Salmonella Typhimurium lacking the Znuabc transporter is attenuated and immunogenic in pigs

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ABSTRACT

Meat contamination by Salmonella spp. is emerging as a major cause of human enteric infections in industrialized countries. The attempts to reduce human cases of salmonellosis encompass pre- and post-harvest interventions. In this context, vaccination of pigs may represent an effective instrument in eliminating/reducing Salmonella burden through the food chain. We have previously demonstrated that Salmonella Typhimurium lacking the ZnuABC transporter (S. Typhimurium ΔznuABC) is a promising candidate live vaccine in different mouse models of Salmonella Typhimurium infection. In this study, we confirmed in pigs the attenuation of S. Typhimurium ΔznuABC. Moreover, we evaluated the safety and immunogenicity of S. Typhimurium ΔznuABC administered to pigs by the oral route. We monitored clinical conditions of animals and we conducted a microbiological culture and a quantification of the humoral and cellular immune response, respectively, on fecal and blood samples of pigs. After vaccination with attenuated S. Typhimurium ΔznuABC, pigs showed a modest degree of hyperthermia. In addition, fecal shedding of S. Typhimurium ΔznuABC could not be detected 28 days after the inoculum. Furthermore, vaccination with S. Typhimurium ΔznuABC elicited a distinct production of anti-Salmonella antibodies and IFN-γ. Taken together, these results suggest that S. Typhimurium ΔznuABC is attenuated and immunogenic in pigs. Although the vaccine dosages do not guarantee complete safety there is ample margin to set up better conditions of use, suggesting that S. Typhimurium ΔznuABC could be a promising attenuated strain to be used as live mucosal vaccine for oral delivery.

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1. Introduction

Nontyphoidal salmonellosis infections pose a significant threat to human health and are responsible for high economic losses worldwide [1]. Moreover, many Salmonella strains are resistant to a number of antimicrobial agents, narrowing the therapeutic alternatives in cases of severe human infection [2,3]. Pork is the main source of Salmonella infection for humans, accounting for 26.9% of the human cases officially reported in the EU [4]. The application of strict hygiene practices and rational husbandry management have been effective in Scandinavian countries, where the prevalence of Salmonella in animals and carcasses at slaughter is nearly zero. However, this approach is hardly feasible in countries where high prevalence of infection is observed. In these settings, vaccination is considered as a major tool to minimize Salmonella contamination at the early stages of meat production. Although live vaccines provide better protection against Salmonella infections compared to inactivated ones [5–8], only a live attenuated vaccine for Salmonella Typhimurium is commercially available in Europe at the moment [5]. Nevertheless, several strains showed promising results vaccine in experimental settings [6–8]. Recently, we found that a Salmonella enterica serovar Typhimurium mutant strain, deleted of the whole znuABC operon (S. Typhimurium ΔznuABC) encoding for a high affinity zinc importer necessary for metal recruitment within the infected host, is significantly attenuated in mice [9–11] and able to protect mice against both systemic and enteric salmonellosis [12,13]. Based on these results, compared the virulence of S. Typhimurium ΔznuABC with those of wild type Salmonella Typhimurium ATCC 14028 in pigs (Exp. 1). In a second experiment (Exp. 2), we established the safety and immunogenicity of S. Typhimurium ΔznuABC administered to pigs as live vaccine. The results reported here demonstrate that S. Typhimurium...
\(\Delta\text{znuABC}\) is attenuated in pigs. Moreover, administered by the oral route, S. Typhimurium \(\Delta\text{znuABC}\) elicits a short-lasting and immunogenic infection that does not affect the animal health status and production performances in nearly all animals.

## 2. Materials and methods

### 2.1. Salmonella spp. cultures

The virulent Salmonella Typhimurium ATCC 14028 and its isogenic \(\text{znuABC}\) mutant strain produced according to the method previously reported [9], were used throughout the study. Strains were grown overnight at 37 °C in Brain Heart Infusion (Oxoid Ltd., UK), harvested by centrifugation and then washed twice in ice-cold phosphate buffer solution (PBS) (Sigma–Aldrich, Italy). A bacterin from S. Typhimurium ATCC 14028 was obtained by inactivating the bacteria with formalin and absorbing them on aluminum hydroxide.

### 2.2. Animals

Twenty-eight commercial hybrid pigs aging ~ 80 days were used in the comparative virulence experiment (Exp. 1). Animals were split in two groups of 10 (Groups A and B) and a group of 8 (Group C). Groups A and B were intragastrically administered with 20 ml of sodium bicarbonate buffer containing \(5 \times 10^9\) CFU of S. Typhimurium \(\Delta\text{znuABC}\) (Group A) or \(5 \times 10^9\) CFU of S. Typhimurium ATCC 14028 (Group B). Group C received only sterile sodium bicarbonate buffer and served as control group. Collection of fecal samples of each pig (0, 1, 3 and 7 days) and registration of rectal temperature (0, 1, 2 and 7 days) were performed. For the safety and immunogenicity experiment (Exp. 2), hybrid pigs born by cesarean section and aging 80–100 days were used. Animals were split into 4 groups of 6 (Groups A, B and C) and 8 (Group D) animals/group. Groups A and B were intragastrically vaccinated, respectively, with a suspension of \(5 \times 10^8\) (Group A) and \(5 \times 10^7\) (Group B) CFU of S. Typhimurium \(\Delta\text{znuABC}\) in 20 ml of sodium bicarbonate buffer. Group C was intramuscularly (upper part of the neck, 16G needle, 40 mm length) vaccinated with \(2 \times 10^8\) CFU of inactivated S. Typhimurium ATCC 14028. Group received an equal booster dose 14 days later. Group D was administered sterile sodium bicarbonate buffer and was left as naive controls. Individual blood and fecal samples were collected before and after immunization (on day 0, 1, 2, 7, 8, 9, 14, 21, 28 and 35 days). Body temperature was registered on the same dates. On day 0 and at 5 weeks, each animal was weighed to evaluate the growth rate. All the pigs used throughout the study (Exp. 1 and Exp. 2) were proved to be Salmonella–free by culture of feces of each animal, and individual sera were checked to exclude Salmonella antibodies. Each group was maintained in separate isolation units under natural day–night rhythm with access to feed (FAMAVIT, Italy) and water ad libitum.

All the experiments were authorized by national authority and were conducted according to national regulation (D.L.116/92).

### 2.3. Fecal shedding of S. Typhimurium

Fecal samples of each pig were collected to assess the elimination of bacteria. The microbiological analysis was conducted according to the ISO 6579:2002/Amendment 1:2007 protocol. This is a semi-quantitative approach that allowed determining the concentration of Salmonella in a sample within a tenfold band (detection limit 1 CFU/g feces). Suspect Salmonella colonies were subjected to biochemical identification by BBL Enterotube II (BD Franklin Lakes, USA) and serological identification using Salmonella group-specific antisera (Remel, Lenexa, USA).

### 2.4. Persistence of S. Typhimurium \(\Delta\text{znuABC}\) in the environment and pig feces

Environmental swabs collected from the pen floor of each group were qualitatively cultured following the protocol indicated in section 2.3 to assess the persistence of Salmonella in the environment. In another set of experiments, we compared the viability of S. Typhimurium \(\Delta\text{znuABC}\) and S. Typhimurium ATCC 14028 in feces. Approximately 2 kg of fresh pig feces were collected from a group of sows serologically negative for Salmonella. Prior to use, the feces were confirmed negative for the presence of Salmonella by culture. A quantity of 27 g of feces was placed into each of 22 sterile 50 ml plastic tubes for inoculation. Three ml of sterile saline containing S. Typhimurium \(\Delta\text{znuABC}\) or S. Typhimurium ATCC 14028 were added to 20 tubes (10 tubes for each strain), to yield a final concentration of \(5 \times 10^6\) CFU/g of feces. Finally, 3 ml of sterile saline were added to 2 tubes, to be used as controls. Tubes were incubated at 20 °C for three weeks. Sampling for culture was performed at 0, 2, 7, 14 and 21 days post-inoculation. After gently mixing, an aliquot of 1 g of feces was taken from each tube for enumeration of Salmonella microorganisms using a miniaturized MPN (Most Probable Number) method based on ISO 6579:2002 [14].

Typical colonies were confirmed serologically as Salmonella by polyvalent antiserum (Salmonella Test Serum; Siemens Healthcare Diagnostics, Italy) and API rapid 20 E (Api Rapid 20E; Biomerieux, Italy). MPN was calculated according to the ISO 7218:2007.

### 2.5. Serological examination

The serological exams were performed using a commercial indirect ELISA test capable of detecting antibodies against lipopolysaccharide antigens of Salmonella serogroups B, C1 and D (Herd-Check Swine Salmonella Antibody Test Kit, Idexx Laboratories Inc., Switzerland). The test was carried out according to the producer’s instructions and read at an optical density of 450 nm. Results were expressed as sample to positive ratio (S:P ratio = (OD of sample – OD of negative control)/(OD of positive control – OD of negative control)).

### 2.6. Statistical analysis

Exp. 1. Differences in body temperature were estimated using 2 ways analysis of variance with a Tuckey’s multiple comparison test and considered significant when \(P-value < 0.05\). Fecal shedding of group A and group B were compared using non parametric Mann–Whitney test.

Exp. 2. Differences between the groups were analyzed by a Two-way ANOVA and Bonferroni corrections were applied. Statistical analysis was performed using a Graphpad PRISM 6.0 software (GraphPad Software Inc., USA). \(P-value \leq 0.05\) was considered significant.

## 3. Results

### 3.1. Pathogenicity of S. Typhimurium \(\Delta\text{znuABC}\)

Our hypothesis was that the deletion of the whole \(\text{znuABC}\) operon encoding for a zinc importer necessary for metal recruitment within the infected host was able to reduce virulence of S. Typhimurium in pigs. So, in Exp.1, we compared the values of body temperature and the duration of fecal shedding of group A (S. Typhimurium \(\Delta\text{znuABC}\)) with those of Group B (S. Typhimurium ATCC 14028) and Group C (controls). Two days after the inoculation, group A and group B displayed statistically significant higher values of body temperature than controls. Nevertheless the increase body temperature was more evident in group B (Fig. 1A). S. Typhimurium
\( \Delta znuABC \) was fecally shed in lower amount than \( S. Typhimurium \) ATCC 14028 (Fig. 1B). At 1 day after the inoculation this difference reached statistically significance. These results confirmed our hypothesis a \( S. Typhimurium \) deleted of whole \( znuABC \) operon has a reduced virulence in pigs.

### 3.2. Effect of \( S. Typhimurium \) \( \Delta znuABC \) oral administration on body temperature

Then, in Exp. 2, we decided to explore how the oral vaccination with \( S. Typhimurium \) \( \Delta znuABC \) (our candidate vaccine strain mutated in \( znuABC \)) modifies the physiologic values of body temperature in pigs, considered to be between 38 and 40 °C [15]. At 1 day after vaccination (DAV), three animals of group B and one animal of group A displayed a body temperature ranging from 40.3 °C and 40.7 °C (Fig. 2 and Fig. S1A). At 2 DAV, three animals of group A and one animal of group B had a body temperature varying from 40.1 °C and 41 °C (Fig. 2 and Fig. S1A). At the following time points (7, 14, 21, 28, 35 DAV), the body temperature of both groups of pigs was comparable with controls. Group C, vaccinated with \( 2 \times 10^9 \) CFU of bacterin, and group D (controls) did not show any alteration of temperature at any time points considered (Fig. 2 and Fig. S1B). These data indicate that the oral administration of \( S. Typhimurium \) \( \Delta znuABC \) at currently used inoculation doses increases body temperature of a limited number of pigs.

### 3.3. Influence of vaccination on growth rate

Next, we evaluated if vaccination with either \( S. Typhimurium \) \( \Delta znuABC \) or inactivated \( S. Typhimurium \) ATCC 14028 interferes with the growth of animals. At 35 DAV, one animal of group B and one of group C did not gain weight, a decrease of body weight was registered in a pig of group A. Our observations suggest pigs of groups vaccinated either with \( S. Typhimurium \) \( \Delta znuABC \) or inactivated \( S. Typhimurium \) ATCC 14028 showed an heterogeneous growth rate. However, no statistically significant difference was noted between the growth rate of vaccinated pigs and controls (Fig. 3 and Fig. S2).

### 3.4. Fecal shedding of \( S. Typhimurium \) \( \Delta znuABC \)

At 1 DAV, stools of group A contained approximately \( 10^5 \) CFU/g of \( S. Typhimurium \) \( \Delta znuABC \) and stools of group B contained less than \( 10^4 \) CFU/g (Fig. 4). Twenty-four hours later, the concentration decreased by ~2 logs in feces of pigs from both groups. At 7 DAV, group A continued to excrete approximately \( 10^2 \) CFU/g. However, at 7 DAV, shedding of group B dropped to less than 10 CFU/g, with 3 animals in this group that had not detectable \( S. Typhimurium \) \( \Delta znuABC \) in feces. At 21 DAV, one animal of group B shed less than 10 CFU/g of \( S. Typhimurium \) \( \Delta znuABC \). At 14 DAV, two pigs of group A shed less than 10 CFU/g and at 21 DAV, two other pigs excreted 10
and $10^2$ CFU/g of S. Typhimurium ΔznuABC each. The shedding of S. Typhimurium ΔznuABC by animals of group A was not detectable at 28 DAV. These data indicate that at 2 DAV, the fecal shedding of S. Typhimurium ΔznuABC decreases to approximately $10^1$–$10^2$ CFU/g and continues to comparable values till day 28.

3.5 Viability of S. Typhimurium ΔznuABC in the environment and pig feces

As indicator of the viability of S. Typhimurium ΔznuABC, we assessed the environmental contamination of pig units after vaccination. As expected, S. Typhimurium ΔznuABC was not detected in pen of controls (group D) and animals of group C, which were vaccinated with the bacterin. Environmental swabs collected from pens of groups A and B revealed that S. Typhimurium ΔznuABC contaminated the environment till 7 DPI. To corroborate these findings we set out to determine the survival of S. Typhimurium ΔznuABC in comparison with S. Typhimurium ATCC 14028 in a fecal matrix. In this setting, the concentration of S. Typhimurium ΔznuABC dropped from $4 \times 10^6$ CFU/g (Standard Deviation $1.4 \times 10^4$) to 844 (Standard Deviation $8.9 \times 10^2$) in two days and was under the detection limit at 14 days after inoculation in the majority of the samples. In contrast, S. Typhimurium ATCC 14028 was still detectable at a concentration of $1.4 \times 10^6$ at 14 days after inoculum (Fig. 5). These data suggest that S. Typhimurium ΔznuABC does not persist long in conditions resembling the natural environment.

3.6 Vaccination induces antibody specific immune response

The antibody response of pigs after vaccination with S. Typhimurium ΔznuABC or inactivated S. Typhimurium ATCC 14028 was investigated. All vaccinated animals mounted a humoral immune response regardless of the type of vaccine administrated (Fig. 6). Anti-Salmonella antibodies raised in group A, group B and group C at 7 DAV and remained high throughout the observational period. At 7, 14, 21, 28 and 35 DAV, antibody titers of groups A, B and C were statistically higher than those of controls. These data demonstrate that administration of S. Typhimurium ΔznuABC or inactivated S. Typhimurium ATCC 14028 is effective in inducing a humoral immune response in pigs.

4. Discussion

The here reported results show that the mutant strain S. Typhimurium lacking the ZnuABC transporter inoculated in pigs by oral route shows characteristics of attenuation if compared with virulent S. Typhimurium ATCC 14028. Moreover, vaccination with S. Typhimurium ΔznuABC (our candidate vaccine strain mutated in znuABC) caused an increase of body temperature for two days but there was no difference in body temperature between the groups by 7 DAV. A heterogeneous growth rate within the vaccinated groups was noticed. However, mean of the weights of vaccinated groups were comparable with those of controls. S. Typhimurium ΔznuABC primed a specific humoral and cellular immune response (Supplementary Data). On the whole, these findings demonstrate that S. Typhimurium ΔznuABC has appreciable characteristics of safety and immunogenicity in pigs.

Vaccination is one of the methods suggested to decrease the burden of S. Typhimurium at swine farm level and prevent its dissemination through the pork production chain [5,16,17]. To assess the safety of S. Typhimurium ΔznuABC in pigs we took into account several parameters such as body temperature, weight gain, serum concentration of TNF-α and fecal shedding of the vaccine strain.

Some vaccinated animals showed a rise in body temperature at 1 and 2 DAV, however it ended within one week. The administration of S. Typhimurium ΔznuABC caused stunted growth of some pigs regardless of vaccine dose. Nevertheless, body weight means of the experimental groups were comparable. However conclusive assessment requires further studies enrolling larger groups of animals with an homogeneous initial size and age, since a previous study reported that the administration of Salmonella live vaccines decreased the growth rate of vaccinated pigs [18].
The serum concentration of TNF-α in pigs inoculated with S. Typhimurium ΔznuABC showed a significant increase compared to controls only when the highest vaccinal dose is administered (Fig. S4). These data are consistent with previous observations that report reduced variations of serum concentration of TNF-α even in pigs fed a diet containing virulent S. Typhimurium [19].

Isolation of S. Typhimurium ΔznuABC from environmental swabs yielded from pens of vaccinated pigs ended within two weeks. Furthermore, S. Typhimurium ΔznuABC evidenced a shorter viability in pig feces when compared with S. Typhimurium ATCC 14028. These findings suggest that S. Typhimurium ΔznuABC expresses a limited persistence in the environment.

The serum concentration of IgG and the antigen specific ex vivo production of IFN-γ (supplementary data) by whole blood samples were chosen as parameters to assess the immunogenicity of S. Typhimurium ΔznuABC. It is known that B-cell and antibodies play a role in the protection from infection in mouse, pigs and humans [20, 21]. In particular, antibodies opsonize Salmonella in the early stages of infection, hindering microorganisms to reach their intracellular niche within phagocytes [21, 22]. In pigs [23], as well as in humans [24] a humoral immune response is crucial in the early phase of life when an immune response against Salmonella is not developed yet. Nevertheless, in pigs maternal antibodies tend to disappear at the time of weaning, leaving piglets susceptible to Salmonella infection [25]. On that account, S. Typhimurium ΔznuABC is reported to prove a humoral immune response inducing high serum levels of IgG.

Live vaccines are believed to be more effective than inactivated ones in controlling S. Typhimurium infection because of their pronounced ability to stimulate the cellular immune compartment and to determine the production of mucosal IgA [16, 25, 26].

A Th1-oriented immune response is crucial for the complete clearance of Salmonella microorganisms, with activated IFN-γ producing CD4+ T-cells fostering bacteria killing by monocytes [21]. Although the ex vivo antigen specific release of IFN-γ by the whole blood samples of animals vaccinated with S. Typhimurium ΔznuABC or inactivated Salmonella vaccine suggests a similar Th1 antigen specific immune response (Fig. S3), we have evidence that after an oral virulent challenge with S. Typhimurium ATCC14028, groups vaccinated with S. Typhimurium ΔznuABC displayed a greater and more prompt IFN-γ production if compared to animals vaccinated with inactivated Salmonella vaccine (data in publication). Oral vaccination of pigs with S. Typhimurium ΔznuABC induced serum IgG and evoked a cellular immune response. These data suggest that S. Typhimurium lacking the ZnuABC transporter is a promising strain to be used as live vaccine for mucosal delivery in pigs. Furthermore, live vaccines can be administered through drinking water, offering a welfare safe-guarding method of vaccination avoiding stressful handling and adverse reactions frequently observed when parenterally injected [27].

Although we proved S. Typhimurium ΔznuABC to be immunogenic, vaccinated pigs showed an increase of body temperature and a heterogeneous weight gain that may raise some concerns about its safety. Therefore, the effects in pigs of a reduction of the vaccine dose or the use as vaccines of S. Typhimurium strains deleted of ZnuABC and of Znt [10] and Znpt [28] two other zinc uptake systems, should be investigated. Overall, our data support the safety and immunogenicity of oral administration of S. Typhimurium ZnuABC in pigs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.04.032.

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