Development and evaluation of candidate recombinant *Salmonella*-vectored *Salmonella* vaccines

R. E. Wolfenden,* S. L. Layton,* A. D. Wolfenden,* A. Khatiwara,* G. Gaona-Ramírez,* N. R. Pumford,* K. Cole,† Y. M. Kwon,* G. Tellez,* L. R. Bergman,‡ and B. M. Hargis*[1]

*Department of Poultry Science, University of Arkansas, Fayetteville 72701; †Department of Animal Sciences, The Ohio State University, Columbus 43210; and ‡Department of Poultry Science, Texas A&M University, College Station 77843

ABSTRACT Attenuated *Salmonella* Enteriditis (ΔSE) recombinant vaccine vectors incorporating a *Salmonella* flagellar filament protein (fliC) subunit, a putative cell-mediated epitope, for expression of the *lamB* gene (encoding a maltose outer membrane porin), with or without co-expression of a putative immune-enhancing CD154 oligopeptide, were developed and compared with wild-type *Salmonella* Enteriditis (experiments 1 and 2) or the attenuated ΔSE empty vector (experiment 3) as initial vaccine candidates against *Salmonella* infection. A total of 3 experiments were performed to assess the infection and clearance rate of each of these constructs. Each construct or *Salmonella* Enteriditis was orally administered to broiler chicks at day of hatch by oral gavage (~10^8 cfu/chick). In experiments 1 to 3, liver-spleen and cecal tonsils were removed aseptically for recovery of wild-type *Salmonella* Enteriditis or ΔSE mutants. These experiments suggested that cell surface expression of fliC alone markedly increased the clearance rate of the vector at or before 21d postvaccination in all 3 experiments. In a fourth experiment, broilers were vaccinated with one of the vaccine constructs or the ΔSE empty vector and then challenged with wild-type *Salmonella* Typhimurium. At 19 d posthatch, 16 d postinfection, neither candidate protected against challenge significantly better than the ΔSE empty vector, although there was significantly less *Salmonella* recovered from vaccinated chickens as compared with nonvaccinated controls. These experiments indicate that these experimental vaccines did not protect against heterologous challenge or enhance clearance after *Salmonella* Typhimurium challenge; as such, their value as vaccines is limited. The increased clearance of the candidate vaccines, particularly the vector expressing fliC alone, may have value in that the fliC epitope may decrease the clearance time of other recombinant vectored *Salmonella* vaccines.

Key words: *Salmonella*, recombinant, vaccine, poultry

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INTRODUCTION

Each year in the United States, an estimated 1.4 million cases of nontyphoidal salmonellosis occur, leading to 600 deaths and an estimated economic loss of over $3 billion (WHO, 2005; CDC, 2008). Cases of human salmonellosis have often been linked to contaminated egg and poultry products and are a leading source of *Salmonella* outbreaks both in the United States and Europe, leading both the poultry industry and government agencies to look for ways to reduce the amount of *Salmonella* in commercial poultry (Hennessy et al., 2004; Gillespie et al., 2005; Straver et al., 2007; White et al., 2007). These *Salmonella* intervention strategies can broadly be broken down into preslaughter and postslaughter interventions. Preslaughter *Salmonella* intervention strategies include biosecurity, therapeutic antibiotics, probiotics and competitive exclusion products, organic acids, and vaccination.

Killed whole-cell bacterins and live attenuated vaccines are the 2 most common types of vaccines currently used in the poultry industry. Both types of vaccines have been shown to be useful in some applications, but both also have limitations. Killed vaccines have been primarily used to protect against systemic infections, and although they have been shown to reduce colonization and shedding, the protection provided by these vaccines has only limited ability to stop intestinal colonization (Nakamura et al., 2004; Deguchi et al., 2009). Carcasses and eggs can still be contaminated by *Salmonella* because these vaccines reduce, but do

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[1]Corresponding author: bhargis@uark.edu

not eliminate, shedding or prohibit colonization of the gastrointestinal tract. Live attenuated vaccines have been shown to colonize the gut, and in young chicks or hens in molt, this colonization by the vaccine strain can competitively exclude heterologous strains of *Salmonella* from colonizing the gastrointestinal tract (Holt and Gast, 2004; Van Immerseel et al., 2005; Barrow, 2007). The adaptive immune response elicited by live vaccines is composed of a humoral as well as cell-mediated response similar to that seen in a natural infection (Mastroeni et al., 2001; Van Immerseel et al., 2005; Barrow, 2007). This is in contrast to a killed vaccine, which primarily stimulates a humoral response. After vaccination, protection against homologous strains of *Salmonella* is almost complete (Chacana and Terzolo, 2006; Abd El Ghany et al., 2007), but little protection is afforded against a challenge by a heterologous serotype (Barrow, 2007; Young et al., 2007).

The ideal criteria for an effective *Salmonella* vaccine are as follows: protection against mucosal and systemic infection, avirulence of vaccine to both man and animals, reduction of intestinal colonization to reduce shedding and egg contamination, congruence with other control measures, and low cost of application (Van Immerseel et al., 2005; Barrow, 2007). Although these criteria are indeed important in selection and development of *Salmonella* vaccines, conspicuously left off the list is protection against all or even most serovars of *Salmonella* capable of causing foodborne illness in humans. Currently, no vaccine or vaccination program is capable of providing this type of protection. For a vaccine to meet all of the above criteria, it is likely that a highly conserved, immunoprotective epitope or epitopes will have to be identified and presented to the host immune system in such a way as to generate an effective immune response.

In recent years, the rapid increase in molecular biological techniques has led to the development of more sophisticated vaccines, of which live recombinant bacterial vectored vaccines are one of the most promising. This type of vaccine uses a genetically modified bacterium to express a heterologous antigen. To date, several species of bacteria including *Escherichia coli*, *Lactobacillus casei*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Typhi, *Bacillus subtilis*, and *Bacillus thuringiensis*, among others, have been manipulated to express protein antigens to protect against bacterial, viral, and protozoal pathogens as well as toxins (Yang et al., 1990; Denich et al., 1993; Tacket et al., 1997; Duc and Cutting, 2003; Arnold et al., 2004; Kotton and Hohmann, 2004; Mauriello et al., 2004; Ashby et al., 2005; Zhang et al., 2006; Duc et al., 2007; Kajikawa et al., 2007; Uyen et al., 2007; Yang et al., 2007; Huang et al., 2008; Liu et al., 2008; Ceragioli et al., 2009; Deguchi et al., 2009). These vaccines have an advantage over many other types of vaccines in that they are able to be delivered directly to a mucosal surface via nasal, ocular, or oral administration. Because most pathogens invade the host through a mucosal surface, an enhanced mucosal immune response is potentially beneficial to an effective immune response.

McSorely et al. (2000) found that mice that survived infection by *Salmonella* Typhimurium had a high percentage of CD4+ T cells, which recognized a highly conserved region of a flagellar filament protein (**fliC**); fliC is part of the flagella of *Salmonella* as well as other related species. This region of fliC was found to be highly conserved across several serovars of *Salmonella* as well as *E. coli*. When naïve mice were immunized with purified fliC, they were found to be protected against a lethal dose of *Salmonella* Typhimurium (McSorley et al., 2000). A recombinant *L. casei* expressing fliC was used to vaccinate mice and was found to provide some protection against colonization of *Salmonella* Enteritidis (Kajikawa et al., 2007).

As vaccines have become more advanced, the need for more advanced adjuvants to potentiate those vaccines has developed. It has been shown that CD154, also known as CD40 ligand, is present on activated T cells (Grewal and Flavell, 1998) and is important for stimulating both B cells and dendritic cells (Barr et al., 2003; Quezada et al., 2004). Although the role of B-cell stimulation is interesting, the stimulation of the antigen presenting dendritic cell to more proficiently present antigen and to produce cytokines is more important in regard to the adjuvanting properties of CD154 (Quezada et al., 2004). The cytokine production and antigen presentation by CD154-stimulated dendritic cells creates a feedback loop with T cells leading to clonal expansion and further differentiation of T cells, leading to a more robust and long-lasting immune response. Recently, a putative CD154 homolog has been found in chickens that could be used to potentiate a recombinant vectored vaccine for chickens (Tregaskes et al., 2005).

In the present study, double-attenuated recombinant *Salmonella* vaccines expressing fliC alone or in conjunction with a putative immunopotentiating region of CD154 were evaluated. Vector clearance, antibody production, and delayed-type hypersensitivity reaction were evaluated as parameters to characterize these vaccines. Additionally, vaccinated chicks were challenged by a heterologous strain of *Salmonella* to determine the efficacy of these vaccines.

MATERIALS AND METHODS

Attenuation of Salmonella Vaccine Candidate Strains

A primary poultry isolate of *Salmonella enterica* serovar Enteritidis phage type 13A (wild-type *Salmonella* Enteritidis, **wtSE**), originally obtained from the USDA National Veterinary Services Laboratory (Ames, IA), was attenuated by introducing defined, irreversible deletion mutations in the *aroA* gene (encoding 5-enolpyruvylshikimate-3-phosphate synthetase), the *htrA* gene (encoding a serine protease), or both,

of the wtSE genome as described previously (Husseiny and Hensel, 2005). Briefly, the target gene sequence in the bacterial genome of wtSE was replaced with the kanamycin-resistant (***KmR***) gene sequence. This was performed using 3-step PCR (**3S-PCR**) and electroporation of the 3S-PCR products into electrocompetent wtSE cells containing the pKD46 plasmid. The resulting cell mixture was plated on Luria-Bertani (**LB**) agar (240110, Becton Dickinson, Sparks, MD) plates supplemented with kanamycin (**Km**), 50 μg/mL (K-1876, Sigma, St. Louis, MO), to select for positive clones containing a *KmR* gene. The *KmR* gene was inserted into the genomic region containing the genes of interest (*aroA* or *htrA*) by flanking the *KmR* gene with sequences homologous to the genes of interest. Once *KmR* mutants were obtained, the deletion mutations were confirmed by PCR and DNA sequencing (data not shown; Cox et al., 2007).

Construction of Recombinant fliC and fliC-CD154 Inserts

Recombinant strains of *Salmonella* Enteritidis with stable integrated copies of a fliC (**SEΔfliC**) insert (VQNRFNSAITNLGNT), a fliC-human CD154 (**SEΔfliC-CD154H**) insert (VQNRFNSAITNLGNT-WAEKGYYTMS), and a fliC-chicken CD154 (**SEΔfliC-CD154C**) insert (VQNRFNSAITNLGNT-WMTTSYAPTS) were constructed using the method of Cox et al. (2007). Briefly, an I-SceI enzyme site along with a *KmR* gene was introduced into loop 9 of the *lamB* gene (encoding a maltose outer membrane porin) of the previously described attenuated *Salmonella* Enteritidis empty vector strain (**ΔSEEV**) by design of a PCR product that contains the I-SceI enzyme site and *KmR* gene flanked by approximately 200 to 300 bp of homologous DNA complementary to the up- and downstream regions directly flanking the insertion site on loop 9 of the *lamB* gene. The PCR product was electroporated into electrocompetent attenuated *Salmonella* Enteritidis cells containing the plasmid pKD46, and the resulting cell mixture was plated on LB agar plates supplemented with Km to select for positive clones now containing a *KmR* gene. After the SceI-Km mutation was made in loop 9, this region was replaced by a codon-optimized (Burns and Beacham, 1985) fliC or fliC-CD154 DNA sequence insert flanked by the loop 9 up- and downstream region, creating an additional 3S-PCR product. These newly created PCR products were electroporated into electrocompetent *Salmonella* Enteritidis containing the I-SceI-Km mutation along with plasmid pBC-I-SceI. The plasmid pBC-I-SceI produces the I-SceI restriction enzyme, which recognizes and cleaves a sequence creating a gap at the I-SceI site in the loop 9 region of the *lamB* gene where the previously described sequences were inserted into the *Salmonella* Enteriditis genome (Kang et al., 2004). The plasmid pBC-I-SceI also carries with it a chloramphenicol-resistant gene as the insert. This will replace the *KmR* gene because the mutations must have a new counterselection marker to select against the previous I-SceI-Km mutation. After electroporation, cells were plated on LB agar plates containing chloramphenicol (C0378, Sigma) at 34 μg/mL for the selection of positive mutants. Once positive mutation-inserts were identified, PCR and DNA sequencing was performed to confirm that the insertion sequences were present and correct (data not shown).

Amplification and Preparation of Salmonella

The *Salmonella* Enteriditis isolate described above and a primary poultry isolate of *Salmonella enterica* serovar Typhimurium, which had been previously selected for resistance to 25 μg/mL of novobiocin (**NO**) and 20 μg/mL of nalidixic acid (**NA**) or the vaccine constructs had previously been grown overnight in tryptic soy broth (**TSB**; 211822, Becton Dickinson), had 30% glycerol added and was stored at −80°C until needed. An aliquot was used to inoculate a fresh 10-mL tube of TSB, which was incubated overnight at 37°C. This culture was continuously incubated at 37°C for another 24 h and was passaged into fresh TSB at 8-h intervals. The cells were then washed 3 times with 0.9% sterile saline by centrifugation at 1,864 × *g*. The approximate concentration of *Salmonella* was estimated using a spectrophotometer and dilutions were made to get to an approximate final concentration of 4×10^8 cfu of vaccine/mL. Actual concentrations were determined retrospectively by serially diluting the vaccine solution and plating on brilliant green agar (**BGA**; 228530, Becton Dickinson, Sparks, MD) plates containing 25 μg/mL of NO (N1628, Sigma) and 20 μg/mL of NA (N4382, Sigma).

Recovery of Salmonella from Organ Samples

In all experiments, the ceca or cecal tonsils, liver, and spleen were aseptically removed from chickens humanely killed by CO_2 asphyxiation. The cecal tonsils as well as the liver and spleen were placed into tetrathionate broth (210420, Becton Dickinson) for enrichment of *Salmonella* or a *Salmonella* vector then incubated for 24 h at 37°C. The samples were then streaked onto BGA agar plates with NO and NA and incubated for 24 h at 37°C. The plates were examined for the presence of lactose negative colonies typical of *Salmonella* and the *Salmonella* vectors. In experiment 3 and experiment 4, the ceca were aseptically removed, manually homogenized, and diluted 1:4 by weight with sterile saline. Total viable counts of *Salmonella* or the *Salmonella* vectors were determined by making 10-fold dilutions and plating on BGA plates with NO and NA after 24 h of incubation at 37°C.

Determination of Serum IgG Antibody Levels to fliC

In experiments 1 and 2, approximately 1 mL of venous blood was drawn from a sample of chickens from each group. Blood was drawn from the jugular vein in chickens 14 d of age or younger and from the wing vein of older chickens. Serum was collected from each blood sample and stored at −80°C until further testing was performed. Standard ELISA procedures similar to those used by Layton et al. (2009) were followed for determination of serum levels of anti-fliC IgG.

Delayed-Type Hypersensitivity Reaction Test

A delayed-type hypersensitivity test adapted from Corrier (1990) was performed at d 30 of experiment 4. Briefly, the thickness of the outside toe webs of both the left and right feet of the remaining 9 to 11 chickens per group were measured using a pinch micrometer (7308, Mitutoyo, Aurora, IL). One hundred micrograms per 0.1 mL of *Salmonella* Typhimurium was injected (subdermally) into the toe web of the left foot, whereas an equal volume of 0.9% sterile saline was injected into the right toe web. The thickness of the toe webs was measured at 24 and 48 h postinjection. The change in thickness was calculated by the equation: (left toe web thickness end − left toe web thickness initial) − (right toe web thickness end − right toe web thickness initial).

Experiment 1

Experiment 1 was conducted to assess the clearance rate of SEΔfliC and SEΔfliC-CD154H in comparison to wtSE through d 21 after oral gavage at hatch. Commercial male Cobb chicks were obtained on day of hatch, randomized, tagged, and orally gavaged with 0.25 mL of 0.9% sterile saline containing approximately 1×10^8 cfu of SEΔfliC, SEΔfliC-CD154H, or wtSE (Table 1). The 4 groups of 40 chicks were placed into pens measuring approximately 2.2 m^2. All groups in this experiment and subsequent experiments had fresh pine shavings as bedding material and had free access to feed and water throughout the duration of the trial. Additionally, chicks in all experiments were kept at an age-appropriate temperature. A sample of 10 chicks per group was humanely killed, and the cecal tonsils, liver, and spleen were aseptically removed for individual recovery of wtSE or vectors at d 1, 7, and 21. Blood samples were drawn from 15 chicks per group on d 14 and 21.

Experiment 2

Experiment 2 was conducted to assess the colonization rate of wtSE and the *Salmonella* Enteriditis vectors at d 3 as well as the clearance of wtSE and *Salmonella* Enteriditis vectors at 21 d. Commercial female Cobb chicks were obtained on day of hatch, randomized, tagged, and orally gavaged with approximately 1×10^8 cfu of SEΔfliC, SEΔfliC-CD154C, or wtSE (Table 1). The 3 groups of 60 chicks were placed into pens measuring approximately 4.4 m^2. On d 3 and 21, ten chicks per group were humanely killed, and the cecal tonsils, liver, and spleen were aseptically removed for individual recovery of wtSE or *Salmonella* Enteriditis vectors. The ceca were also aseptically removed at d 21 for enumeration of wtSE or vectors from cecal content. Blood samples were drawn from 15 chicks per group on d 10 and 21.

Experiment 3

The purpose of experiment 3 was to assess the clearance rate of *Salmonella* Enteriditis vector strains in comparison to ΔSEEV. Cobb eggs were obtained from a local hatchery at d 18 of incubation and were hatched at the University of Arkansas. On day of hatch, the chicks were counted, randomized, tagged, and orally gavaged with 0.9% sterile saline or approximately 1×10^8 cfu of SEΔfliC, SEΔfliC-CD154C, or ΔSEEV (Table 1). The 4 groups of 62 chicks were placed into pens measuring approximately 4.4 m^2. The cecal tonsils, liver, and spleen were aseptically removed from a sample of 5 chicks per group for individual recovery of ΔSEEV or the appropriate vector 3 d posthatch. Additionally, 15 chicks per group were sampled for recovery of ΔSEEV or vector strain from their cecal tonsils,

Table 1. Confirmed dose of *Salmonella* Enteritidis (wtSE), *Salmonella* Enteritidis empty vector strain (ΔSEEV), or vaccine candidate strains of *Salmonella* Enteritidis[1] administered at day of hatch in experiments 1 to 4

Group	Experiment 1	Experiment 2	Experiments 3 and 4
wtSE	1.5×10^8	2×10^8	
ΔSEEV			0.75×10^8
SEΔfliC	0.75×10^8	0.75×10^8	0.625×10^8
SEΔfliC-CD154C	0.5×10^8		0.5×10^8
SEΔfliC-CD154H		0.5×10^8	

[1]Modified variants of the *Salmonella* Enteritidis mutant empty vector strain expressing a flagellar filament protein (fliC) antigen alone (SEΔfliC) or with the putative immunopotentiating compound CD154 of either human or chicken origin (SEΔfliC-CD154H and SEΔfliC-CD154C, respectively).

liver, and spleen at d 10, 19, and 30. From d 10 and 19 samples, the ceca were also aseptically removed for enumeration of ΔSEEV or vector strain.

Experiment 4

Experiment 4 was conducted to evaluate the ability of the *Salmonella* Enteriditis vector strains to provide protection against a heterologous challenge by *Salmonella* Typhimurium. Cobb embryos were obtained at d 18 of incubation and were hatched at the University of Arkansas poultry hatchery. On the day of hatch, the chicks were counted, randomized, tagged, and orally gavaged with 0.9% sterile saline or approximately 1×10^8 cfu of SEΔfliC, SEΔfliC-CD154C, or ΔSEEV (Table 1). The 4 groups of 62 chicks were placed into pens measuring approximately 4.4 m^2. The cecal tonsils, liver, and spleen were aseptically removed from a sample of 5 chicks per group for individual recovery of ΔSEEV or the appropriate vector 3 d posthatch. After the d 3 samples were obtained, all groups were challenged with 1.25×10^7 cfu of *Salmonella* Typhimurium per chick. Additionally, 15 chicks per group were individually sampled for recovery of *Salmonella* Typhimurium and ΔSEEV or vector strain from their cecal tonsils, liver, and spleen at d 10, 19, and 30. From the d 10 and 19 samples, ceca were also removed for enumeration of ST and ΔSEEV or vector strain. At d 30, a delayed-type hypersensitivity reaction test was performed to evaluate response to *Salmonella* Typhimurium.

Statistical Analysis

Numerical data from these studies were subjected to ANOVA using JMP7 (JMP Software, SAS Institute Inc., Cary, NC) and partitioned treatment means with a *P*-value of less than 0.05 indicated statistical significance. The percentage of recovery of *Salmonella* was compared using the χ^2 test of independence testing all of the possible group combinations to determine significance ($P < 0.05$) for these studies (Zar, 1984).

RESULTS

Colonization and Clearance of wtSE or ΔSE

In experiment 1, the initial colonization and organ invasion at 24 h by the experimental vaccine vector SEΔfliC was lower ($P \leq 0.05$) than that of wtSE (Table 2). This is in contrast to SEΔfliC-CD154H, which at 24 h was statistically similar to that of the positive control group receiving wtSE. By d 7 postvaccination, both vaccine vectors were recovered at a reduced rate ($P \leq 0.05$) from the liver and spleen as compared with wtSE. However, at this same time point, only SEΔfliC was recovered at a significantly reduced rate from the cecal tonsil. By d 21 postvaccination, the SEΔfliC vector could not be recovered from the liver, spleen, or cecal tonsils from any of the chickens sampled in this group, whereas wtSE and SEΔfliC-CD154H were recovered from the cecal tonsils at 100 and 50%, respectively, on d 21. The results of the second experiment (Table 3) were similar in that by d 21, wtSE and SEΔfliC-CD154C were recovered at a higher frequency ($P \leq 0.05$) than SEΔfliC. The major difference in this experiment was that the initial colonization of SEΔfliC was not significantly lower than that of either of the other 2 vaccinated groups. Additionally, the ceca of chickens from all groups in experiment 2 were removed at d 21 for enumeration of wtSE or ΔSE per gram of cecal content. The SEΔfliC was present at less than 0.77 $\log_{10}$ cfu/gram, as compared with wtSE and SEΔfliC-CD154C, which were recovered at a rate of 2.69 and 2.36 $\log_{10}$ cfu/g, respectively (Table 3). In the final colonization and clearance study, experiment 3, the positive control of wtSE was replaced with the ΔSEEV. Although initial colonization of the cecal tonsils on d 3 by the 3 experimental groups was not significantly different, less *Salmonella* was recovered from the livers and spleens of the SEΔfliC-CD154C group at this same time point (Table 4). By d 10 of the third experiment, *Salmonella* was recovered from the cecal tonsils in 6.7 and 60% from the SEΔfliC-CD154C and SEΔfliC groups, respectively, whereas 100% of the chickens in the ΔSEEV group were still colonized. Enumeration of cecal content at this time point also showed approximately 3 orders of magnitude less *Salmonella* recovered from ΔfliC-CD154C as compared with ΔSEEV. By 19 d postvaccination, 13.3% or less of the chickens in any group were positive for *Salmonella* and no significant differences in either percentage of recovery or enumeration of *Salmonella* were noted between any groups.

Serum Levels of Anti-fliC IgG

The fliC-specific IgG was detected in the serum of the immunized broilers as well as those to which wtSE was administered. The highest levels of fliC-specific antibody were detected from the group receiving wtSE in experiment 1, but no group in experiment 2 produced significant levels of antibody (data not shown).

Challenge by Heterologous Salmonella

In the final experiment, the broilers were challenged with a wild-type strain of *Salmonella* Typhimurium 3 d after immunization with vaccine candidate SEΔfliC-CD154C or SEΔfliC or with ΔSEEV strain. Colonization on d 3 of the ceca by the vaccine strains and ΔSE was greater than or equal to 90% (Table 5). Ten days after vaccination and 7 d post-*Salmonella* Typhimurium challenge, only SEΔfliC-CD154C exhibited a lower colonization rate than the positive control, ΔSEEV. At d 19, although there was not a significant reduction in terms of percentage of chickens colonized by *Salmonella*, there was a significant decrease in the colony-forming units of *Salmonella* per gram of cecal content recovered from SEΔfliC-CD154C and SEΔfliC when

Table 2. Percentage of *Salmonella* recovered[1] from the liver and spleen (L/S) and cecal tonsil (CT) of *Salmonella* Enteritidis (wtSE) or vaccine candidate strains of *Salmonella* Enteritidis (SEΔ)[2] from experiment 1

	24 h		7 d		21 d	
Group	L/S	CT	L/S	CT	L/S	CT
Negative control	0% (0/10)^{b}	0% (0/10)^{c}	0% (0/10)^{b}	0% (0/10)^{b}	0% (0/7)^{b}	0% (0/7)^{c}
wtSE	50% (5/10)^{a}	100% (10/10)^{a}	100% (10/10)^{a}	100% (10/10)^{a}	63% (5/8)^{a}	100% (8/8)^{a}
SEΔfliC	0% (0/10)^{b}	60% (6/10)^{b}	30% (3/10)^{b}	20% (2/10)^{b}	0% (0/9)^{b}	0% (0/9)^{c}
SEΔfliC-CD154H	50% (5/10)^{a}	70% (7/10)^{ab}	20% (2/10)^{b}	70% (7/10)^{a}	50% (4/8)^{a}	50% (4/8)^{b}

$^{a–c}$Different superscripts within a column indicate differences between treatments ($P < 0.05$).

[1]Day-of-hatch broiler chicks were administered sterile saline (negative control) or challenged with one of the above strains of *Salmonella*.

[2]Wild-type *Salmonella* Enteritidis or a modified variant expressing a flagellar filament protein (fliC) antigen alone (SEΔfliC) or with the putative immunopotentiating compound CD154 (SEΔfliC-CD154H).

compared with the unvaccinated control group (Table 5). Enumeration also showed a numerical reduction of *Salmonella* when comparing the experimentally vaccinated groups to the group receiving the naked vector strain before challenge. By d 30 postvaccination, there was a further reduction in the recovery of *Salmonella* from all groups, and no significant variation between any group either in terms of organ invasion or cecal colonization were noted.

Delayed-Type Hypersensitivity Reaction

Salmonella Typhimurium was injected subcutaneously into the toe web of chickens from experiment 4 thirty days postvaccination to elicit a delayed-type hypersensitivity reaction. The subsequent increase in toe web thickness was measured. No statistically significant differences were noted in terms of increase in toe web thickness, but all 3 groups that had been vaccinated with either one of the experimental vaccines or the empty vector strain had numerically greater swelling than the unvaccinated but challenged group at 24 h postinjection (Table 6). At 48 h postinjection, both groups vaccinated with the vaccine vector strains showed a numerical increase in toe web thickness, but the group vaccinated with the ΔSEEV strain no longer had numerically increased toe web thickness as compared with the negative control group. Both groups vaccinated with one of the experimental vaccine strains had a numerically greater response than the group receiving the empty vector strain.

DISCUSSION

The data from experiments 1 and 2 clearly demonstrate that the vaccine candidate SEΔfliC was cleared from both the internal organs as well as the gastrointestinal tract significantly more quickly than wtSE or the other vaccine candidates SEΔfliC-CD154C or SEΔfliC-CD154H (Tables 2 and 3). Because ΔSEEV has been attenuated by double gene deletion of the *aroA* and *htrA* genes, it may not be surprising that the SEΔfliC candidate cleared more quickly because these deletions were intended to decrease its viability within the host. What is surprising is that in comparison to the CD154-expressing vectors, which were constructed from the same empty vector strain, this modification cleared more slowly than the naked vector and similarly to the wtSE (Tables 2 and 3). These *Salmonella* recombinants were created for evaluation as candidates for vaccination of chickens, which ultimately would be used for human consumption. As such, clearance of the vaccine before processing is necessary for regulatory approval as well as consumer acceptance. Because these vaccines were constructed using wtSE as an antigen-expressing vehicle, the vaccine itself could potentially cause foodborne illness unless it has completely cleared from the animal before slaughter. Additionally, because such vaccines would be considered genetically modified organisms, they should ideally not be present when the poultry are marketed.

In the third experiment, the clearance of SEΔfliC and SEΔfliC-CD154C was compared with the clearance rate of the empty vector (Table 4). The empty

Table 3. Percentage of *Salmonella* recovered[1] from the liver and spleen (L/S) and cecal tonsil (CT) and enumeration of *Salmonella* Enteritidis (wtSE) or vaccine candidate strains of *Salmonella* Enteritidis (SEΔ)[2] from experiment 2

	Day 3		Day 21		
Group	L/S	CT	L/S	CT	Ceca ($\log_{10}$ cfu/g ± SEM)
wtSE	60% (6/10)	100% (10/10)^{a}	50% (5/10)^{a}	80% (8/10)^{a}	2.69 ± 0.34^{a}
SEΔfliC	20% (2/10)	70% (7/10)^{ab}	0% (0/10)^{b}	20% (2/10)^{b}	0.77 ± 0.55^{b}
SEΔfliC-CD154C	20% (2/10)	60% (6/10)^{b}	40% (4/10)^{a}	70% (7/10)^{a}	2.35 ± 0.44^{a}

^{a,b}Different superscripts within a column indicate differences between treatments ($P < 0.05$).

[1]Day-of-hatch broiler chicks were challenged with one of the above strains of *Salmonella*.

[2]Wild-type *Salmonella* Enteritidis or modified variants expressing a flagellar filament protein (fliC) antigen alone (SEΔfliC) or with the putative immunopotentiating compound CD154 (SEΔfliC-CD154C).

Table 4. Percentage *Salmonella* recovered[1] from the liver and spleen (L/S) and cecal tonsil (CT) and enumeration of *Salmonella* Enteritidis empty vector strain (ΔSEEV) or vaccine candidate strains of *Salmonella* Enteritidis (SEΔ)[2] from experiment 3

	Day 3		Day 10		Ceca (log_{10} cfu/ g ± SEM)	Day 19		Ceca (log_{10} cfu/ g ± SEM)	Day 30	
Group	L/S	CT	L/S	CT		L/S	CT		L/S	CT
Negative control	0% (0/10)^{b}	0% (0/10)^{b}	0% (0/15)^{b}	0% (0/15)^{c}	0.0 ± 0.0^{c}	0% (0/15)	0% (0/15)	0.0 ± 0.0	0% (0/15)	0% (0/15)
ΔSEEV	80% (8/10)^{a}	90% (9/10)^{a}	20% (3/15)^{ab}	100% (15/15)^{a}	4.16 ± 0.37^{a}	0% (0/15)	13.3% (2/15)	0.36 ± 0.24	0% (0/15)	6.7% (1/15)
SEΔfliC	100% (10/10)^{a}	100% (10/10)^{a}	33% (5/15)^{a}	60% (9/15)^{b}	2.33 ± 0.58^{b}	0% (0/15)	6.7% (1/15)	0.18 ± 0.18	0% (0/15)	0% (0/15)
SEΔfliC-CD154C	30% (3/10)^{b}	100% (10/10)^{a}	0% (0/15)^{b}	6.7% (1/15)^{c}	1.37 ± 0.55^{b}	0% (0/15)	13% (2/15)	0.50 ± 0.36	0% (0/15)	0% (0/15)

[a–c]Different superscripts within a column indicate differences between treatments ($P < 0.05$).

[1]Day-of-hatch broiler chicks were administered sterile saline (negative control) or challenged with one of the above strains of *Salmonella*.

[2]An attenuated empty vector strain of *Salmonella* Enteriditis (ΔSEEV) or modified variants expressing a flagellar filament protein (fliC) antigen alone (SEΔfliC) or with the putative immunopotentiating compound CD154 (SEΔfliC-CD154C).

Table 5. Percentage of *Salmonella* recovery[1] from the liver and spleen (L/S) and cecal tonsil (CT) and enumeration of *Salmonella* Enteritidis empty vector strain (ΔSEEV), vaccine candidate strains of *Salmonella* Enteritidis (SEΔ)[2] and *Salmonella* Typhimurium[3] from experiment 4

	Day 3		Day 10		Ceca (log_{10} cfu/ g ± SEM)	Day 19		Ceca (log_{10} cfu/ g ± SEM)	Day 30	
Group	L/S	CT	L/S	CT		L/S	CT		L/S	CT
Negative control	0% (0/10)^{b}	0% (0/10)^{b}	40% (6/15)^{ab}	73.3% (11/15)^{ab}	3.12 ± 0.53	13.3% (2/15)	66.7% (10/15)	2.61 ± 0.39^{a}	0% (0/15)	20% (3/15)
ΔSEEV	80% (8/10)^{a}	90% (9/10)^{a}	53.3% (8/15)^{a}	93.3% (14/15)^{a}	3.50 ± 0.42	20% (3/15)	53.3% (8/15)	1.91 ± 0.44^{ab}	0% (0/15)	13.3% (2/15)
SEΔfliC	100% (10/10)^{a}	100% (10/10)^{a}	53.3% (8/15)^{a}	66.7% (10/15)^{ab}	2.74 ± 0.57	13.3% (2/15)	40% (6/15)	1.21 ± 0.41^{b}	0% (0/15)	13.3% (2/15)
SEΔfliC-CD154C	30% (3/10)^{b}	100% (10/10)^{a}	13.3% (2/15)^{b}	60% (9/15)^{b}	2.18 ± 0.51	20% (3/15)	46.7% (7/15)	1.42 ± 0.41^{b}	0% (0/15)	30% (6/15)

[a,b]Different superscripts within a column indicate differences between treatments ($P < 0.05$).

[1]Day-of-hatch broiler chicks were administered sterile saline (negative control) or challenged with one of the above strains of *Salmonella*.

[2]An attenuated empty vector strain of *Salmonella* Enteriditis (ΔSEEV) or modified variants expressing a flagellar filament protein (fliC) antigen alone (SEΔFliC) or with a the putative immunopotentiating compound CD154 (SEΔfliC-CD154C).

[3]Day-of-hatch chicks were vaccinated with ΔSEEV or one of the SEΔ vaccine candidates. On d 3 postvaccination, all groups were challenged with wild-type *Salmonella* Typhimurium.

vector strain was used in these studies as the backbone for construction of the recombinant vaccine candidates, so like the vaccine candidates, it has been attenuated by the deletion of the *aroA* and *htrA* genes. Both vaccine candidates as well as the empty vector strain were initially well colonized within the gastrointestinal tract; however, by d 19, both vaccine candidates were recovered at a lower rate from the gastrointestinal tract both in terms of percentage of chickens colonized as well as number of colony-forming units of *Salmonella* per gram of cecal content (Table 4). This was in contrast to the previous experiments because the SEΔfliC-CD154C vaccine candidate was recovered at a significantly reduced rate as compared with SEΔfliC.

In the final experiment, chicks were vaccinated with SEΔfliC, SEΔfliC-CD154C, or the ΔSEEV and then challenged 3 d later with *Salmonella* Typhimurium (Table 5). Commercial poultry are often infected with *Salmonella* within the first week of life when the infectious dose required is several orders of magnitude less than that of an adult chicken (Cox et al., 1990; Byrd et al., 1998). Being able to reduce this primary infection with *Salmonella,* although important for all poultry, is especially important for broilers and commercial layers. Some broilers are slaughtered at approximately 4 wk of age and most broilers are slaughtered at or before 8 wk of age. This short time period often does not allow enough time for the clearance of *Salmonella* by a natural immune response and can lead to the contamination of poultry meat by these foodborne pathogens. Because some strains of *Salmonella*, particularly *Salmonella* Enteritidis, are able to go into a carrier state, recrudescent *Salmonella* infection often occurs at onset of lay due to immune suppression by hormonal and physiological changes (Chappell et al., 2009). These recrudescent infections may lead to colonization of the reproductive tract by *Salmonella*, which in turn can lead to contaminated eggs (Guard-Petter, 2001; Chappell et al., 2009). To try and prevent early infection by *Salmonella* and to best replicate likely commercial use of these recombinants as candidate vaccines, all groups were challenged with the heterologous serovar *Salmonella* Typhimurium. Because an adaptive immune response takes at least 10 d to develop, we did not anticipate protection to this early challenge to be mediated by this type of response. It has been noted that once colonized, *Salmonella* strains are able to competitively exclude heterologous strains, and it was through this mechanism that we hypothesized early protection could be achieved (Holt and Gast, 2004). Both vaccine candidates as well as the empty vector strain were well colonized within the gastrointestinal tract as indicated by 90 to 100% colonization within the cecal tonsils (Table 5). By d 19, both vaccinated groups had a greater than 10-fold reduction of *Salmonella* as compared with the nonvaccinated control. Although both vaccinated groups were numerically less infected than the group vaccinated with ΔSEEV, the reduction was not statistically significant. By 30 d postvaccination, all groups remained weakly positive for *Salmonella* and no significant differences were noted between groups. These results indicate that neither vaccine candidate is more protective against a heterologous *Salmonella* challenge than ΔSEEV

In conclusion, the groups inoculated with the experimental recombinants evaluated in these experiments tended to clear infections at a faster rate than either wtSE or ΔSEEV. It has been shown that FliC interacts with toll-like receptor 5 (**TLR5**) to stimulate a proinflammatory response (Tallant et al., 2004). Both poultry-specific serovars of *Salmonella*, Pullorum, and Gallinarum fail to illicit a substantial innate immune response largely because these unflagellated strains do not trigger an innate immune response via TLR5 (Iqbal et al., 2005). It is thought that this is one of the main reasons for the increased virulence of the host-specific strains as compared with other known invasive strains like *Salmonella* Enteritidis and *Salmonella* Typhimurium (Iqbal et al., 2005). Because the fliC-expressing vaccine strains present more fliC antigen than either the wtSE or the empty vector strain, it is likely that these strains are more easily recognized by TLR5 expressing cells such as heterophils and are destroyed at a faster rate than strains expressing less fliC (Genovese et al., 2007). In the present experiments, these experimental vaccine candidates did not protect against heterologous challenge or enhance clearance after *Salmonella* Typhimurium challenge. As such, their potential value as vaccines is extremely limited, although carriage of increased expression of the fliC epitope did increase clearance of the vaccine vector in the present studies (Tables 2 to 5). This lack of protection and clearance after challenge may be due to the lack of adaptive immune response. In these experiments, there was no evidence that an adaptive immune response was induced by administration of recombinants with increased expression of fliC, with or without the immunostimulatory molecule CD154. Several strains of *Salmonella* have been used to express various heterologous epitopes to elicit a protective immune response against viral, bacterial, and protozoal

Table 6. Evaluation of delayed-type hypersensitivity reaction to *Salmonella* Typhimurium injected subcutaneously in the toe web 30 d postvaccination with *Salmonella* Enteritidis empty vector strain (ΔSEEV) or vaccine candidate strains of *Salmonella* Enteritidis[1] (SEΔ) from experiment 4

	Increase in toe web thicknesss (mm)	
Group	24 h	48 h
Negative control	1.6 ± 0.7	4.5 ± 0.9
ΔSEEV	2.2 ± 0.7	4.0 ± 0.6
SEΔfliC	3.6 ± 0.5	4.9 ± 0.4
SEΔfliC-CD154C	3.1 ± 0.5	5.8 ± 0.7

[1]An attenuated empty vector strain of *Salmonella* Enteriditis (ΔSEEV) or modified variants expressing a flagellar filament protein (fliC) antigen alone (SEΔfliC) or with the putative immunopotentiating compound CD154 (SEΔfliC-CD154C).

pathogens as well as toxins. A problem with using these vaccines is that recombinant *Salmonella* are then shed for a period of time into the environment and could potentially contaminate food or water and lead to human or animal salmonellosis. Addition of the fliC epitope to these vaccines may decrease both the duration of shedding as well as the number of organisms shed into the environment. This would decrease the probability of humans or animals acquiring an infection by these vaccines, making their use in human and veterinary medicine perhaps more feasible. Nevertheless, the authors believe that the observed persistence of infection of even these highly attenuated and modified recombinants, along with general lack of efficacy for prevention of heterologous challenge, essentially disqualifies these as commercial vaccine candidates.

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