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An attenuated *Salmonella enterica* serovar Typhimurium strain lacking the ZnuABC transporter induces protection in a mouse intestinal model of *Salmonella* infection

M. Pesciaroli[a], F. Aloisio[b], S. Ammendola[c], C. Pistoia[a], P. Petrucci[a], M. Tarantino[a], M. Francia[a], A. Battistoni[c], P. Pasquali[a,*]

[a] *Department of Food Safety and Veterinary Public Health, Istituto Superiore di Sanità, 00161 Rome, Italy*
[b] *Institute of Animal Pathology Vet-Suisse Faculty University of Bern, Switzerland*
[c] *Department of Biology, Università di Roma Tor Vergata, 00133 Rome, Italy*

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ABSTRACT

Salmonella enterica serovar Typhimurium has long been recognised as a zoonotic pathogen of economic significance in animals and humans. Attempts to protect humans and livestock may be based on immunization with vaccines aimed to induce a protective response. We recently demonstrated that the oral administration of a *Salmonella enterica* serovar Typhimurium strain unable to synthesize the zinc transporter ZnuABC is able to protect mice against systemic salmonellosis induced by a virulent homologous challenge. This finding suggested that this mutant strain could represent an interesting candidate vaccine for mucosal delivery. In this study, the protective effect of this *Salmonella* strain was tested in a streptomycin-pretreated mouse model of salmonellosis that is distinguished by the capability of evoking typhlitis and colitis. The here reported results demonstrate that mice immunized with *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) SA186 survive to the intestinal challenge and, compared to control mice, show a reduced number of virulent bacteria in the gut, with milder signs of inflammation. This study demonstrates that the oral administration a of *S.* Typhimurium strain lacking ZnuABC is able to elicit an effective immune response which protects mice against intestinal *S.* Typhimurium infection. These results, collectively, suggest that the streptomycin-pretreated mouse model of *S. typhimurium* infection can represent a valuable tool to screen *S. typhimurium* attenuated mutant strains and potentially help to assess their protective efficacy as potential live vaccines.

1. Introduction

Salmonella enterica serovar Typhimurium is a Gram-negative bacterium representing an important cause of human diarrhoeal disease worldwide. Human infections are generally acquired by consuming food of animal origin and are mainly limited to the intestinal tract, with limited risk of dissemination in systemic organs [1].

Salmonella Typhimurium can also infect a wide range of hosts and causes diseases with different features depending on the host species. In humans and cattle, *S. typhimurium* leads to a self-limiting diarrheal enterocolitis without systemic symptoms in the vast majority of cases. In contrast, mice develop a highly lethal systemic disease, named "mouse typhoid". This is considered an excellent model for human "typhoid fever" induced by *Salmonella typhi*, but is poorly useful to study the enteric forms of human and animal salmonellosis.

To overcome such limitation, other models have been proposed. Studies on the pathogenesis of the enteric disease conducted on tissue cultures [2], intestinal organ cultures [3] and infection of legated murine and rabbit ileal loops [4] have allowed the discovery and the analysis of many *S. typhimurium* virulence factors. Nevertheless, a suitable experimental animal model useful to understand and disclose the mechanisms of pathogenicity of either human or animal enteric salmonellosis is still strongly needed.

An interesting advancement in the possibility to investigate *Salmonella*-induced enteritis has been provided by the recent development of a mouse model characterized by the ability to evoke an intestinal inflammation [3]. In fact, it has been shown that pretreatment with streptomycin 24 h before infection by oral route modifies murine intestinal flora [5,6], allowing *S. typhimurium* to evoke colitis and typhlitis. In particular, after a first phase, intestinal colonization is paralleled by a systemic infection whose destiny largely depends on the genetic background of the host. In fact,

* Corresponding author. Tel.: +39 06 4990 2326; fax: +39 06 4938 7077.
E-mail address: paolo.pasquali@iss.it (P. Pasquali).

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BALB/c and C57BL/6 mice (*Ity*^{s}), which are highly susceptible to *S. typhimurium* infection, usually succumb within 5–6 days after infection, permitting only to investigate the acute phase of inflammation. Conversely, DBA/2 and 129Sv/Ev mice (*Ity*^{R}), which are resistant to *S. typhimurium* infection, are able to control more efficiently infection, showing delayed mortality in 10–11 days (DBA/2) or being able to survive to the infection (129Sv/Ev) [7]. For its peculiarity, this model can therefore represent a valuable approach to investigate the pathogenesis of intestinal infection.

S. typhimurium is a major cause of food borne zoonoses in developed countries and pork meat consumption is addressed as the main source of human cases of *S. typhimurium* infection [8]. In order to reduce the burden of *S. typhimurium* gastroenteritis on human population it is pivotal to control the bacterial load in disease-free carrier pigs that are the predominant cause of *Salmonella* introduction into the food production chain [9–11]. The use of live *S. typhimurium* vaccines is believed to be a potential valuable instrument in achieving this goal [12]. Furthermore *S. typhimurium* infections represent an animal health concern causing significant economic loss in livestock industry. Due to the patterns of infections in farm animals, it is important to test new vaccines in enteric models of salmonellosis. In this respect, the aim of this study was to test the efficacy of a candidate *Salmonella* live vaccine to be used by the oral route in a murine intestinal model of *Salmonella* infection (streptomycin-pretreated mouse model). On that account we used a mutant strain denominated *S. typhimurium* SA186 [13], which is significantly attenuated due to deletion of the whole *znuABC* operon encoding for a high affinity zinc importer necessary for metal recruitment within the infected host [14]. In fact, we have previously demonstrated that this mutant strain is a good candidate mucosal vaccine, being able to reduce colonization of systemic organs and to protect mice against challenge infections [13,14]. Here we demonstrate that *S. typhimurium* SA186 administered by the oral route is able to elicit an effective immune response and protects against *Salmonella* infection. This study encourages further investigations to confirm that the enteric model could indeed represent a valuable instrument to replace, reduce and refine the use of farm animals to assess the efficacy of candidate mucosal vaccines.

2. Materials and methods

2.1. Salmonella spp. cultures

Virulent *S. typhimurium* ATCC 14028 and its isogenic znuABC mutant strain denominated *S. typhimurium* SA186 [13,14] were used throughout the study. *S. typhimurium* strains were grown overnight at 37 °C in Brain Heart Infusion (Oxoid Ltd., Basingstoke, UK), harvested by centrifugation and then washed twice in ice cold phosphate buffer solution (PBS) (Sigma–Aldrich, Milan, Italy).

2.2. Animals

Age matched (8–9 weeks old) SPF DBA/2 (*Ity*^{r}) female mice were purchased from Charles River (Calco, Italy) Experiments with mice, according to national (D.L.116/92) and European (86/609/EEC) regulation, were previously authorised by national authority (Decreto 225/2009 – B) and were carried out under the supervision of certified veterinarians. Mice were fed were fed with a commercial maintenance diet for rats and mice (Altromin-R diet, A. Rieper S.p.A., Vandoies, Italy) and tap water was provided ad libitum. All mice were acclimatized for a minimum of 1 week prior to experimentation. Mice were inoculated orally, using a gastric gavage needle, with different Salmonella strains suspended in 10% sterile sodium bicarbonate buffer in order to raise the pH of the stomach and increase the survival ratio of administered microorganisms.

2.3. Immunization and challenge infection

Groups of four up to five mice were orally inoculated with 2×10^7 CFU of the attenuated strain *S. typhimurium* SA186 or were kept as controls by receiving only sodium bicarbonate buffer. On 39th day after the inoculum, immunized and control animals orally received 20 mg of streptomycin (200 μl of sterile solution or sterile saline) 24 h prior of being intragastrically challenged with 2×10^8 CFU of virulent *S. typhimurium* ATCC14028. Some immunized and control animals were maintained unchallenged and used only for histopathological measurements. Animals were fasted for 4 h before the administration of bacterial or antibiotic suspension, drinking water and food were supplied again 2 h post inoculation.

2.4. Protection assay

Vaccinated and control mice, challenged as described above, were monitored daily to assess mortality rate. When animals became moribund they were opportunely euthanized.

2.5. Evaluation of the infection

Mice were euthanized by cervical dislocation at 4 and 11 days after challenge infection and spleen, cecum and colon were aseptically removed, weighed and homogenized in a 10-fold volume of saline. An aliquot of the organ homogenates were prelevated and conserved at −20 °C to be used to assess the production of cytokines. The number of CFU in the spleen was determined by plating appropriate dilutions of the resulting cell suspensions on Triptone Agar Plates (Oxoid Ltd., Basingstoke, UK). Enumeration of virulent bacteria in cecum and colon was achieved partially modifying a semi-quantitative culture technique previously reported [15]. Briefly, 0.5 ml of each enteric trait homogenate was transferred in 4.5 ml of peptone water (PW) (Oxoid Ltd., Basingstoke, UK). This solution was used as first to perform a serial decimal dilution carried out by systematically transferring an aliquot of 0.5 ml of each consequent dilution in 4.5 ml of PW. All PW samples were incubated at 37 °C for 24 h. Afterwards, 1 ml of each diluted mixture was transferred in 9 ml of Rappaport-Vassiliadis broth (Oxoid Ltd., Basingstoke, UK) and incubated a 41.5 ° C for 24 h for selection and enrichment of *Salmonella*. After the incubation, presence of *Salmonella* organisms was assessed by streaking 1 μl loop of enrichment broth suspension onto Brilliant Green Agar plates (Oxoid Ltd., Basingstoke, UK) and incubating them overnight at 37 °C. The identity of *Salmonella* colonies grown was serologically confirmed by performing a slide agglutination test using *Salmonella* O-antigen murine antisera (Remel, Lenexa, KS, USA).

2.6. Ex vivo production of IFN-γ

Aliquots of organ previously homogenates in saline were used *in toto* in order to ex vivo assess the production of IFN-γ in spleen, cecum and colon. The level of IFN-γ was calculated using a commercial ELISA kit (Quantikine M kit; R&D Systems, Milan, Italy).

2.7. Histopathology

Random segments of cecum and colon were formalin fixed, embedded in paraffin and stained with hematoxylin and eosin according to standard procedures. Each organ from each animal was sampled in double. To measure the intensity of the tissue inflammation, a semiquantitative scoring system was developed. Features taken in consideration were submucosal edema, leukocyte infiltrate, and epithelial damage. A numeric value from 0 to 3 was given to the severity and distribution of the pathologic features considered as follows: