Salmonella DNA Adenine Methylase Mutants Elicit Protective Immune Responses to Homologous and Heterologous Serovars in Chickens

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Salmonella DNA adenine methylase (Dam) mutants that lack or overproduce Dam are highly attenuated for virulence in mice and confer protection against murine typhoid fever. To determine whether vaccines based on Dam are efficacious in poultry, a Salmonella Dam− vaccine was evaluated in the protection of chicken broilers against oral challenge with homologous and heterologous Salmonella serovars. A Salmonella enterica serovar Typhimurium Dam− vaccine strain was attenuated for virulence in day-of-hatch chicks more than 100,000-fold. Vaccination of chicks elicited cross-protective immune responses, as evidenced by reduced colonization (10- to 10,000-fold) of the gastrointestinal tract (ileum, cecum, and feces) and visceral organs (bursa and spleen) after challenge with homologous (Typhimurium F98) and heterologous (Enteritidis 4973 and S. enterica O6,14,24:e,h-monophasic) Salmonella serovars that are implicated in Salmonella infection of poultry. The protection conferred was observed for the organ or the maximum CFU/tissue/bird as a unit of analysis, suggesting that Dam mutant strains may serve as the basis for the development of efficacious poultry vaccines for the containment of Salmonella.

Salmonellosis resulting from the consumption of contaminated eggs and poultry meat poses a significant public health risk to consumers worldwide. The Centers for Disease Control and Prevention has estimated that there are approximately 2 million cases of human nontyphoid salmonellosis per year in the United States, resulting in up to 2,000 deaths (1). Most cases of salmonellosis in developed countries are zoonotic in origin and not due to person-to-person contamination. This disease is caused by exposure to products contaminated with Salmonella, e.g., animal products (such as eggs, milk, poultry), or the ingestion of food products that have been exposed to animal feces. Economic constraints associated with improved management of production and slaughter facilities suggest that on-farm control of Salmonella via a combination of antibiotics, competitive exclusion products, and/or vaccination may be the most practical and economically feasible methods toward reducing contamination of poultry products (34). Such a reduction in preharvest pathogen load may provide a means for decreasing the potential for transmission to humans.

Dam− Salmonella is attenuated for virulence in day-of-hatch chicks. Salmonella DNA adenine methylase (Dam) mutants are attenuated for virulence in mice and elicit protective (10, 16) and cross-protective (15) immune responses against murine typhoid fever. To examine whether Dam− Salmonella cells were attenuated for virulence, we challenged day-of-hatch chicks with either Dam− or Dam+ Salmonella enterica serovar Typhimurium UK-1 (Table 1). All chicks (15 out of 15) survived that were infected on the day of hatch with 1010 Dam− UK-1 (MT2313) cells (Table 2). In contrast, 8 of 15 chicks survived after challenge with 107 Dam+ UK-1 (MT2315) cells. These data indicate that a mutation in dam attenuated the virulence of serovar Typhimurium UK-1 in day-of-hatch chickens by ≥100,000-fold.

Immunization with Dam− Salmonella elicits protective immunity. To determine whether Dam− serovar Typhimurium elicits protective immune responses, chicks were orally vaccinated with 107 CFU of Dam− Typhimurium UK-1 (MT2313) within 10 h of hatching and again at 1 week of age. Chicks were challenged at 5 weeks of age with 109 CFU of serovar Typhimurium F98 (MT2318). Vaccine efficacy was determined by comparison of vaccinates (n = 62) and control (n = 62) quantitative cultures of the spleen, bursa of Fabricius, ileum, cecum, and feces; cultures were performed at 2, 3, 5, 7, 9, 12, and 14 days postchallenge. The mean log10 CFU of homologous challenge with Typhimurium F98 by organ and day of termination for vaccinated birds relative to controls are presented in Fig. 1A. Vaccination with Dam− UK-1 (MT2313) resulted in significantly lower CFU (P < 0.05) in the spleen and feces of vaccinates on all 7 postchallenge days examined. Significantly lower CFU in vaccinates relative to controls were observed in the bursa on 4 out of 7 termination days, in the ileum on 5 of 7 days, and in the cecum on 3 of 7 days. Vaccinated birds also had significantly lower CFU than controls following homologous challenge with serovar Typhimurium F98 (MT2318) with maximum CFU/tissue/bird as the unit of analysis (Fig. 2). All control birds had at least 40 CFU of challenge organism in at least one organ following challenge. However, no salmonellae were isolated in any organ from 7 out of 62 (11%) vaccinates; an additional 7 out of 62 (11%) vaccinates had only 10 CFU isolated from at least one organ. In vaccinates, 32 out of 62 (52%) birds had ≥105 CFU
in at least one organ, compared to 2 (3%) out of 62 controls in this
category. Birds with \( \leq 10^8 \) CFU in at least one organ
included 46 (74%) out of 62 vaccines compared to only 7
(11%) out of 62 controls. Taken together, these data indicate
that immunization of chicks with Dam\(^{-}\) serovar Typhimurium
confers protection against homologous challenge with the
organ or maximum CFU/tissue/bird as the unit of analysis.

**Immunization of chicks with Dam\(^{-}\) Salmonella elicits cross-
protective immunity.** Next, we examined the cross-protective
capacity of chicks immunized with Dam\(^{-}\) Salmonella. Sixty
chicks were orally vaccinated with \( 10^7 \) Dam\(^{-}\) UK-1 (MT2313)
cells within 10 h of hatching and again at 1 week of age; 60
additional chicks remained as nonvaccinated controls. All
chicks were challenged at 5 weeks of age with \( 10^8 \) CFU of
serovar Enteritidis 4973 (serogroup D) or \( 10^9 \) CFU of
serovar S. enterica O6,14,24:e,h-monophasic (sero-
group H; MT2339). Figure 1B and C show the mean \( \log_{10} \) CFU
of serovars Enteritidis 4973 and S. enterica O6,14,24:e,h-mono-
phasic challenge organisms by organ and day of termination
for vaccinated birds relative to nonvaccinated controls. Six days
postchallenge with serovar Enteritidis 4973 (MT2314), vacci-
nates had significantly lower CFU in the spleen, bursa, cecum,
and feces: no challenge organisms were recovered from any
vaccinated bird organs, whereas 50 to 60% of control organs
were positive for Salmonella. No challenge organisms were
recovered from the spleens of vaccines on day 6 or 7 post-
challenge with S. enterica serovar O6,14,24:e,h-monophasic
(MT2339); in contrast, salmonella were recovered from 19
out of 20 control spleens on these 2 days.

Vaccinated birds had significantly lower CFU than controls
following heterologous challenge when the maximum CFU/
tissue/bird was used as the unit of analysis (Fig. 2). No serovar
Enteritidis 4973 was recovered from 18 out of 30 (60%) chal-
nenged vaccines compared to only 7 out of 30 (23%) nonin-
fected control birds. Vaccinated birds also showed protection
against challenge with serovar S. enterica O6,14,24:e,h-monophas-
ic, as 7 out of 30 (23%) vaccines had \( \leq 100 \) CFU in at
least one organ; no control birds were in this category. Taken
together, these data indicate that chicks vaccinated with Dam\(^{-}\)
serovar Typhimurium UK-1 elicited cross-protective immune
responses to challenge with serovars Enteritidis 4973 and S.
enterica O6,14,24:e,h-monophasic for the organ or maximum
CFU/tissue/bird as the unit of analysis.

The safety of the food supply can be compromised by large-
scale animal husbandry, agricultural methods, and distribution
practices that are prone to microbial contamination. This pub-
lie health problem has been recently exacerbated by the emer-
gence of pathogens that are resistant to multiple antibiotics
and/or cause more debilitating forms of disease (e.g., Esche-

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**TABLE 1. Bacterial strains and phage used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source and/or reference(s)</th>
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</thead>
<tbody>
<tr>
<td>S. enterica serovar Typhimurium</td>
<td>Wild-type x(3761)</td>
<td>Curtiss (12)</td>
</tr>
<tr>
<td>UK-1</td>
<td>Wild-type y(4433)</td>
<td>Curtiss (2, 12)</td>
</tr>
<tr>
<td>MT2057</td>
<td>ATCC 14028 z(f)-(7506);Mu(d)</td>
<td>5</td>
</tr>
<tr>
<td>MT2116</td>
<td>ATCC 14028 dam(102);Mu(d)-(Cm)</td>
<td>John Roth; 16</td>
</tr>
<tr>
<td>MT2313</td>
<td>UK-1 dam(102);Mu(d)-(Cm)</td>
<td>This work</td>
</tr>
<tr>
<td>MT2315</td>
<td>UK-1 z(f)-(7504);Mu(d)</td>
<td>This work</td>
</tr>
<tr>
<td>MT2318</td>
<td>F98 z(f)-(7504);Mu(d)</td>
<td>This work</td>
</tr>
<tr>
<td>S. enterica serovar Enteritidis</td>
<td>Wild-type x(3700)</td>
<td>Curtiss (12, 19)</td>
</tr>
<tr>
<td>4973</td>
<td>4973 z(f)-(7504);Mu(d)</td>
<td>This work</td>
</tr>
<tr>
<td>S. enterica O6,14,24</td>
<td>Serogroup H, O6,14,24:e,h-monophasic</td>
<td>California Animal Health and Food Safety Laboratory</td>
</tr>
<tr>
<td>3670</td>
<td>K(0)-(670)</td>
<td>20; this work</td>
</tr>
<tr>
<td>MT2339</td>
<td>K(0)-(670) (\Delta)z(f)-(7506);Kn</td>
<td>31</td>
</tr>
<tr>
<td>Bacteriophage P22</td>
<td>HT105/1(\text{int-}201)</td>
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</tr>
</tbody>
</table>

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**TABLE 2. Dam\(^{-}\) Salmonella is attenuated for virulence in chickens**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Challenge dose (CFU)</th>
<th>No. of survivors</th>
</tr>
</thead>
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<tr>
<td>MT2313</td>
<td>Dam(^{-}) UK-1</td>
<td>(10^8)</td>
<td>15/15</td>
</tr>
<tr>
<td>MT2315</td>
<td>Dam(^{-}) UK-1</td>
<td>(10^5)</td>
<td>8/15</td>
</tr>
</tbody>
</table>

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\(a\) Chicks within 10 h of hatching were infected with the S. enterica serovar Typhimurium strain indicated. Survival was assessed until 3 weeks of age. Strains used in infection studies were grown overnight in Luria broth at 37°C, and serial dilutions were performed in 0.2 M Na\(2\)HPO\(_4\) buffered to pH 8.0 to the desired cell density for infection. Fertile eggs obtained from White Leghorn chickens (Specific Pathogen Free Avian Services, Charles River) were incubated and hatched in several Marsch Automatic incubators (Lyon Electric Co., Inc., Chula Vista, Calif.).
FIG. 1. Dam− Salmonella elicits protective responses in chicken tissue sites. The protective capacity of Dam− serovar Typhimurium was assessed by orally immunizing 62 chicks with 10⁷ CFU of Dam− UK-1 (MT2313) within 10 h of hatch and boosted with the same dose at 1 week of age (hatched bars); 62 additional chicks remained as nonvaccinated controls (filled bars). All chicks were challenged at 5 weeks of age with 10⁸ CFU of serovar Typhimurium F98 (MT2318). Data are depicted as mean log₁₀ CFU by organ. Nine control and nine vaccinated chickens were terminated at 2, 3, 5, 7, 9, and 12 days postchallenge; 8 birds per group were terminated 14 days postchallenge. Cross-protective immunity was
Salmonella serovars Enteritidis and Typhimurium DT104. Vaccination of chickens offers a practical and economically feasible approach to reducing contamination of poultry products. Here, we show that an S. enterica serovar ‘Typhimurium Dam’ mutant was severely attenuated for virulence in day-of-hatch chicks. Additionally, chicks immunized with this Salmonella Dam- vaccine strain exhibit protective immune responses against homologous and heterologous Salmonella serotypes that are implicated in Salmonella infection of poultry. Vaccines based on altered levels of Dam activity may prove effective in controlling Salmonella contamination of poultry, meat, and dairy products derived from animals susceptible to Salmonella infection and colonization.

Enumeration of salmonellae from the visceral organs and intestinal tract of vaccinated and nonvaccinated chickens challenged with virulent serovars Typhimurium or Enteritidis or S. enterica O6,14,24:e,h-monophasic was used to determine the degree of protection associated with vaccination. Significantly lower mean log_{10} CFU were observed in visceral organs and the gastrointestinal tract of vaccines versus nonvaccinates. Comparison of these results with challenge studies for other vaccines is problematic as the outcome of infection varies greatly with challenge strain, inoculation and immunization dose, use of multiple (booster) immunizations, the age of the birds at vaccination and challenge, statistical analysis of the data, etc. Previous studies using live attenuated Salmonella arsA (11, 17, 18) and Δcya Δcrp mutants (12) showed reduced visceral invasion and colonization of the gastrointestinal tract in chickens by homologous and, to a lesser extent, heterologous challenge strains. Oral vaccination with attenuated ΔarsA mutants of serovar Typhimurium (3) or Enteritidis (6, 7) reduced fecal shedding following homologous challenge, but not heterologous challenge (8). Vaccination with serovar Typhimurium Δcya Δcrp conferred protection against intestinal and visceral invasion by both homologous and heterologous challenge serotypes (12). Moreover, this vaccine also provided protection against intestinal, visceral, reproductive tract and egg colonization by Salmonella for at least 11 months postvaccination, with no effect on egg production (13).

Results of this study are promising in that significant protection was observed following homologous and heterologous challenge at high challenge doses. It should be noted that a single challenge may not reflect the field situation wherein animals can be exposed to various doses of several virulent serovars alone and in combination. That said, multiple and/or continuous exposures to several serovars in the field situation do not necessarily result in susceptibility of immunized animals to disease: repeated exposures may contribute to the maintenance of heightened levels of protection in vaccinated hosts.

The data presented here suggest that vaccines based on altered DNA methylation may reduce preharvest Salmonella contamination in poultry, ultimately decreasing the potential for food-borne transmission of this pathogen to humans. DNA

assessed as follows. Sixty chicks were orally vaccinated with 10^{7} Dam- UK-1 (MT2313) cells within 10 h of hatching and again at 1 week of age; 60 additional chicks remained as nonvaccinated controls. All chicks were challenged at 5 weeks of age with either 10^{9} CFU of serovar Enteritidis 4973 (MT2314) or O6,14,24:e,h-monophasic (MT2339). Ten control and 10 vaccinated chickens were terminated at 4, 5, and 6 days postchallenge for the serovar Enteritidis challenge or 5, 6, or 7 days postchallenge for the S. enterica serovar O6,14,24:e,h-monophasic challenge. For each experiment, a separate cohort of 8 to 14 nonvaccinated, nonchallenged negative control birds were maintained and necropsied as described below. Approximately 1 g of each organ was obtained in the following order: spleen, bursa of Fabricius, ileum and ileal contents, cecum and cecal contents, and rectum and feces. Organs were weighed, homogenized, and serially diluted, and 100 μl of each dilution was plated on 1% lactose MacConkey agar plates containing kanamycin. For detection of salmonellae at concentrations below 40 CFU/g, the 1:4 dilution homogenate sample was incubated for 24 h in 9 ml of tetrahydroxionate solution, streaked for single colonies on lactose MacConkey agar, and incubated for 24 h at 37°C. Samples positive by selective enrichment in tetrahydroxionate broth were recorded as 10 CFU, and negative samples were recorded as 0 CFU. For tissue experiments, bacterial titers were confirmed via serial dilutions plated on 1% lactose MacConkey agar; colony serotype was confirmed via standard biochemical tests (urea, triple sugar iron, and ONPG [O-nitrophenyl-β-D-galactopyranoside]) and agglutination with appropriate antisera on two randomly selected colonies from each organ of each bird. *, significant difference (P < 0.05) between vaccines and controls as assessed by the Mann-Whitney test.
methylation plays a role in the virulence of a wide variety of pathogens of the gamma subdivision of proteobacteria, including Salmonella (murine typhoid; 10, 15), Yersinia pseudotuberculosis (murine bacteremia; 21), and Vibrio cholerae (cholera; 21). Additionally, DNA methylation is required for the virulence of Brucella abortus (fetal calf abortion; 30) via CcrM, a cell-cycle regulated DNA adenine methyltransferase present in members of the alpha group of proteobacteria (28, 33). Since Dam and CcrM affect the virulence of such distantly related pathogens, the function of DNA methylation in virulence may emerge as a general theme in bacterial pathogenesis.

The role of DNA methylation in virulence and the elicitation of protective immune responses may rely on its capacity as a global regulator of gene expression (16, 22, 25, 26, 28, 29). Dam regulates the production of a number of adhesins in E. coli (23, 32) and Salmonella (27), as well as several genes required for Salmonella infection (14, 16). Such ectopic gene expression may result in the production of an expanded repertoire of antigens that contribute to the heightened immunity seen in vaccinated animals (15, 16, 21). Thus, dysregulation of Dam activity may be a means to elicit protective immune responses directed against diverse pathogens that infect a wide variety of animal hosts (24, 25).

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REFERENCES