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(Article begins on next page)

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Parenteral administration of attenuated *Salmonella* Typhimurium *ΔznuABC* is protective against salmonellosis in piglets

- J. Ruggeri[a, 1], M. Pesciaroli[b, c, d, 1], B. Gaetarelli[a], F.E. Scaglione[e], P. Pregel[e], S. Ammendola[f], A. Battistoni[f], E. Bollo[e], G.L. Alborali[a], P. Pasquali[b, c, ,

Highlights

• Parenteral administration of Salmonella Typhimurium ΔznuABC is safe and effective versus pig salmonellosis.

• Natural and experimental infections with Salmonella Typhimurium wild type have different patterns of response.

• Efficacy of Salmonella Typhimurium ΔznuABC is dose dependent.

Abstract

A major cause of salmonellosis in humans is the contamination of pork products. Infection in pigs can be controlled using bio-security programs, but they are not sufficient in countries where a high level of infection is recorded. In this context, the use of vaccines can represent a valid supplementary method of control. Recently, we have demonstrated that an attenuated strain of Salmonella enterica serovar Typhimurium (Salmonella Typhimurium ΔznuABC) is protective against systemic and enteric salmonellosis in mouse and pig infection models, candidating this strain as an oral attenuated vaccine. In this study, we compared the efficacy of this attenuated Salmonella Typhimurium strain when administered orally or parenterally. Furthermore, in order to reproduce a pseudo-natural infection model, vaccinated pigs were allocated in the same pen with animals shedding virulent Salmonella Typhimurium. Animals were monitored weekly after vaccination and contact with infected piglets. Diarrhea and ataxia were recorded and Salmonella shedding was tested individually through bacterial culture.

After four weeks of cohousing, piglets were euthanized, after which lymph nodes reactivity and gross lesions of the gut sections were scored at necropsy. Organs were submitted to microbiological and histological analyses.

The data reported herein show that parenterally vaccinated animals do not shed the attenuated strain, and at the same time the absence of symptoms and decrease in virulent strain shedding in feces from day 6 after challenge demonstrated protection against infection induced by virulent Salmonella Typhimurium. In conclusion, our findings suggest that this is an alternative route of Salmonella Typhimurium ΔznuABC administration, without ignoring the advantages associated with oral vaccination.

Keywords
Vaccine;
Salmonella;
Piglets;
Pseudo-infection model

1. Introduction

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a Gram-negative bacterium, responsible for food borne zoonotic infections. The disease is usually characterized by self-limiting gastroenteritis in humans, but may occasionally cause fever and systemic infection [1] and [2]. Salmonellosis along with being a public health concern, is a economic problem as it also affects livestock breeding [3] and [4]. 26.9% of the human cases in the EU are caused by contaminated pork meat [5], [6], [7] and [8]. The prevalence of the main serovars in pigs, S. Typhimurium and S. Derby, is estimated approximately to be 10.3% at the slaughterhouse [6]. Application of bio-security programs, the use of probiotics and organic acids in feed, pest control and good farming practices are the most common actions applied to reduce Salmonella spp. at a farm level [9] and [10]. Nevertheless, there is not yet an easy, economic and effective strategy ensuring the full eradication of Salmonella from pig farms. In this context, the use of vaccines could represent a valuable solution to increase the resistance of pigs to

infection and minimize the spread of Salmonella spp. in the environment [11] and [12]. Attenuated vaccines are known to be more effective than those based on killed-microorganisms, being able to induce both mucosal and systemic immune responses [4], [13], [14] and [15]. Unfortunately, safe and effective live S. Typhimurium vaccines for swine are available only in few European countries [16].

Recently, we produced a mutant strain of S. Typhimurium deleted from the znuABC operon, which encode for the high affinity zinc importer ZnuABC (S. Typhimurium ΔznuABC) [17]. This Salmonella strain is attenuated due to its inability to efficiently recruit zinc in the infected host and it is protective in both mouse and pig models of salmonellosis [1], [17], [18], [19] and [20].

Here, we have assessed whether S. Typhimurium ΔznuABC is able to exert a protective effect also when it is parenterally administered in piglets. In addition, we used a new challenge model in order to simulate a natural infection, when vaccinated and naïve piglets were co-housed with piglets that were shedding wild type S. Typhimurium. More specifically, this challenge model was different from the challenge model used in our previous study in which vaccine and virulent strain of S. Typhimurium were intragastrically administered to pigs [19] and [20].

2. Materials and methods

2.1. Salmonella spp. cultures

S. Typhimurium ATCC14028 and its isogenic mutant strain denominated S. Typhimurium ΔznuABC were used in this study. Attenuated strain (S. Typhimurium ΔznuABC) was produced according to the method previously described [17], [18] and [19]. Wild type and mutant strains were cultured 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. Experimental design

Twenty-five weaned piglets were enrolled in the study. They were born in a Salmonella free farm and were individually checked to be Salmonella-free through microbiological and serological methods. In addition, a serological analysis was conducted on provenance sows in order to exclude the presence of Salmonella antibodies.

These were allocated at the animal facilities of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna and acclimatized for a week before the onset of the experiments. Experiments were authorized by national authority (protocol number 118/2012) in accordance to Italian and European regulations (D.L. 116/92) and were carried out under the supervision of certified veterinarians.

Piglets were divided into 5 groups. Each group was kept in a separate room under natural day-night rhythm and with free access to water and feed. Group A (5 piglets) was intramuscularly vaccinated with 104 CFU of S. Typhimurium ΔznuABC suspended in 1 ml of PBS; groups B (5 piglets) and C (4 piglets) were intragastrically vaccinated with 5 × 107 and 5 × 105 CFU of S. Typhimurium ΔznuABC, respectively. Salmonella cells were administered in 20 ml of sodium bicarbonate buffer in order to increase gastric pH and to aid bacterial survival. Group D (6 piglets) was untreated and used as a control group. Five seeder piglets composed Group E.

Fecal samples were collected at 1, 2, 7, 14, 21 and 42 days after vaccinations (DAV). Six weeks after vaccination, the piglets of group E were infected with 4 × 108 CFU of wild type S. Typhimurium ATCC 14028 and all groups of piglets enrolled in the study were hence allocated in the same barn for two weeks in order to allow contact between vaccinated, naïve and shedder animals (seeder infection model). In order to estimate the spread of wild type S. Typhimurium, the feces of each animal were collected once a week until the end of the experiment. Piglets were euthanized four weeks after contact with shedder piglets. Samples of tonsils, ileocecal lymph nodes, ileum, caecum and colon were collected and submitted for microbiological and histological analyses.

2.3. Post mortem analyses

During necropsy, gross lesions of lymph nodes and ileum were scored by an arbitrary scale.

The lymph nodes scores ranged from 0 to 5, based on the hyperplastic reaction (0 no reaction, 5 severe hyperplasia and hyperaemia).

Ileum was scored according to hyperaemic reaction and erosions (0 no lesions and 5 severe lesions, erosion and hyperaemia).

2.4. Microbiology

Fecal shedding and organ colonization of wild-type S. Typhimurium and S. Typhimurium ΔznuABC were determined using ISO 6579: 2002/Amendment 1: 2007 protocol. Feces, intestinal contents and organ samples were weighed and homogenized in 9 parts of Buffered Peptone Water (BPW) (Oxoid Ltd., UK). This solution was used at 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 BPW. All BPW samples (diluted or not) were incubated at 37 °C for 18 ± 3 h. Afterwards, 0.1 ml of BPW cultures was seeded on semisolid modified Rappaport-Vassiliadis agar (MSRV) plates (Oxoid Ltd., UK) and incubated at 41.5 °C for 48 h for selection and enrichment of Salmonella. The semisolid medium aids motility of S. Typhimurium detected as halos of growth around the original point of inoculation, no distinct colonies were observed. A loopful of growth on a MSRV plates was streaked onto Xylose-Lysine-Desoxycholate Agar (Oxoid Ltd., UK) and Brilliant Green Agar (Oxoid Ltd., UK) plates and hence incubated at 37 °C overnight. Suspect Salmonella colonies underwent biochemical identification by BBL Enterotube II (BD Franklin Lakes, NJ, USA) and serological identification using Salmonella group specific antisera. This semi-quantitative approach enabled the determination of the likely concentration of Salmonella in each sample [17], [18] and [19].

2.5. Histology

Samples of ileocecal lymph nodes, ileum, caecum and colon were fixed in 10% buffered formalin according to standard procedures. For histological investigations, tissue samples were wax-embedded, sectioned at 4 μm using a microtome (Leica Microsystems, Wetzlar, Germany) and stained with hematoxylin and eosin (HE). In order to perform a statistical analysis, a numerical value based on the degree of lesions observed was assigned to each examined intestinal section, according to the scores reported in Table 1, taking into account villi, submucosa and Peyer's patch status, congestion and lesions pattern.

2.6. Statistical analyses

All statistical analyses were performed using GraphPad InStat (vers. 3.05) and GraphPad Prism (vers. 3.05) software (GraphPad Inc., San Diego, CA, USA). For villi necrosis, and lymphocytic and eosinophilic infiltration, the data obtained for the four study groups and for each of the examined tract were analyzed using the Kruskal–Wallis test (non-parametric one-way analysis of variance – ANOVA).

The same parameters were analyzed by Friedman Test (non-parametric repeated measures ANOVA), followed by Dunn's Multiple Comparison post test, comparing the different intestinal tracts in each group, to identify the most affected intestinal district.

Epithelial conglutination and vasal congestion distribution among groups were analyzed by Chi-squared Test for Independence. Differences in the microbiological results were estimated using the non-parametric Mann-Whitney test. A P-value of <0.05 was considered to indicate statistically significant differences.

3. Results

3.1. The vaccination route determines a different fecal shedding pattern of S. Typhimurium ΔznuABC

The first task was to determine the fecal shedding of S. Typhimurium ΔznuABC in vaccinated piglets. We determined the presence and the amount of bacteria in stools at different time-points after vaccination (1, 2, 7, 14, 21 and 42 DAV). Stools of group A (parenterally vaccinated) and group D (naïve animals) did not contain S. Typhimurium ΔznuABC at any of the time points considered (Fig. 1). On the whole, Group B shed a greater amount of S. Typhimurium ΔznuABC than group C throughout the observation period (B and C, orally vaccinated groups). At 42 DAV one animal of group C was shedding less than 10 CFU/g. Conversely, our results show that parenteral vaccination is not followed by an environmental shedding of S. Typhimurium ΔznuABC through feces during the first 6 weeks after vaccination.

3.2. Vaccination with S. Typhimurium ΔznuABC reduces fecal shedding of wild type S. Typhimurium

Six weeks after vaccination of groups A–C, five piglets of group E were orally inoculated with wild type S. Typhimurium ATCC14028. Vaccinated and naïve animals were allocated in the same pen with the five piglets inoculated with wild type S. Typhimurium (seeder pigs). Our aim was to reproduce a situation of co-housing in which both naïve and vaccinated animals were in contact with infected animals shedding wild type S. Typhimurium into the environment (seeder infection model). As depicted in Fig. 2, naïve piglets (group D) acquired the infection; wild type S. Typhimurium was detected in their feces two days after the contact and shedding increased throughout the observation period reaching a mean value of 103 CFU/g at 26 days after contact with seeder pigs. Vaccinated groups (A–C) also acquired the infection, showing a shedding peak mean of approximately 10 CFU/g of wild type S. Typhimurium at 8 (group A and B) and 19 (group C) days after contact with seeder pigs. At the next time point (26 days), all vaccinated groups (A–C) shed wild type S. Typhimurium to a lesser extent than the control group (group D). The differences were statistically significant. Vaccination with S. Typhimurium ΔznuABC reduces fecal shedding of wild type S. Typhimurium after infection acquired by close contact with seeder piglets.

3.3. Vaccination with S. Typhimurium ΔznuABC reduces colonization and lesions caused by wild type S. Typhimurium in organs

Animals were euthanized four weeks after contact with inoculated piglets. Tonsils, ileocecal lymph nodes, ileum, caecum and colon of each animal were analyzed as natural targets during infections [20]. Tonsils and lymph nodes of group D were more colonized than those of vaccinated groups (Fig. 3). However, differences between the vaccinated groups and controls were not statistically significant.

No differences were found in caecum colonization. Colon colonization was different between groups; animals of group D had a colon colonization ranging from 10 to 104 CFU of wild type S. Typhimurium. In contrast, animals of vaccinated groups showed a lower degree of colonization. The difference reached the statistical significance in the case of group B.

Post mortem analyses are reported in Fig. 4 and Fig. 5. Naïve piglets showed ileum wall thickening and reactivity of ileocecal lymph nodes. Piglets orally vaccinated with the lower dose (group C) showed a reaction similar to that observed in naïve piglets. Parenterally vaccinated piglets (group A) showed an intermediate degree of reaction, mainly characterized by ileum wall thickening.

Lastly, piglets vaccinated by the oral route with the higher dose (group B), showed no reactions after infection and gut and ileocecal lymph nodes were macroscopically normal. No macroscopical or microscopical lesions were observed in the tonsils of animals of any groups (A–D). Histologically, naïve piglets showed the most severe lesions in all the examined intestinal tracts. Epithelial conglutination and necrosis were associated with vasal congestion and lymph node depletion. In addition to the gross findings, Group C, being the most affected of the vaccinated groups, showed microscopical lesions similar to that observed in naïve piglets. In fact, epithelial conglutination was present in 94.44% of the examined tracts in group D and in 50.00% in group C, whereas only 26.67% and 20.00% of intestinal tracts of group A and B were involved ($p < 0.0001$). Vasal congestion distribution did not vary significantly among groups.

Group A and B seemed to show a milder degree of lymphocytic and eosinophilic inflammation (Fig. 5 and Fig. 6).

4. Discussion

In previous studies, we showed that S. Typhimurium lacking the ZnuABC transporter is an effective oral vaccine in preventing disease and in reducing colonization of gut and systemic organs of pigs experimentally challenged with virulent Salmonella microorganisms [19] and [20]. The results presented herein, corroborate and extend these findings, and also show that pigs intramuscularly vaccinated with S. Typhimurium ΔznuABC exhibit a degree of protection against virulent S. Typhimurium infection comparable to that observed in orally vaccinated animals. Group A (intramuscularly

vaccinated) shed wild type S. Typhimurium with a pattern similar to orally vaccinated groups (B and C) showing a peak of shedding at 8 days and a decline thereafter. Moreover, colonization and organs lesions observed in organs of animals of group A were minor to those observed in group D.

Nontyphoidal salmonellosis (NTS) still has an important impact on public health in developed countries [3] and the consumption of pork is increasingly referred to as a major source of human infection [5] and [6]. Vaccination is one of the methods suggested to decrease the burden of S. Typhimurium at the swine farm level and to prevent dissemination of bacterial microorganisms through the pork production chain. Vaccine efficacy could benefit from the association with other prophylaxis measures such as the administration of prebiotics, probiotics and organic acids in feed or water [11] and [12].

The application of Salmonella spp. vaccines was implemented in the EU at the poultry farm level over recent years to avoid Salmonella infection in humans, which often was caused by consumption of contaminated avian products. The positive results produced by this approach, led our team to pursue similar prophylactic tools applicable in pig farms. With this regard, we aimed at developing a safe vaccine and hence investigating its efficacy according to the different administration routes.

In this study, we assessed the ability of an intramuscular administration of attenuated S. Typhimurium ΔznuABC to induce immune protection in piglets against a virulent S. Typhimurium infection. To test our hypothesis, we took advantage of an experimental protocol, in which piglets shedding wild type S. Typhimurium (seeder pigs) were co-housed with vaccinated and naïve animals. Interestingly, all animals rapidly acquired infection after contact with the infected animals, regardless of the vaccination protocol undergone. Nevertheless, they showed a different progression of infection, as assessed by the analysis of bacterial shedding in feces. Unvaccinated piglets showed a progressive increase of S. Typhimurium shedding throughout the observational period, while piglets vaccinated either intramuscularly or orally showed a reduced and self-limiting shedding

of wt S. Typhimurium, although different degrees between groups were noted. These findings, obtained in an experimental setting resembling farm conditions, corroborate and extend that reported earlier both in mice [1], [17] and [21] and in pigs [20]. On that account, it is interesting to point out that the infection, acquired by direct contact with piglets shedding wt S. Typhimurium, showed marked differences to the pattern observed when we infected animals by an oral challenge [19] and [20]. In this study, we observed a clear involvement of the ileum, with evident signs of reactivity that were absent in the previous case. We can infer that the response of piglets is probably different and conditioned by the different protocols used. When wt S. Typhimurium is intragastrically administered, a high number of salmonellae reaches the gut and rapidly invades the mucosa of colon and caecum. On the contrary, when piglets are infected by continuous contact with environmental S. Typhimurium released by shedding piglets, they are exposed to a reduced number of Salmonellae, but for a protracted period of time. It is possible, therefore, to hypothesize that, in such conditions, ileum represents a preferential route of Salmonella entry, through the M cells of the mucosa [22].

Moreover, these findings suggest that S. Typhimurium ΔznuABC intramuscularly administered to pigs is able to increase the resistance of animals, protecting them from clinical salmonella infection. This evidence is notable since it contributes to broaden the possible use of S. Typhimurium ΔznuABC as a vaccine. In field conditions, vaccines administered by oral route have a series of advantages over those parenterally administered [23]. Nevertheless, singular parenteral vaccination can represent a useful approach in particular conditions, for examples when other vaccines are administered parenterally such as those for Porcine Enzootic Pneumonia or Aujeszky disease, or when it is necessary to limit the vaccination only to a group of animals [24]. We observed that piglets vaccinated using an intramuscular administration did not shed attenuated S. Typhimurium ΔznuABC after vaccination. This finding is not surprising because it is possible to hypothesize that the attenuated strain S. Typhimurium ΔznuABC does not colonize the gut when administered parenterally. However, a further set of experiments are needed to evaluate the clearance kinetics of S. Typhimurium ΔznuABC from organs of pigs vaccinated either orally or parenterally in order to exclude the possible

development of vaccine carrier animals. Intramuscularly vaccinated piglets showed a reduced shedding of wt S. Typhimurium. This finding is intriguing because it suggests the involvement of an immune-based protection either at mucosal and systemic level, implying a migration of functional immune cells to the gut, considered to some extent a secluded and privileged immunological district [25]. Although it is known that mucosal vaccines have the capability to prime better mucosal immune responses as they better mimic the natural infection, parenteral vaccines are still able to induce a certain degree of protection over mucosal infections at mucosal sites [26]. The immune system at the mucosal sites can be considered a functionally distinct compartment with unique characteristics [27]. In fact, once stimulated, orchestrates a complex response not only in the site of priming but also in other mucosal sites and even at systemic level [25].

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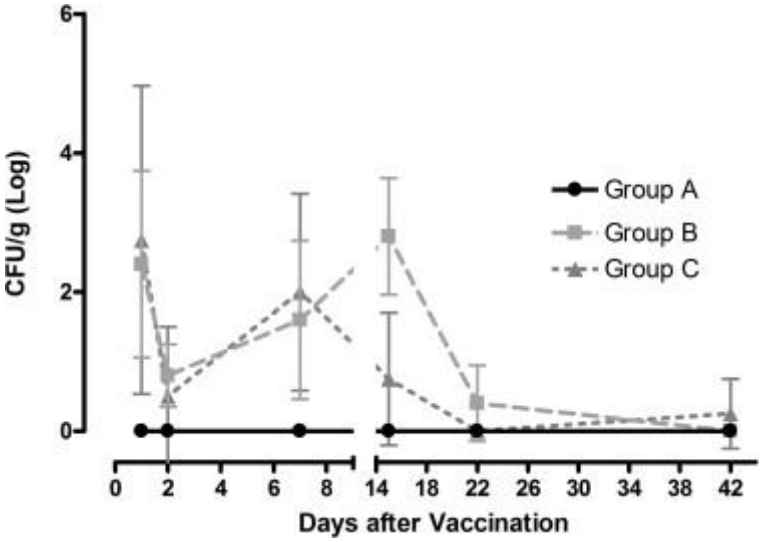

Fig. 1.

Attenuated *S.* Typhimurium *ΔznuABC* is shed for a limited period of time only after oral administration. Fecal shedding of *S.* Typhimurium *ΔznuABC* in piglets intramuscularly injected (group A), or orally inoculated (group B and group C), with a dose of 1×10^4, 5×10^7 or 5×10^5 CFU, respectively. Symbols represent group means and bars standard deviation.

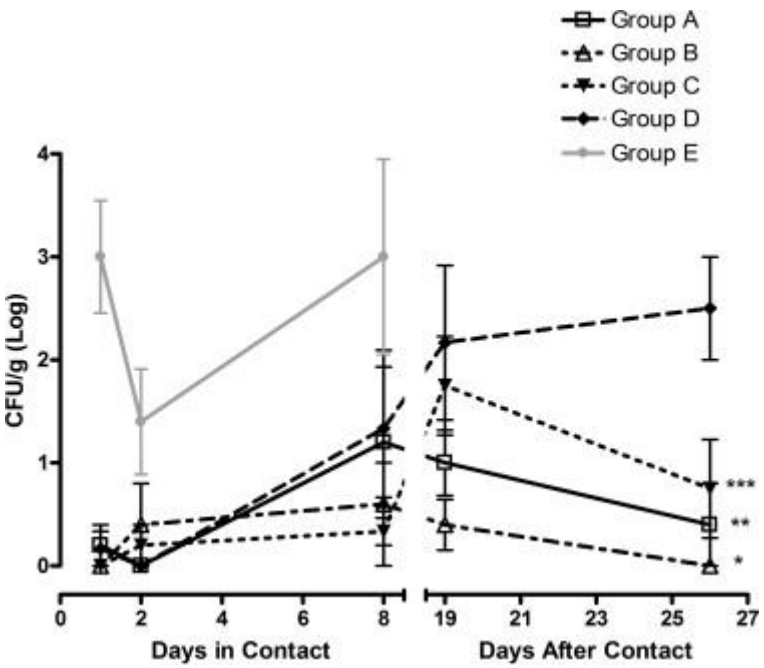

Fig. 2.

S. Typhimurium *ΔznuABC* vaccination reduces wild type *Salmonella* fecal shedding in infected piglets. *Salmonella* fecal shedding, at different time points after contact with piglets shedding virulent *Salmonella*. Results of group D (unvaccinated control piglets) were compared with results of group A (piglets vaccinated intramuscularly with 10^4 CFU), group B (vaccinated with an oral dose of 5×10^7 CFU) and group C (vaccinated with an oral dose 5×10^5 CFU). Differences were estimated using non-parametric Mann-Whitney test and were considered significant when * $P \leq 0.05$. Symbols represent group means and bars standard deviation.

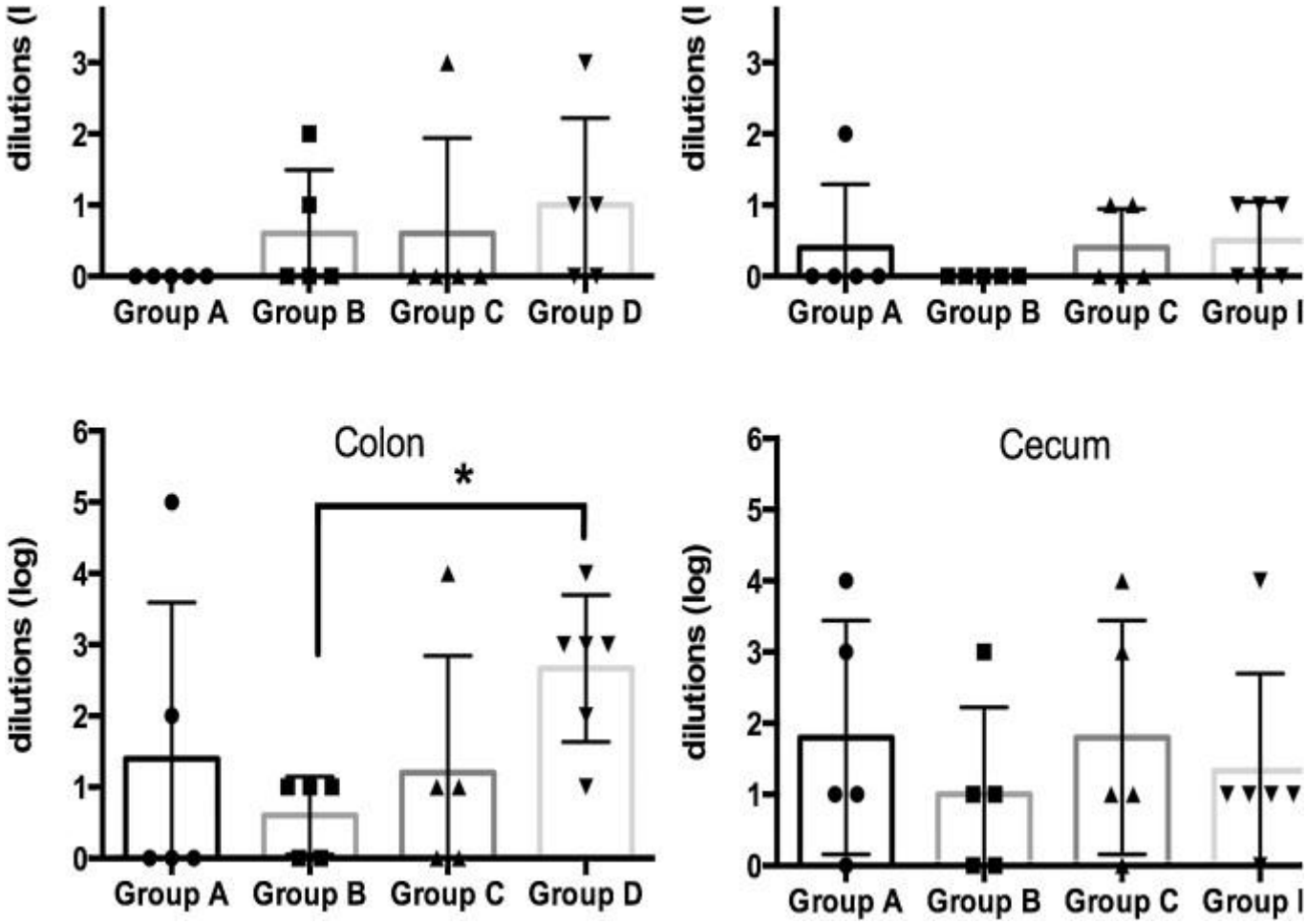

Fig. 3.

S. Typhimurium *ΔznuABC* vaccination reduces wild type *Salmonella* organ colonization in infected piglets. Recovery of wild type *Salmonella* from lymph nodes, tonsils, caecum and colon of piglets of group A-D after contact with wild type *S.* Typhimurium shedding piglets. Results of group D (unvaccinated control piglets) were compared with results of group A (piglets vaccinated intramuscularly with 10^4 CFU), group B (vaccinated with an oral dose of 5×10^7 CFU) and group C (vaccinated with an oral dose 5×10^5 CFU). Differences were estimated using non-parametric Mann-Whitney test and were considered significant when * $P \leq 0.05$. Each symbol represents one animal and the column represents the mean value of the group and bars standard deviation.

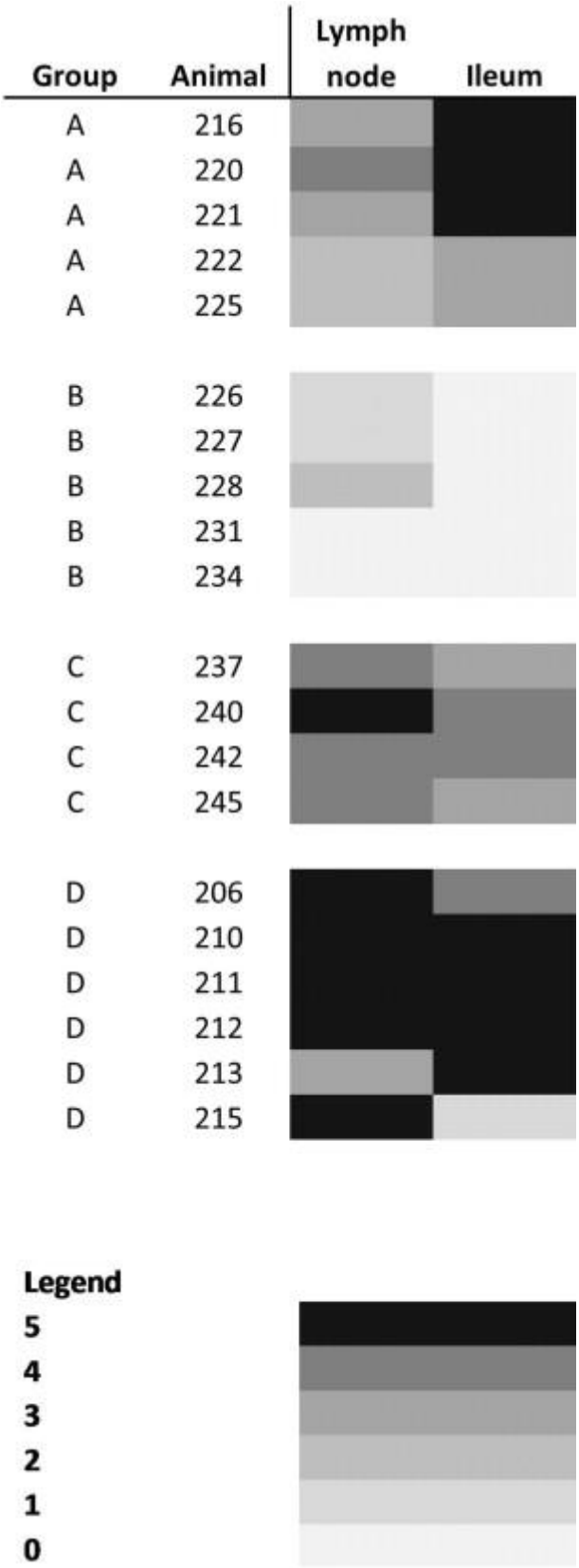

Fig. 4.

Vaccination prevents *Salmonella*-induced lesions in the gut. Macroscopical lesions of piglets vaccinated intramuscularly with 10^4 CFU (group A), vaccinated with an oral dose of 5×10^7 (group B), or with 5×10^5 (group C) of attenuated *S.* Typhimurium *ΔznuABC*, or unvaccinated control pigs (group D) after contact with wild type *S.* Typhimurium shedding

piglets, were reported using an arbitrary scale from an independent examiner (0: no lesions; 5: severe lesions).

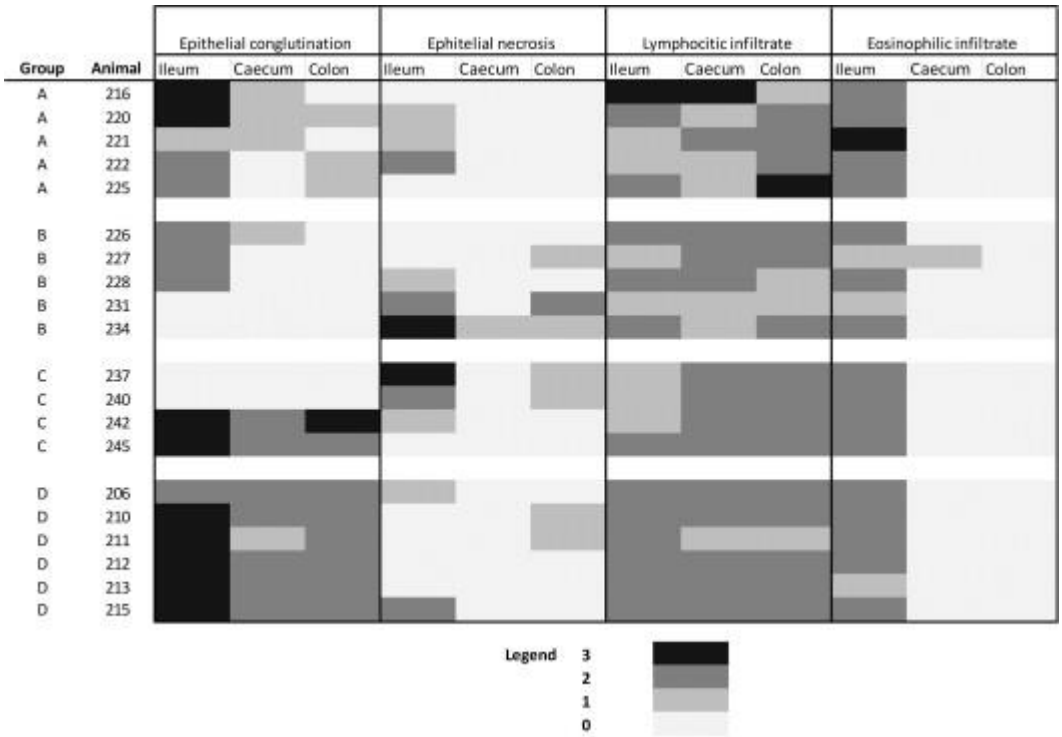

Fig. 5.

Parenteral vaccination and oral vaccination with higher dose reduce epithelial conglutination and necrosis caused by wild type *Salmonella* Typhimurium infection. Epithelial conglutination, necrosis, lymphocitic and eosinophilic infiltrate in ileum, caecum and colon of piglets vaccinated intramuscularly with 1×10^4 CFU (group A), vaccinated with an oral dose of 5×10^7 (group B), or with 5×10^5 (group C) of attenuated *S.* Typhimurium *ΔznuABC*, or unvaccinated control pigs (group D), after contact with wt *S.* Typhimurium shedding piglets. The severity of microscopical lesions is represented by a colorimetric scale (0: no lesions; 3: severe lesions).

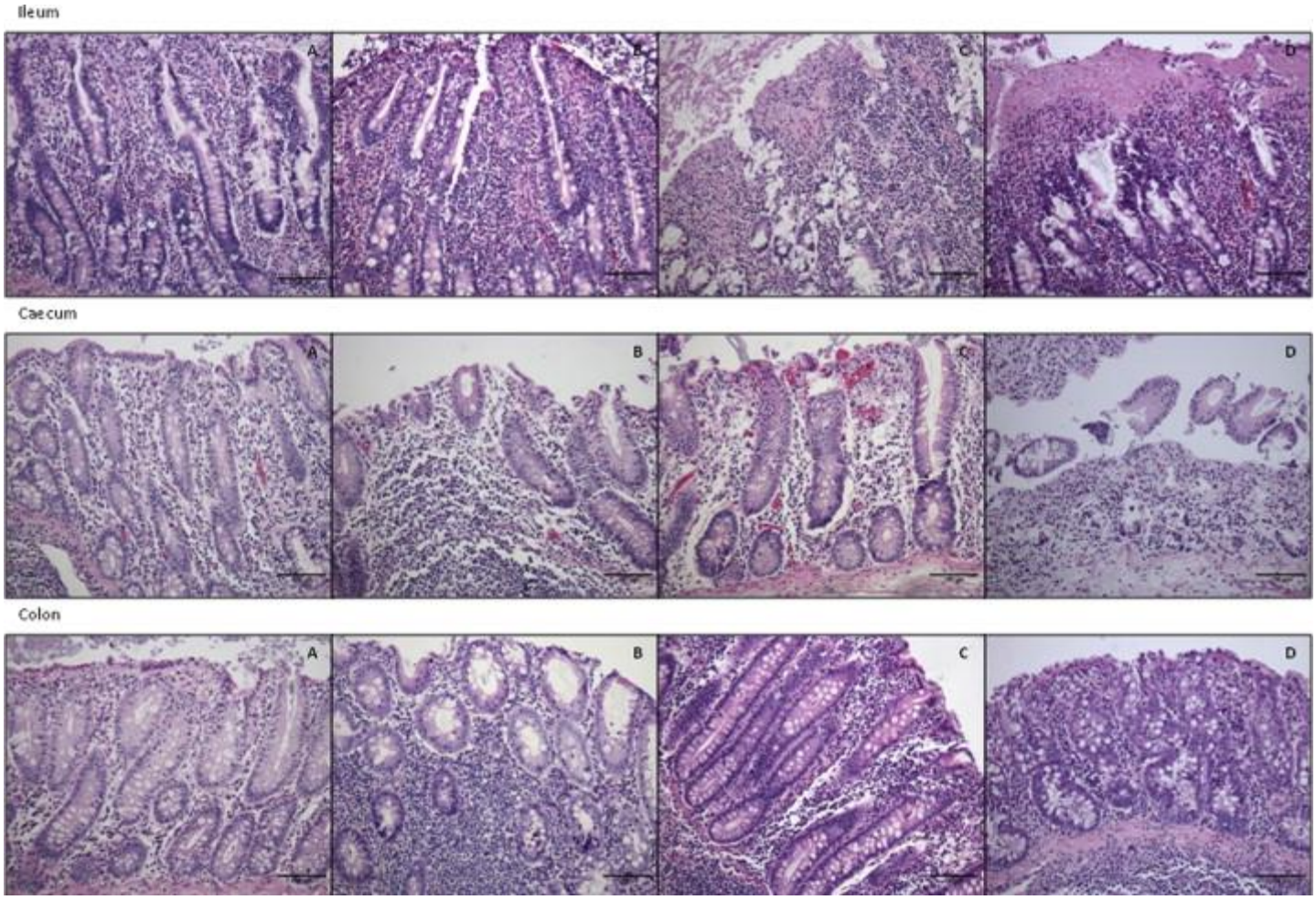

Fig. 6.

Wild type *Salmonella* Typhimurium cause serious lesion in ileum, caecum and colon. Epithelial conglutination, necrosis, lymphocitic and eosinophilic infiltrate in ileum, caecum and colon of piglets vaccinated intramuscularly with 1×10^4 CFU (group A), vaccinated with an oral dose of 5×10^7 (group B), or with 5×10^5 (group C) of attenuated *S.* Typhimurium *ΔznuABC*, or unvaccinated control pigs (group D), after contact with wild type *S.* Typhimurium shedding piglets.