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Orally administered attenuated *Salmonella enteritidis* reduces chicken cecal carriage of virulent *Salmonella* challenge organisms

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Abstract

Chickens were immunized orally with 10^9 cfu of the temperature-sensitive (T_s) mutant E/1/3 of *Salmonella enteritidis* at 1, 2, 3 and 7 days of age. The animals were challenged with wild-type strains of *Salmonella* of different serotypes 7 or 14 days following immunization. Chickens receiving multiple oral doses of the vaccine strain showed no signs of disease. Immunized animals shed the vaccine strain for at least 2 weeks after the last inoculation; on the other hand, colonization by the attenuated mutant of internal organs such as spleen and liver was limited. Early exposure of the immunized animals to the virulent bacteria resulted in a reduced cecal colonization by the pathogen. Visceral invasion by the wild-type strain of *S. enteritidis* or *S. gallinarum* was drastically diminished in birds challenged 14 days after immunization. Significant differences in the number of these *Salmonella* were found in the cecal contents, spleen and liver of immunized birds compared with the control animals. In addition, cecal colonization by the virulent strain was reduced in birds challenged with *S. typhimurium.* These results demonstrate that immunization of newly hatched chickens with live attenuated T_s mutant E/1/3 of *S. enteritidis* is safe and reduces *Salmonella* shedding.

Keywords: *Salmonella enteritidis*; Vaccine strain; Temperature-sensitive mutant; Chickens

1. Introduction

Salmonellosis is one of the most important food-borne diseases. The World Health Organization (WHO) has reported 1.3 billion cases per year of acute gastroenteritis due to non-typhoid salmonellosis with 3 million fatal cases (Gómez et al., 1997). Poultry meat

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and eggs contaminated with *Salmonella enteritidis* or *S. typhimurium* account for a large percentage of human s*almonellae* infections worldwide (Centers for Disease Control, 1991, 1993). Chicken cecal carriage of *Salmonella* can lead to horizontal transmission of the infection and contamination of eggshell with feces, carcass contamination during slaughter and probably retrocontamination of ovaries (Keller et al., 1995). Disease control measures should, therefore, be aimed at providing protection of poultry against pathogenic bacterial colonization. In this regard, the World Health Organization (1988) has recommended the development of live *Salmonella* vaccines as part of an overall control strategy to contain the pathogen in food animals. *Salmonella*-killed vaccines fail to control intestinal colonization, that is why live attenuated *Salmonella* vaccines are becoming more attractive and acceptable. Live avirulent strains of *Salmonella* given orally initially colonize the gut-associated-lymphoid-tissue (GALT), prior to colonization deeper tissues such as spleen and liver (McCaughan and Basten, 1983). Attenuated temperature-sensitive (T_s) mutant E/1/3 of *S. enteritidis* produced previously in our laboratory is non-virulent and induced excellent protection against lethal challenge, in vaccinated mice and chickens (Cerquetti et al., 1990; Gherardi et al., 1993; Cerquetti and Gherardi, 2000). In this study, we showed that newly hatched chickens can be safely inoculated with multiple oral doses of the T_s mutant E/1/3 to reduce wild-type *Salmonella* colonization of the gut and internal organs.

2. Materials and methods

2.1. Animals, bacteria and bacterial cultures

One-day-old male Shaver 579 chickens were obtained from the School of Veterinary Medicine, University of Buenos Aires, and were kept in our facilities.

Temperature-sensitive mutant E/1/3 of *S. enteritidis* was produced earlier in our laboratories and is fully described elsewhere (Cerquetti et al., 1990; Gherardi et al., 1993). Bacteria were cultured in trypticase soy broth (TSB, Difco Laboratories, Detroit, MI) in a water shaker incubator at the appropriate temperature (28 or 37°C) and at 220 cycles per min (cpm). Trypticase soy agar (TSA, Difco) plates were used for routine propagation of bacteria. The T_s mutant E/1/3 was grown overnight as a standing culture at 28°C in TSB. The culture was diluted 1:20 in TSB and incubated with aeration at 28°C to an absorbance of 0.6 at 600 nm. Bacterial cultures were pelleted by centrifugation, at 4°C and $6500 \times g$, and suspended to the appropriate density in buffered saline. *S. enteritidis* (O1, 9, 12), *S. typhimurium* (O1, 4, 12) and *S. gallinarum* (O1, 9, 12) were included in the challenge experiments. Challenge bacteria were grown at 37°C in TSB to an absorbance of 0.25 at 600 nm.

2.2. Experimental design

Chickens were deprived of food and water for 4 h before oral vaccination with the attenuated mutant E/1/3. Animals received 10^9 cfu of the vaccine strain at 1, 2, 3 and 7 days of age. Vaccinated animals were divided in two groups and were challenged orally at

day 7 (Group A) or day 14 (Group B) after the last immunizing dose. Non-immunized animals were used as controls. The challenge dose was 10^8 cfu per animal contained in 100 μl of saline. Fecal samples were taken immediately before challenge, swabs were incubated in selenite broth at 28°C for 18 h, then after incubation the broth was streaked on XLT4 agar plates and incubated at 28°C overnight. Animals were sacrificed 14 days after challenge. Ceca, spleens and livers were removed aseptically. Cecal contents were collected in pre-weighed sterile tubes. Three milliliters of saline were added to each tube and samples were homogenized immediately. Spleens and livers were weighed and homogenized in 1 and 5 ml of saline, respectively. Appropriate dilutions of the homogenates were plated on XLT4 agar. All samples were inoculated in selenite broth for enrichment. Plates were incubated overnight at 37°C, and examined for bacterial growth. *Salmonella* colonies were confirmed by biochemical and serological tests.

2.3. Statistical analysis

Analysis of variance was used to compare bacterial counts among vaccinated groups. Further, χ^2 test was utilized to determine significant differences in percentage of positive samples between groups. Values of $p<0.05$ were considered significant.

3. Results

3.1. Safety and persistence of the vaccine strain of S. enteritidis

The effect of multiple oral inoculations of the vaccine strain to newly hatched chickens was analyzed. Animals received four doses of 10^9 cfu within the first week of life. Vaccinated chickens were observed for clinical signs of infection such as diarrhea, lethargy or anorexia. Birds showed no signs of disease and survived the time course of the experiments. T_s mutant E/1/3 was isolated from 54% (38/70) of the animals 1 week after the last immunizing dose. The number of birds shedding the attenuated *Salmonella* was reduced to 28% (10/35) by the end of the second week. Colonization of internal organs such as spleen and liver was limited. In fact, the mutant was isolated from liver in one out of five animals only after enrichment in selenite broth.

3.2. Effect of vaccination on challenge with virulent S. enteritidis, S. gallinarum and S. typhimurium

Multiple oral doses of vaccine strain E/1/3 protected birds from challenge with wild-type *S. enteritidis.* Early exposure of the immunized animals to the virulent bacteria resulted in a reduced cecal colonization by the pathogen. Colonization of spleen and liver was also reduced at this time; the number of *S. enteritidis* (O1, 9, 12) recovered from immunized birds, however, was not statistically different from the amount isolated from the control group (Fig. 1, Group A). Visceral invasion by the wild-type strain of *S. enteritidis* was significantly diminished in immunized birds challenged 14 days after the last oral dose of the vaccine strain. Significant differences in the number of cfu were

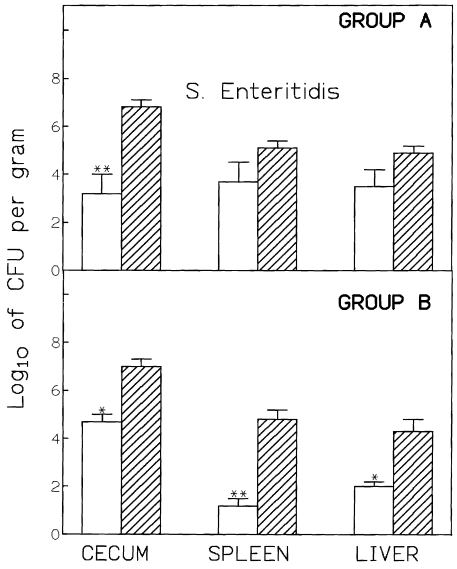

Fig. 1. Chickens were inoculated with 10^9 cfu of the T_s mutant E/1/3 at days 1, 2, 3 and 7 post-hatch. Animals were challenged with 10^8 cfu of *S. enteritidis* wild-type strain 7 (Group A) or 14 days (Group B) after the last immunizing dose. Birds were sacrificed 14 days after the challenge. Open bars, immunized animals; shaded bars, control animals; *, $p<0.05$; **, $p<0.01$.

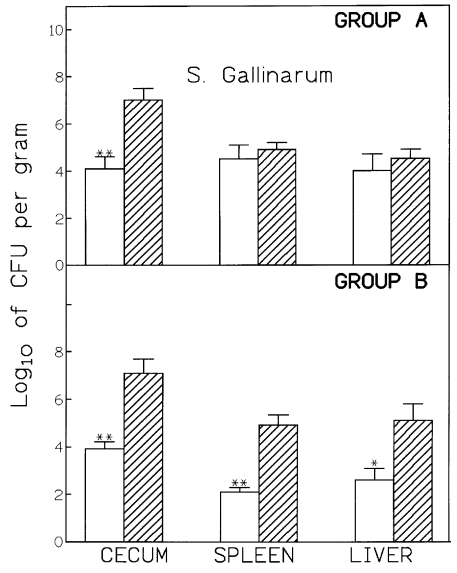

Fig. 2. Chickens were inoculated with 10^9 cfu of the T_s mutant E/1/3 at days 1, 2, 3 and 7 post-hatch. Animals were challenged with 10^8 cfu of *S. gallinarum* wild-type strain 7 days (Group A) or 14 days (Group B) after the last immunizing dose. Birds were sacrificed 14 days after the challenge. Open bars, immunized animals; shaded bars, control animals; *, $p<0.05$; **, $p<0.01$.

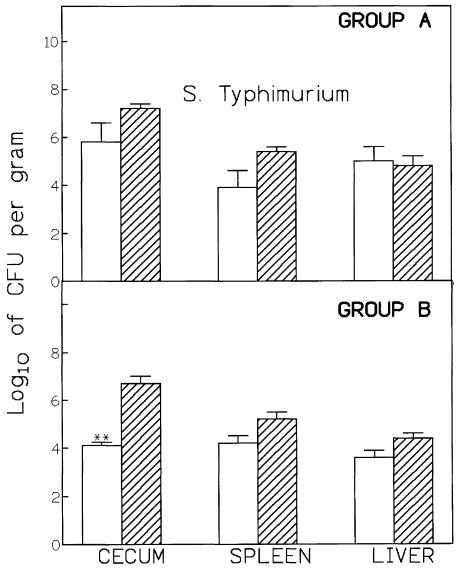

Fig. 3. Chickens were inoculated with 10^9 cfu of the T_s mutant E/1/3 at days 1, 2, 3 and 7 post-hatch. Animals were challenged with 10^8 cfu of *S. typhimurium* wild-type strain 7 days (Group A) or 14 days (Group B) after the last immunizing dose. Birds were sacrificed 14 days after the challenge. Open bars, immunized animals; shaded bars, control animals; **, $p<0.01$.

found in the cecal contents, spleen and liver of immunized birds compared with the control animals (Fig. 1, Group B). Multiple oral doses of the vaccine strain E/1/3 also protected chickens from the challenge with virulent *S. gallinarum* (O1, 9, 12). Again, immunized birds showed reduced cecal colonization by the pathogen after early challenge (Fig. 2, Group A). Animals challenged 14 days after immunization showed reduced amount of the pathogen isolated from cecum, spleen and liver (Fig. 2, Group B). Cross protection induced by E/1/3 vaccine strain was analyzed after the challenge with a strain of *S. typhimurium* (O1, 4, 12). Results showed that vaccination of young chickens with mutant E/1/3 did not reduce significantly the amount of pathogen recovered from cecum, spleen or liver after early exposure of the birds to *S. typhimurium* (O1, 4, 12) (Fig. 3, Group A). Cecal colonization by the heterologous *Salmonella* strain was reduced ($p<0.01$) in animals challenged 14 days after immunization (Fig. 3, Group B).

4. Discussion

The outcome of *Salmonella* infection in young chickens is rapid multiplication and excretion of the pathogen (Turner et al., 1998). Although extensively studied, the development of the carrier status is not well understood. In this regard, it was reported that intestinal colonization could be enhanced by the transient lymphocyte depletion of lymphoid organs observed following the early exposure of chickens to virulent *Salmonella* (Hassan and Curtiss III, 1994). We demonstrated that inoculation of newly

hatched chickens with high doses of T_s mutant of *S. enteritidis* E/1/3 is not only safe, but is beneficial for the birds.

The World Health Organization (1988) has recommended a program for the effective prevention and control of salmonellosis that includes the development of an efficacious live vaccine. Bacterins are effective in protecting chickens against invasion of internal organs by *Salmonella*, but fail to control the intestinal colonization (Germanier, 1972; Barrow et al., 1990; Gast et al., 1992, 1993). Live vaccines, on the other hand, confer better protection than killed vaccines. Chickens vaccinated orally with live homologous *Salmonella* showed a reduction in visceral invasion by the challenge strains and diminished colonization of the gastrointestinal tract (Barrow et al., 1990). Recently, it was demonstrated that live vaccines of *Salmonella* can induce protection against challenge with homologous and heterologous strains (Hassan and Curtiss III, 1997). Oral vaccination of chickens at 14 and 28 days of age with a live avirulent *S. typhimurium* vaccine prevented colonization and invasion of internal organs by *S. typhimurium* and *S. enteritidis* wild-type strains. The authors reported that this significant degree of protection lasted for 11 months after vaccination. We have recently reported that immunization of chickens with T_s mutant of *S. enteritidis* E/1/3 by different routes at day 1 of age, followed by a booster at day 14 reduced colonization of the cecum after challenge with virulent *Salmonella* strain (Cerquetti and Gherardi, 2000). The best results were observed in animals receiving the first dose of the vaccine strain (at day 1 of age) by the oral route. Undoubtedly, there is also a need to prevent intestinal colonization by *Salmonella* in newly hatched chickens. It was suggested that potential vaccine candidates should be tested for their capacity to prevent intestinal colonization in 1-day-old chickens (Methner et al., 1997). These authors showed that pretreatment of 1-day-old chickens with attenuated *S. typhimurium* strains resulted in a weak but significantly reduced colonization by the isogenic strain. Colonization by *S. enteritidis*, however, could not be diminished by either of the *S. typhimurium* vaccine strains tested. In the present work, we showed that immunization of chickens with T_s mutant E/1/3 of *S. enteritidis* within the first week of age prevented colonization of the cecum and invasion of spleen and liver after challenge with virulent *S. enteritidis* and *S. gallinarum*. Immunization also reduced colonization by an heterologous virulent strain of *S. typhimurium*.

Live vaccine strains of *Salmonella* are able to replicate, colonize and invade the spleen and liver of immunized animals, thereby leading to the induction of strong immunity (Germanier, 1972; Vielitz et al., 1992). Nevertheless, extended colonization and invasion of internal organs by the vaccine strain, may be undesirable. We have demonstrated earlier that T_s mutant E/1/3 of *S. enteritidis* conferred excellent protection to immunized mice with the additional advantage of producing limited invasion of the deep tissues (Gherardi et al., 1993). Results presented here show that the beneficial effects of immunization with T_s mutant of *S. enteritidis* E/1/3 started relatively soon after oral inoculation. Animals challenged 1 week after immunization showed reduced colonization of the cecum by the virulent strains. At this time, the vaccine strain could still be recovered from feces. It is unlikely, however, that an effective immune response was already induced in the challenged birds. Therefore, we cannot rule out that early intestinal colonization by the attenuated mutant played a role in the reduction of cecum colonization by the wild-type strains after challenge.

In conclusion, immunization of chickens with live attenuated T_s mutant E/1/3 is safe and reduces cecum colonization and visceral invasion by wild type *Salmonella*. Studies are currently underway to identify the mechanisms involved in the reduction of the intestinal colonization of birds inoculated at the day of hatch with multiple oral doses of T_s mutant E/1/3 of *S. enteritidis.*

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References

Barrow, P.A., Hassan, J.O., Berchieri Jr., A., 1990. Reduction in faecal excretion of *Salmonella typhimurium* strain F98 in chickens vaccinated with live and killed *Salmonella typhimurium* organisms. Epidemiol. Infect. 104, 413–426.

Centers for Disease Control, 1991. Outbreak of *Salmonella enteritidis* infection associated with consumption of raw shell eggs. Morbid Mortal Weekly Report 41 (1992) 369–372.

Centers for Disease Control, 1993. Outbreaks of *Salmonella enteritidis* gastroenteritis-California. Morbid. Mortal. Weekly Report 42 (1993) 793–797.

Cerquetti, M.C., Gherardi, M.M., Sordelli, D.O., 1990. Evaluation of different temperature-sensitive mutant phenotypes of *Salmonella enteritidis* as vaccine potentials. Curr. Microbiol. 21, 225–228.

Cerquetti, M.C., Gherardi, M.M., 2000. Vaccination of chickens with a temperature-sensitive mutant of *Salmonella enteritidis*. Vaccine 6, 1140–1145.

Gast, R.K., Stone, H.D., Holt, P.S., Beard, C.W., 1992. Evaluation of the efficacy of an oil-emulsion bacterin for protecting chickens against *Salmonella enteritidis*. Avian Dis. 36, 992–999.

Gast, R.K., Stone, H.D., Holt, P.S., 1993. Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of *Salmonella enteritidis* by laying hens. Avian Dis. 37, 1085–1091.

Germanier, R., 1972. Immunity in experimental salmonellosis. III. Comparative immunization with viable and heat-inactivated cells of *Salmonella typhimurium*. Infect. Immun. 5, 792–797.

Gherardi, M.M., García, V.E., Sordelli, D.O., Cerquetti, M.C., 1993. Protective capacity of a temperature-sensitive mutant of *Salmonella enteritidis* after oral and intragastric inoculation in a murine model. Vaccine 11, 19–24.

Gómez, M., Motarjemi, Y., Miyagawa, S., Kaferstein, F.R., 1997. Food-borne salmonellosis. World Health Stat. Q. 50, 81–89.

Hassan, J.O., Curtiss III, R., 1994. Virulent *Salmonella typhimurium*-induced lymphocyte depletion and immunosuppression in chickens. Infect. Immun. 62, 2027–2036.

Hassan, J.O., Curtiss, R.III, 1997. Efficacy of a live avirulent *Salmonella typhimurium* vaccine in preventing colonization and invasion of laying hens by *Salmonella typhimurium* and *Salmonella enteritidis*. Avian Dis. 41, 783–791.

Keller, L.H., Benson, C.E., Krotec, K., Eckroade, R.J., 1995. *Salmonella enteritidis* colonization of the reproductive tract and forming and freshly laid eggs of chickens. Infect. Immun. 63, 2443–2449.

McCaughan, G., Basten, A., 1983. Immune system of the gastrointestinal tract. Int. Rev. Physiol. 28, 131–157.

Methner, U., Barrow, P.A., Martin, G., Meyer, H., 1997. Comparative study of the protective effect against *Salmonella* colonization in newly hatched SPF chickens using live, attenuated *Salmonella* vaccine strains, wild-type *Salmonella* strains or a competitive exclusion product. Int. J. Food Microbiol. 35, 223–230.

Turner, A.K., Lovell, M.A., Hulme, S.D., Zhang-Barber, L., Barrow, P.A., 1998. Identification of *Salmonella typhimurium* genes required for colonization of the chicken alimentary tract and for virulence in newly hatched chicks. Infect. Immun. 66, 2099–2106.

Vielitz, E., Conrad, E.C., Vob, M., Lohren, U., Bachmeier, J., Hahn, I., 1992. Immunization against *Salmonella* infections using live and inactivated vaccine preparations. World Poultry Congress, Amsterdam, pp. 435–438.

World Health Organization, 1988. Recommendations for research, WHO Tech. Rep. Ser. 774, pp. 65–69.