\Delta pmrG-HM-D$ strain was comparable in the in vitro growth characteristics with respect to wild type *Salmonella*. This suggests that the LPS modifications and loss of regulation of other virulence genes, may be responsible for the attenuation of this strain in the mouse model. Vaccination of the mice with $\Delta pmrG-HM-D$ strain not only decreased the organ burden of wild type *Salmonella* but also conferred 100% protection against the challenge. Being an intracellular pathogen, development of strong cell mediated immune response will a primary requisite to clear the infection. The vaccinated mice exhibited appreciable increase in CD8+ T cells suggesting its role in protection. Further in vitro studies are in progress to look more closely into the functionality of these CD8+ T cells, in terms of CTL response and IFN γ production. Also, studies are underway to elucidate the memory population of CD8+ T cells, which can contribute to the long-term protective immunity against *Salmonella*. One more important observation was the induction of abortion in pregnant mice by the WT *Salmonella* and not by vaccine strain, $\Delta pmrG-HM-D$. Moreover, the pups which were delivered from the WT infected mice also died a day after delivery. The vaccine strain neither induced abortion nor caused death of the pups. These finding strongly suggests that the vaccine strain $\Delta pmrG-HM-D$ appears to be safe even in pregnant individuals.

An ideal live vaccine strain combines efficient immunogenicity with safety. In this study, we have shown that a $\Delta pmrG-HM-D$ mutant of *S. enterica* serovar Typhimurium fulfills both of these criteria. The mutant becomes attenuated and elicits protective immunity against a subsequent challenge with the wild type strain, requires a single low dose and is very safe even in the pregnant animal model.

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References

- [1] Synder JW, Check W. Bioterrorism threats to our future. A report from American Society of Microbiology; 2000.
- [2] Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. Bull World Health Organ 2004;82:346–53.

- [3] Bhan MK, Bahl R, Bhatnagar S. Typhoid and paratyphoid fever. *Lancet* 2005;366:749–62.
- [4] Kirkpatrick BD, McKenzie R, O'Neill JP, et al. Evaluation of *Salmonella enterica* serovar Typhi (Ty2 aroC-ssaV-) M01ZH09, with a defined mutation in the Salmonella pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers. *Vaccine* 2006;24:116–23.
- [5] Khan SA, Stratford R, Wu T, et al. *Salmonella typhi* and *S typhimurium* derivatives harbouring deletions in aromatic biosynthesis and Salmonella Pathogenicity Island-2 (SPI-2) genes as vaccines and vectors. *Vaccine* 2003;21:538–48.
- [6] Tacket CO, Szein MB, Lososky GA, Wasserman SS, Nataro JP, Edelman R, et al. Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htrA* and *aroC* and immune response in humans. *Infect Immun* 1997;65:452–6.
- [7] Ivanoff B, Levine MM, Lambert PH. Vaccination against typhoid fever: present status. *Bull WHO* 1994;72:957.26.
- [8] Miller SI, Loomis WP, Alpuche-Aranda C, Behlau I, Hohmann E. The PhoP virulence regulon and live oral Salmonella vaccines. *Vaccine* 1993;11(2):122–5.
- [9] Yrlid U, Svensson M, Hakansson A, Chambers BJ, Ljunggren HG, Wick MJ. In vivo activation of dendritic cells and T cells during *Salmonella enterica* serovar Typhimurium infection. *Infect Immun* 2001;69:5726–35.
- [10] Sebastiani G, Blais V, Sancho V, Vogel SN, Stevenson MM, Gros P, et al. Host immune response to *Salmonella enterica* serovar Typhimurium infection in mice derived from wild strains. *Infect Immun* 2002;70:1997–2009.
- [11] Trezena AG, Souza CM, Borrego A, Massa S, Siqueira M, De Franco M, et al. Co-localization of quantitative trait loci regulating resistance to *Salmonella typhimurium* infection and specific antibody production phenotypes. *Microbes Infect* 2002;4:1409–15.
- [12] Eguchi Y, Utsumi R. A novel mechanism for connecting bacterial two-component signal-transduction systems. *Trends Biochem Sci* 2005;30:70–2.
- [13] Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 2000;97(June (12)):6640–5.
- [14] Huang DB, DuPont HL. Problem pathogens: extra-intestinal complications of *Salmonella enterica* serotype Typhi infection. *Lancet Infect Dis* 2005;5:341–8.
- [15] DeRoeck D, Clemens JD, Nyamete A, Mahoney RT. Policymakers' views regarding the introduction of new-generation vaccines against typhoid fever, shigellosis and cholera in Asia. *Vaccine* 2005;23:2762–74.
- [16] Gunn JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SI. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. *Infect Immun* 2000;68:6139–46.
- [17] Nishino K, Hsu FF, Turk J, Cromie MJ, Wosten MM, Groisman EA. Identification of the lipopolysaccharide modifications controlled by the Salmonella PmrA/PmrB system mediating resistance to Fe(III) and Al(III). *Mol Microbiol* 2006;61:645–54.
- [18] Nagy G, Dobrindt U, Hacker J, Emody L. Oral immunization with an *rfaH* mutant elicits protection against salmonellosis in mice. *Infect Immun* 2004;72:4297–301.
- [19] Tang IK, Ji DD, Chou CF, Lin HC, Liao CL, Sytwu HK, et al. Characterization of a highly attenuated *Salmonella enterica* serovar Typhimurium mutant strain. *J Microbiol Immunol Infect* 2002;35:229–35.
- [20] Valentine PJ, Devore BP, Heffron F. Identification of three highly attenuated *Salmonella typhimurium* mutants that are more immunogenic and protective in mice than a prototypical *aroA* mutant. *Infect Immun* 1998;66:3378–83.