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# Immunogenicity of a *Staphylococcus aureus*-cholera toxin $A_2$/B vaccine for bovine mastitis

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ABSTRACT

*Staphylococcus aureus* causes a chronic, contagious disease of the udder, or mastitis, in dairy cows. This infection is often refractory to antibiotic treatment, and has a significant economic impact on milk production worldwide. An effective vaccine to prevent *S. aureus* mastitis would improve animal health, reduce antibiotic dependence and inform human vaccine approaches. The iron-regulated surface determinant A (IsdA) and clumping factor A (ClfA) are conserved *S. aureus* extracellular-matrix adhesins and target vaccine antigens. Here we report the results of two bovine immunogenicity trials using purified IsdA and ClfA-cholera toxin $A_2$/B chimeras (IsdA-$CTA_2$/B and ClfA-$CTA_2$/B). Cows were intranasally inoculated with IsdA-$CTA_2$/B + ClfA-$CTA_2$/B at dry off and followed for 70 days. Trial 1 utilized three groups with one or two booster doses at a total concentration of 600 or 900 μg. Trial 2 utilized two groups with one booster at a total concentration of 1200 μg. Humoral immune responses in serum and milk were examined by ELISA. Responses in serum were significant between groups and provide evidence of antigen-specific IgG induction after vaccination in both trials. Cellular proliferation was detected by flow cytometry using antigen-stimulated PBMCs from day 60 of Trial 2 and revealed an increase in CD4+ T cells from vaccinated cows. IsdA and ClfA stimulation induced IL-4 expression, but not IFN-γ or IL-17, in PBMCs from day 60 as determined by cytokine expression analysis. Opsonophagocytosis of *S. aureus* confirmed the functional *in vitro* activity of anti-IsdA antibodies from Trial 2 serum and milk. The vaccine was well tolerated and safe, and results support the potential of mucosally-delivered $CTA_2$/B chimeras to protect cows from mastitis caused by *S. aureus*.



## 1. Introduction

The bacterium *Staphylococcus aureus* is a major cause of persistent inflammation of the mammary gland, or mastitis, in bovines. Mastitis is one of the most important diseases affecting dairy cattle worldwide, with an estimated economic impact of $1.7–2 billion annually in the U.S [1]. This disease may be caused by a number of microorganisms, however *S. aureus* is significant for its ability to establish contagious, chronic disease that is difficult or impossible to treat. Improved control practices have reduced incidence, but *S. aureus* remains a leading cause of mastitis in dairy herds worldwide. U.S. studies have estimated that 10% of all dairy cows are infected, and 84% of herds are positive for *S. aureus* [2,3]. While reports indicate that bovine and human isolates are host-adapted, some evidence exists for transmission of antibiotic resistant *S. aureus* between humans and cattle, increasing the urgency to find methods of prevention [4–6]. *S. aureus* can survive on the skin and mucous membranes and is transmitted between cows, most commonly, during the milking process [7]. Importantly, survival of *S. aureus* within bovine mammary cells, including alveolar cells and macrophages, is believed to contribute to chronic disease [8].

Vaccines targeting *S. aureus* have been in development for many years and approaches have included attenuated or inactivated bacteria, toxoids, purified capsule polysaccharide and purified protein subunit. Current vaccines, that include a lysed whole cell vaccine of three prevalent capsular *S. aureus* serotypes (Lysigin®, Boehringer Ingelheim Vetmedica, Inc.) and a polyvalent inactivated vaccine (STARTVAC®, Hippa, Spain) have shown reduction in clinical

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severity, but limited ability to prevent colonization [9–12]. Reduced efficacy may be due to inadequate humoral responses against heterologous isolates [13]. These vaccines also require multiple intramuscular doses and subsequent follow up. It is recognized that future strategies against *S. aureus* must focus on multiple conserved virulence factors to promote strain cross-protection. Inducing mucosal responses to block bacterial attachment, and cellular responses to reduce intracellular infection, are also key for preventing colonization and chronic disease. In this report, we present the immunogenicity and initial safety assessment of a mucosal vaccine to prevent *S. aureus* mastitis as determined by two field trials in dairy cows. The vaccine is based upon the *S. aureus* iron-regulated surface protein A (IsdA) and clumping factor A (ClfA) antigens fused into a *Vibrio cholerae* cholera toxin (CT) $A_2$/B chimera (IsdA-$CTA_2$/B + ClfA-$CTA_2$/B). $CTA_2$/B chimeras are purified holotoxin-like molecules that have the toxic domain of CT genetically removed and replaced with an antigen of interest [14,15]. These molecules have been reported to induce bactericidal mucosal and systemic responses after intranasal delivery [16]. Intranasal vaccine delivery has also recently been shown to induce antigen-specific responses in bovine milk [17]. We hypothesized that a $CTA_2$/B-based *S. aureus* mucosal vaccine would be effective to reduce or eliminate *S. aureus* colonization of the udder. Our results indicate that intranasal delivery of IsdA-$CTA_2$/B + ClfA-$CTA_2$/B during the dry period is well tolerated in dairy cows and induces antigen-specific humoral responses in blood and milk that are active *in vitro* to trigger uptake and killing of *S. aureus*.

## 2. Materials and methods

### 2.1. Bacterial strains, growth conditions and plasmids

*S. aureus* Newbould 305 was used for cloning of *isd*A and *clf*A and opsonophagocytosis assay (OPA) [18,19]. *S. aureus* was grown in Luria Broth (LB) at 37 °C for DNA isolation and in low iron media (LIM) without shaking at 37 °C for OPA [20]. *E. coli* TE1 was used for cloning and protein expression of HIS-tagged antigens from pCK001 and pMaH001. These strains were grown in LB for cloning, or terrific broth (TB) for protein expression, + 100 μg/mL ampicillin at 37 °C. *E. coli* Clear Coli BL21(DE3) (Lucigen, Madison, WI) was used for expression of $CTA_2$/B chimeras from pLR001 and pLR003 and grown in TB + 35 ug/mL chloramphenicol at 37 °C. pLR001 was constructed by amplification of Newbould 305 DNA using *isd*A primers for cloning into the vector pARLDR19 (SphI-ClaI) [21]. pLR003 was similarly constructed into pARLDR19 (SphI-XhoI) using *clf*A primers. pCK001 was constructed by insertion of *isd*A (Newbould 305) into pTRCHIS (BamHI-HindIII) to express HIS-IsdA. pMaH001 was constructed by insertion of *clf*A (Newbould 305) into pBAD18 (NheI-HindIII) to express ClfA-HIS. Strains, plasmid details and primer sequences (with restriction sites underlined) are shown in Supplementary Table 1. All plasmids were sequenced through cloning junctions.

### 2.2. Protein expression and purification

Chimeras were purified as described [22]. Briefly, to express IsdA-$CTA_2$/B and ClfA-$CTA_2$/B, ClearColi® (Lucingen, Madison, WI) transformed with pLR001 or pLR003 was grown to an optical density (O.D.600) of 0.9 and induced for 24 h with 0.2% L-arabinose. Proteins were isolated from the periplasmic extract with 1 mg/mL polymyxin B and purified by affinity chromatography on immobilized D-galactose (Pierce™ D-Galactose Agarose, Thermo Fisher, Waltham, MA). Vaccine proteins were dialyzed into sterile 20% glycerol + 1×PBS and concentrations determined by BCA (Pierce™ BCA, Thermo Fisher). Vaccines were tested to ensure endotoxin levels below 0.05 EU/mL (LAL Endpoint Chromogenic, Lonza, Allendale, NJ), plated for sterility and stored at −80 °C until use. For ELISA, IsdA and ClfA were isolated from the cytosol or periplasm of *E. coli* Top10 (Thermo Fisher) + pCK001/pMAH001 after overnight induction with 1 M IPTG. Proteins were purified on cobalt (HisPur™, Thermo Fisher) and dialyzed into sterile 1×PBS. For use in flow cytometry and OPA, endotoxin was removed (Pierce™ Endotoxin Removal Columns, Thermo Fisher).

### 2.3. Animals, trial design and sample collection

All animal protocols were pre-approved by the Boise State University Institutional Animal Care and Use Committee. Sample size was determined prior to Trial 1 by power analysis based upon predicted immune responses in milk and serum using data from a small pilot study ($n = 3$) (Supplementary Fig. 1). The sample size of 7 cows per group was predicted to provide, at a 95% level of confidence, 92% power to detect a minimum difference between the control and any other group of 15%. This is based on a univariate two-group repeated measures ANOVA, assuming that the between groups error term is 0.13 and the within-groups error term is 0.08 [23]. Healthy Holstein cows in the third or fourth lactation were enrolled in the study (21 total Trial 1, $n = 7$; 22 total Trial 2, $n = 11$). Animals were screened as those with at least one of two previous somatic cell counts (SCC) below $200 \times 10^3$ cells/mL and no clinical evidence of mastitis. Further enrollment criteria included: (1) no growth of *S. aureus* from milk, (2) low baseline milk anti-IsdA responses, and (3) no evidence of bovine leukemia virus (BLV) infection. Cows were randomized into two or three groups (Fig. 2). One cow in Trial 1 (group 2) and four cows in Trial 2 (groups 1 and 2) left the study prior to day 60 due to unrelated complications. Milk was not able to be obtained by day 70 from two additional cows in Trial 1 (groups 1 and 3). The vaccine was delivered in 2.5 mL volumes into each nare using a nasal cannula (Merck & Co., Kenilworth, NJ). Blood was isolated by tail vein and coagulated at room temperature (RT) for 1hr prior to removal of serum and dilution into 1:10 Inhibitor Solution (IS = 1× HALT ™ protease inhibitor, and 5% glycerol in 1× PBS). Whole blood was collected on day 60 of Trial 2 in vacutainer tubes for peripheral blood mononuclear cell (PBMC) isolation (Becton Dickinson, Franklin Lakes, NJ). Composite milk samples were obtained aseptically after washing teat ends with 70% ethanol. Milk was centrifuged at 700g for 20 min at 4 °C and skim milk stored in 1:10 IS at −20 °C prior to use in ELISA. Nasal culture was obtained by swab (Q-swab, Hygiena, Thermo Fisher).

### 2.4. Milk culture, PCR and clinical assessment

100 μL and 10 μL of milk and nasal culture was plated on MP2 (Udder Health Systems, Inc., Meridian, ID). The presence of small, white, esculin-negative colonies was considered presumptive *Staphylococcus*. Genomic DNA from these colonies was analyzed by PCR for staphylococcal species and for *nuc*, *isd*A and *clf*A (primers supplemental Table 3). All animals were scored for clinical mastitis by temperature and observation of udder on days of vaccination and milk sampling [24,25]. SCC analysis was performed on milk using the California Mastitis Test (CMT, Udder Health Systems, Inc.).

### 2.5. IgG and IgA ELISA

IsdA and ClfA specific responses on serum and milk were detected using ELISA, as described [22]. Briefly, 96-well plates (Nunc, Thermo Fisher) were coated with 10 μg of IsdA or ClfA (from pCK001 and pMAH001, as described above) in 50μL 1×PBS, blocked for 2 h at 37 °C in 1% goat milk + 1× PBS, and incubated with twofold dilutions of bovine serum or purified milk IgG from Trials 1 and 2.

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