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Development of a *Salmonella* cross-protective vaccine for food animal production systems

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ABSTRACT

Intensive livestock production is associated with increased *Salmonella* exposure, transmission, animal disease, and contamination of food and water supplies. Modified live *Salmonella enterica* vaccines that lack a functional DNA adenine methylase (Dam) confer cross-protection to a diversity of salmonellae in experimental models of murine, avian, ovine, and bovine models of salmonellosis. However, the commercial success of any vaccine is dependent upon the therapeutic index, the ratio of safety/efficacy. Herein, secondary virulence-attenuating mutations targeted to genes involved in intracellular and/or systemic survival were introduced into *Salmonella dam* vaccines to screen for vaccine candidates that were safe in the animal and the environment, while maintaining the capacity to confer cross-protective immunity to pathogenic salmonellae serotypes. *Salmonella dam mgtC*, *dam sifA*, and *dam spvB* vaccine strains exhibited significantly improved vaccine safety as evidenced by the failure to give rise to virulent revertants during the infective process, contrary to the parental *Salmonella dam* vaccine. Further, these vaccines exhibited a low grade persistence in host tissues that was associated with reduced vaccine shedding, reduced environmental persistence, and induction of cross-protective immunity to pathogenic serotypes derived from infected livestock. These data indicate that *Salmonella dam* double mutant vaccines are suitable for commercial applications against salmonellosis in livestock production systems. Reducing pre-harvest salmonellae load through vaccination will promote the health and productivity of livestock and reduce contamination of livestock-derived food products, while enhancing overall food safety.

1. Introduction

Salmonellosis is a common cause of morbidity and mortality in intensive livestock production systems. Many *Salmonella* infections are subclinical with disease more commonly observed in young, debilitated and parturient animals which are most susceptible to infection [1]. Disease outbreaks compromise animal welfare, promote antimicrobial use and subsequently lead to selection for antimicrobial resistance in zoonotic pathogens, compromising productivity and at times resulting in substantial mortality (20–60% of the susceptible population [2]). There are over 2500 *Salmonella* serotypes, all of which are potentially pathogenic, however, 80% of clinical disease in a given region is frequently attributed to less than 2% of serotypes with those of serogroups B, C, D and E most frequently incriminated [3]. Sources of infection for livestock include feed, water, wildlife, insects, people and contaminated equipment [4]. Infected animals amplify environmental contamination and represent a source of infection for other stock. Effluent from intensive animal production systems is often utilized as a fertilizer to grow crops, and *Salmonella*-contaminated effluent provides a reservoir of infection and a vehicle for contamination of waterways, fruits and vegetables. Thus, *Salmonella* contamination of animal and plant food products by a variety of mechanisms provides an ongoing source of infection for human populations.

Salmonella control efforts continue to be problematic for the following reasons: (1) most livestock infections are subclinical [2,5]; (2) disease outbreaks are sporadic and frequently caused by specific serotypes although many serotypes are endemic to livestock production systems [5–7]; (3) environmental persistence provides an ongoing reservoir for livestock infection [8–11]; (4) the recent emergence of strain variants that are more virulent and can kill vaccinated animals [12]; (5) some strains derived from human salmonellosis patients are distinct from those of animal origin [13]; (6) management and environmental events can increase pathogen exposure and/or compromise host immunity [1,2,8,9,14,15].

Vaccination represents a sustainable approach for promoting animal health, welfare, and food safety through reducing pathogen exposure at the outset of the food production chain [16].

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However, the immunity conferred by conventional vaccines is restricted to a narrow range of closely-related strains, and on-farm control requires the development of vaccines that elicit protection against many pathogenic serotypes [16]. Recent advancements have resulted in the development of modified live *Salmonella* cross-protective vaccines, many of which contain mutations in global regulatory networks that favor antigen production, and that are also suitable for the expression of heterologous antigens [17–21]. The molecular basis of cross-protective vaccine efficacy may be attributed to several parameters including: the expression of multiple antigens shared among pathogenic serotypes; diminished vaccine-induced immunosuppression; targeted removal of immunodominant antigens to expose cross-protective epitopes; type III secretion of recombinant antigens; and/or delayed vaccine attenuation for enhanced stimulation of immune responses (reviewed in [16,18,22,23]). Modified live attenuated *S. enterica* serovar Typhimurium that harbor loss of function mutations in the gene encoding the DNA adenine methylase (*dam*) are capable of eliciting protection against a diversity of salmonellae and are well-tolerated when applied as modified live vaccines in mice [17,24], poultry [25,26], sheep [27] and calves [28–30]. Induction of immunity is rapid and the vaccine can be administered via drinking water for low-cost and low-stress immunization of livestock populations [27,31]. The commercial success of any vaccine is dependent on the therapeutic index, the ratio of safety/efficacy, and safety is of particular concern for modified live vaccines that have the potential to revert to heightened virulence. Herein, we evaluated whether the introduction of attenuating mutations into a *Salmonella dam* mutant vaccine strain significantly improves vaccine safety without compromising efficacy in immunized mice. The vaccine candidates that offer the best safety and protection characteristics are the most suitable for a commercially viable product against livestock salmonellosis.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Salmonella animal isolates were derived from different outbreaks, individual cases, or surveillance submissions to diagnostic laboratories [13]. Virulent *Salmonella Typhimurium* UK-1 was used in all studies for comparison [32]. Unless otherwise specified, bacteria were derived from stationary phase cultures aerated at 37 °C containing Luria-Bertani (LB) medium [33]. Antibiotics were used at the following concentrations: kanamycin (Kn), 50 μg/ml; ampicillin (Ap), 50 μg/ml.

2.2. Construction of S. Typhimurium dam vaccine candidates comprising an additional attenuating mutation

S. Typhimurium UK-1 *Δdam* was constructed by introducing an in-frame 300 bp deletion of defined *dam* sequence, termed *damΔ232* [34], using standard genetic protocols [35]. Secondary virulence-attenuating deletion mutations were introduced into the parental *S. Typhimurium* UK-1 *damΔ232* strain utilizing ampicillin-resistant suicide vector pCVD442 as described [35], resulting in the construction of in-frame deletions of defined coding sequence in the following targeted genes: *aroA* (MT3138; 1056 bp); *htrA* (MT3142; 1341 bp); *mgtC* (MT3146; 606 bp); *sifA* (MT3150; 807 bp); *spiC* (MT3154; 306 bp); *spvB* (MT3158; 1563 bp); and *ssaV* (MT3162; 1959 bp). The resultant genetic constructs were confirmed by PCR using primers that flank the deleted sequences.

2.3. Statistical analysis

Continuous repeated measures data were analyzed using residual (or restricted) maximum likelihood (REML) analysis (GenStat, 15th Edition, VSN International, UK). A single variate, repeated measures model was used to analyze CFU on a log base 10 scale. The fixed effects of the model were the factors time, treatment and their interaction, and repeated observations on the same mouse were allowed for in the residual term specification. The Wald chi-square test was used to determine significant main effects and/or significant interactions between factors. Any non-significant terms were dropped from the model and analysis repeated. Following analysis, data are presented as predicted model-based means, i.e. predicted means are those obtained from the fitted model rather than the raw sample means. *P*-values less than 0.05 were considered to be statistically significant. The number of CFU present in tissues at necropsy was analyzed using analysis of variance (ANOVA, GenStat, 15th Edition, VSN International, UK). Differences between the individual means calculated using REML and ANOVA were determined by calculating an approximate least significant difference (LSD). A difference of means that exceeded the calculated LSD was considered significant.

Binomial data (shedding [yes/no] and outcome [live/dead]) were analyzed using a logistic regression model (GenStat, 15th Edition, VSN International, UK). Vaccine status was specified as a predictor in the model. Overall significance was assessed using the Wald statistic ($P < 0.05$). Significance of fixed effects (vaccine) was assessed according to the *t* parameter estimates relative to the reference group. *P*-values less than 0.05 were considered statistically significant.

2.4. Ethics statement

All animal experimentation was conducted following the National Institutes of Health guidelines for housing and care of laboratory animals and performed in accordance with Institutional regulations after pertinent review and approval by the Institutional Animal Care and Use Committee at the University of California, Santa Barbara.

3. Results

3.1. Evaluation of Salmonella dam double mutant vaccine candidates for colonization and persistence in mucosal and systemic tissues

Colonization/persistence of the *Salmonella dam* double mutant vaccine candidates (*dam aroA*, *dam htrA*, *dam mgtC*, *dam sifA*, *dam spiC*, *dam spvB*, *dam ssaV*) in BALB/c mice was assessed in mucosal (Peyer's patches; mesenteric lymph nodes) and systemic tissues (liver and spleen) at 2 and 4 weeks post oral immunization (Fig. 1a and b). The *Salmonella dam* double mutant candidates were classified into two groups: Class (I) those that showed similar colonization/persistence relative to that of the parental *Salmonella dam* vaccine strain (*dam mgtC*, *dam sifA*, *dam spvB*); and Class (II) those that exhibited reduced colonization/persistence relative to that exhibited by the parental *dam* vaccine (*dam aroA*, *dam htrA*, *dam spiC*, *dam ssaV*). These data indicate that Class I vaccines sustained a low-grade persistence in host tissues that may be necessary for elicitation of robust protection, whereas Class II vaccines showed rapid clearance in vaccinated animals.