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Vaccination of chickens with a temperature-sensitive mutant of *Salmonella enteritidis*

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Abstract

One-day old chickens were inoculated with temperature-sensitive mutant E/1/3 of *S. enteritidis*. Two routes of inoculation were used: oral and intraperitoneal (ip). One group of chickens were given two oral inoculations (oral–oral). A second group received two ip inoculations (ip–ip). A third group received the first dose orally and the second ip (oral–ip) and the fourth group was given the first dose ip and the second dose orally (ip–oral). The vaccine strain was safe even when inoculated at high doses, and induced strong protection against virulent *S. enteritidis* strain after oral challenge. Results show that vaccination with mutant E/1/3 reduced the number of animals shedding the pathogen after challenge. Furthermore, animals immunized oral–oral and oral–ip showed a significant reduction in cecal and spleen colonization by virulent *Salmonella*.

Keywords: *Salmonella enteritidis*; Temperature-sensitive; Live vaccine; Chickens

1. Introduction

Salmonellosis remains one of the three most common food-associated diseases in humans. Poultry meat and eggs contaminated with *Salmonella enteritidis* account for a large percentage of human *salmonellae* infections worldwide [1,2]. Indeed, in the last decades, contamination of poultry products with *S. enteritidis* has been of increasing significance. Chicken cecal carriage of *Salmonella* is an important practical issue because it results in horizontal transmission of the infection and contamination of eggshell with feces, carcass contamination during slaughter and probably retrocontamination of ovaries [3]. Therefore, poultry must have a healthy and functional intestinal tract to maintain the feed efficiency required by modern production standards [4].

The development of an efficacious live vaccine has been acknowledged by the World Health Organization as part of an overall control strategy to contain *Salmonella* in food animals [5]. *Salmonella* killed vaccines can protect chickens against invasion of internal organs but fail to control intestinal colonization responsible for the spread of the pathogen. Thus, vaccination with live attenuated *Salmonella* vaccines is becoming more attractive and acceptable. Live avirulent strains of *Salmonella* given orally initially colonize the gut-associated-lymphoid-tissue (GALT), prior to colonizing deeper tissues such as spleen and liver. It is known that delivery of antigens to the GALT leads to generalized secretory, humoral and cellular immune responses [6].

Several means of attenuation have been used for developing live avirulent strains of *Salmonella* including curing of virulence plasmid and auxotrophic mutations [7,8]. A novel attenuation technique has been recently described for *S. enteritidis*; the pathogen is repeatedly phagocytosed and recovered from neutrophils [9]. As a method of genetic attenuation tempera-

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ture sensitivity may offer certain advantages over other procedures. Temperature-sensitive (Ts) mutants isolated in our laboratory are able to duplicate at 26–28°C but show impaired growth at 37°C (non-permissive temperature). Some Ts mutants cannot duplicate at the non-permissive temperature whereas others are able to sustain limited replication at 37°C before ceasing growth [10]. The latter are known as "coasters" and upon inoculation into a host they resemble the early stages of the natural infection. Ts coaster mutant E/1/3 of *S. enteritidis* produced previously in our laboratory is avirulent and immunogenic. It was demonstrated that Ts mutant E/1/3 of *S. enteritidis* induced excellent protection against lethal challenge in vaccinated mice [11,12].

In this study we showed that Ts mutant E/1/3 protects birds from oral challenge and that it can be safely inoculated intraperitoneally or by the oral route into 1-day-old chickens. These results suggest that vaccination with live attenuated mutant E/1/3 could be useful in the prevention of food poisoning associated with *S. enteritidis*.

2. Materials and methods

2.1. Animals

Male Shaver 579 chickens were obtained from the School of Veterinary Medicine, University of Buenos Aires and were kept in our facilities.

2.2. Bacteria

The Ts mutant E/1/3 of *S. enteritidis* was obtained and characterized earlier in our laboratory [11,12], it can grow well at 28°C but has limited replication at 37°C. The parental strain of *S. enteritidis*, which is resistant to kanamycin (Km^R) was used for challenge experiments. Bacteria were cultured in trypticase soy broth (TSB, Difco Laboratories, Detroit, MI) with aeration, at the appropriate temperature (28°C or 37°C) and at 220 cycles min^{-1}. Trypticase soy agar (TSA, Difco) plates were used for routine propagation of bacteria. The Ts 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 ***g***, and resuspended to the appropriate density in buffered saline. The wild type *S. enteritidis* and *S. enteritidis* Km^R strains were grown at 37°C in TSB to an absorbance at 600 nm of 0.25.

2.3. Immunizations

Chickens were given two immunizing doses; the first at 1 day of age and the second 2 weeks later. Two routes of inoculations were used: oral and intraperitoneal (ip). One group of chickens were given two oral inoculations (oral–oral). A second group received two ip inoculations (ip–ip). A third group received the first dose orally and the second ip (oral–ip) and the fourth group was given the first dose ip and the second dose orally (ip–oral). Non immunized animals were included in the experiments as controls. Chickens were deprived of food and water for 4 h before oral vaccination with the Ts mutant. Animals were inoculated orally with 100 μl of 10^{10} colony forming units (cfu) ml^{-1} of Ts mutant E/1/3 of *S. enteritidis*. Food and water were returned 1 h after vaccination. Intraperitoneal vaccination was achieved injecting chickens with 100 μl of buffered saline containing 10^8 cfu ml^{-1} of the mutant E/1/3.

2.4. Protection studies

Animals were challenged orally with 10^9 cfu of the virulent *Salmonella* Km^R strain 14 days after the last immunizing dose. Protection induced by the vaccine strain E/1/3 was assessed analyzing the ability of the wild type strain of *S. enteritidis* to colonize the gastrointestinal tract and spleen of vaccinated and control chickens. After challenge cloacal swabs were taken from each bird and cultured in 10 ml of selenite broth for enrichment for 18 h before plating on XLT4 agar containing 100 μg μl^{-1} of kanamycin. At different time points after challenge animals were sacrificed for the collection of samples. Spleens were removed aseptically, weighed and homogenized in saline. Cecum contents were collected in pre-weighed sterile tubes and processed immediately. Appropriate dilutions of the samples were plated directly on XLT4 agar plates and XLT4 plates containing 100 μg μl^{-1} of kanamycin and they were also cultured for enrichment in selenite broth.

2.5. Statistical analysis

Significance was calculated at the 0.05 level of probability using two-tailed *t* tests.

3. Results

3.1. Safety of the vaccine strain E/1/3 inoculated to 1-day old chickens

One-day-old chickens were inoculated with the Ts mutant E/1/3 or the wild type strain of *S. enteritidis*.

Table 1
Safety of the Ts mutant E/1/3 of *S. enteritidis* inoculated to newly hatched chickens

Strains	Route	Dose (cfu)	Survivors
Ts mutant E/1/3	Intraperitoneal	10^7	10/10
Wild-type *S. enteritidis*		10^7	0/10
Ts mutant E/1/3	Oral	10^9	10/10
		10^8	10/10
		10^7	10/10
Wild-type *S. enteritidis*		10^9	0/10
		10^8	0/10
		10^7	6/10

Animals received a single ip injection of 10^7 cfu or a single oral inoculation containing 10^7, 10^8 or 10^9 cfu. Animals were observed for clinical signs of infection such as diarrhea, lethargy or anorexia for 21 days after inoculation. Birds inoculated with Ts mutant E/1/3 showed no signs of disease and survived the time course of the experiments. Conversely, animals receiving the wild type strain died within 6 days after challenge, regardless of the route of inoculation. Results are shown in Table 1.

3.2. Persistence of the Ts mutant E/1/3 and the parental wild type strain

Spleen and liver colonization by Ts mutant E/1/3 was studied after a single oral dose containing 10^9 cfu of the attenuated bacteria (Fig. 1). The vaccine strain showed limited ability to colonized internal organs compared with the wild type *S. enteritidis*. Only at day 2 post inoculation the mutant was found in the liver in high numbers (10^4 cfu g^{-1}). In contrast, organs from animals receiving 10^9 cfu of the parental strain of *S. enteritidis* were highly colonized (ca. 10^5 cfu g^{-1}) and all animals died within 6 days after inoculation.

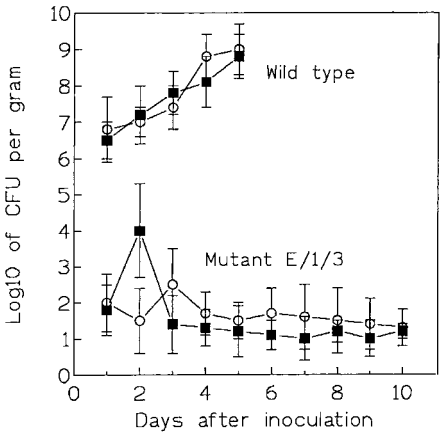

Fig. 1. Internal organ colonization by the wild type strain of *Salmonella enteritidis* and the Ts mutant E/1/3. One-day old chickens received a single oral dose of 10^9 cfu of either bacterial strain. At different time points five animals were sacrificed, the organs removed and the number of cfu per gram of spleen (○) and liver (■) was calculated. Values are means ± SEM.

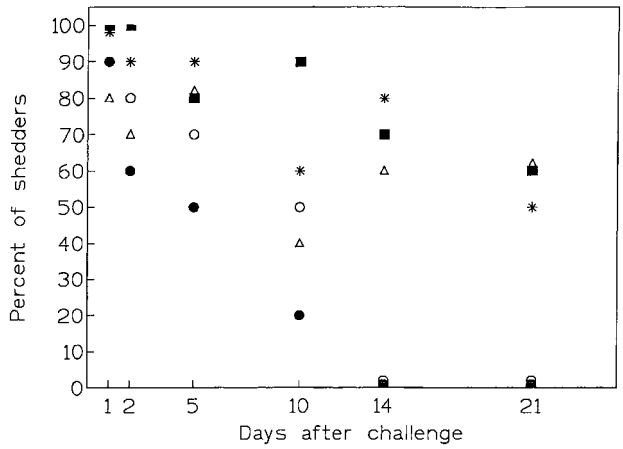

Fig. 2. Effect of vaccination on the shedding of virulent *Salmonella enteritidis*. The percentage of animals shedding the virulent *Salmonella* (shedders) was recorded for 21 days after challenge among chickens immunized: oral–oral (●), oral–ip (○), ip–oral (△), or ip–ip (∗), and control animals (square).

3.3. Protection studies

None of the vaccinated chickens showed signs of disease at anytime after challenge with the virulent strain. Control animals, however, suffered from diarrhea the 2nd day after challenge and 10% of birds (3/30) died (data not shown). Vaccination reduced the number of animals shedding the pathogen after challenge (Fig. 2). Chickens receiving the first immunizing dose by the oral route showed the lowest number of

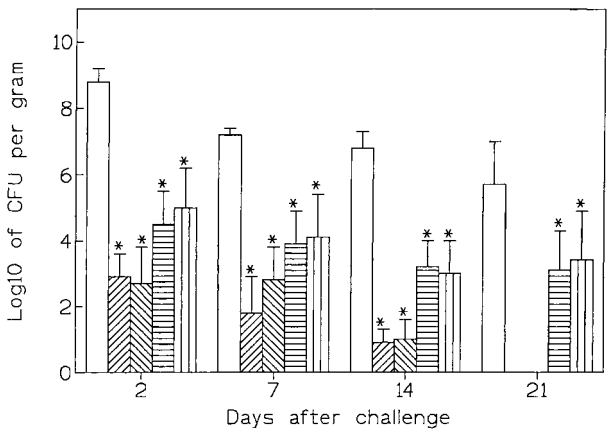

Fig. 3. Effect of vaccination on the cecal content of virulent *Salmonella* in chickens immunized oral–oral (▨), oral–ip (▧),; ip–oral (▤), or ip–ip (▥), and control animals (□). Values are means ± SEM; $n = 5$.

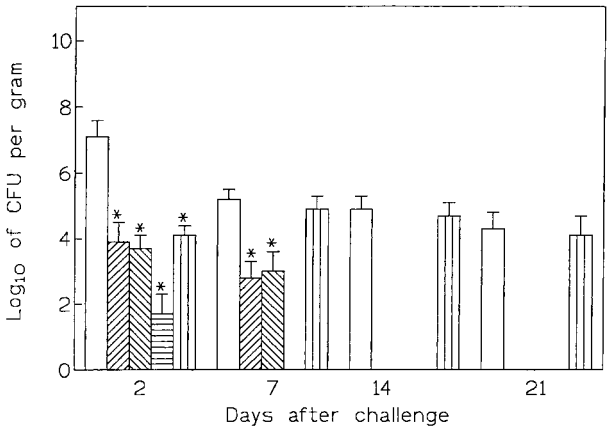

Fig. 4. Effect of vaccination of chickens with mutant E/1/3 on spleen colonization by the virulent strain of *S. enteritidis*. Animals were immunized oral–oral (▨), oral–ip (▧),; ip–oral (▤), or ip–ip (▥), and control animals (□). Values are means ± SEM; $n = 5$.

shedders. On the other hand, the number of shedders among non-immunized birds was never below 60%.

The amount of virulent *Salmonella* in cecal contents after challenge was diminished in vaccinated chickens (Fig. 3). The number of cfu of the pathogen recovered from ceca of immunized animals was significantly lower than the number of cfu from control chickens, for every time point analyzed. Duration of colonization was reduced in animals immunized oral–oral and oral–ip. No *Salmonella* could be recovered from ceca of these animals by day 21 after challenge.

The effect of vaccination of chickens with Ts mutant E/1/3 on spleen colonization is depicted in Fig. 4. Spleens from control animals, and from chickens vaccinated ip–oral, were colonized by the pathogen at least until day 21 after challenge. In contrast, double ip immunization (ip–ip) drastically reduced spleen colonization. In fact, *Salmonella* colonized the spleen of chickens vaccinated ip–ip only at day 2 post infection. Chickens that received double oral (oral–oral) or oral–ip inoculations eliminated the pathogen from spleen by day 14 after challenge (Fig. 4). The number of cfu of *Salmonella* recovered from spleens of orally vaccinated chickens at day 7 was lower than the number of cfu recovered from spleens of control animals, although differences were not statistically significant. Except at day 2 post challenge, no differences were found between control animals and chickens immunized ip–oral on the level of spleen colonization.

4. Discussion

Salmonella infection in young chickens results in rapid multiplication and extensive excretion of the pathogen in the feces [13]. Immunization with killed and live vaccines has been extensively used for the prevention of *Salmonella* infection in birds. Reported results vary depending on the vaccine preparation, challenge bacteria, route of inoculation, immunizing dose, and age at immunization, among other factors [14–21]. In this study 1 day-old chickens were inoculated with high doses of a Ts mutant of *S. enteritidis*. Our results showed that the attenuated mutant E/1/3 is highly avirulent and protective. Indeed, mutant E/1/3 was completely safe as all newly hatched chickens survived oral and intraperitoneal inoculations. Doses used in this study are near 10 times higher than the average immunizing dose used for other attenuated strains of *Salmonella* [15,18,22]. Ts mutant E/1/3 induced strong protection against colonization and invasion by the wild type *S. enteritidis*. Colonization of the cecum was limited in all vaccinated animals; the best protection was achieved when animals received the first immunizing dose orally. In fact birds inoculated oral–oral or oral–ip also showed a reduction in the duration of colonization of the cecum after challenge with the *S. enteritidis* virulent strain. Moreover, the percentage of animals shedding the pathogen after challenge was lower among birds from oral–oral and oral–ip groups. These results show that Ts mutant E/1/3 is more effective than other live *Salmonella* vaccines to prevent ceca colonization by *S. enteritidis* in 1 day-old chickens [15].

Protective capacity of Ts mutant E/1/3 was also evident when spleen invasion by the challenge pathogen was studied. Orally immunized chickens (oral–oral and oral–ip) cleared virulent *S. enteritidis* by day 14 after challenge. Interesting, animals immunized ip–oral (but not those inoculated ip–ip) cleared the virulent strain of *S. enteritidis* even faster. These results are in agreement with those reported recently showing that ip inoculation of *S. typhimurium* to chickens followed by an oral booster dose stimulates a specific secretory IgA response in the intestine [17]. Therefore, although the oral is the natural route for *Salmonella* infection, parenteral administration of *Salmonella* vaccines should not be totally ruled out, and deserves further consideration.

Live vaccines produce better protection than killed ones [23]. It has been demonstrated that killed vaccines can induce effective protection against invasion of visceral organs but they fail to control intestinal colonization by *Salmonella* [17,19,20,24]. Live attenuated strains of *Salmonella* can replicate, colonize and invade intestinal and visceral organs of inoculated chickens, thereby leading to the induction of strong immunity [23,25]. Extended colonization and invasion of internal organs by the vaccine strain, however, may be undesirable. In this report we show that E/1/3 conferred excellent protection with the additional advantage of producing limited invasion of the deep tissues.

The efficacy of immunization may be age-related, failure may occur in some cases because of the presence of interfering antibodies or because of undeveloped responsiveness of the immune system. Oral administration of pathogenic *Salmonella* to newly hatched chickens, for instance, can induce immunosuppression facilitating the establishment of the carrier status. On the other hand, it was reported that inoculation of non-pathogenic gut flora into 1 day-old chickens reduced colonization by *Salmonella* [26,27]. This phenomenon has been termed competitive exclusion. The role of the mutant E/1/3 in providing young birds with an established gut flora was not studied in this work but it can be speculated that inoculation of the Ts mutant E/1/3 early in life is beneficial. Some authors found that vaccination of chickens at 2 weeks of age rather than at hatch induces a better immune response [22]. In this report we show that oral inoculation of E/1/3 to 1 day-old chickens was safe, and followed by an oral or ip booster dose at day 14 it was highly protective.

In summary, attenuated mutant E/1/3 of *S. enteritidis* was used to immunize 1-day old chickens. It was found that the attenuated vaccine strain is safe even after the administration of high immunizing doses. Ts mutant E/1/3 induced strong protection against the virulent strain of *S. enteritidis* after challenge. Oral immunization of chickens at 1 day of age followed by an oral or ip booster dose at day 14 reduced shedding of the pathogen and colonization of cecum and spleen.

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