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Development of Novel Vaccines for Campylobacter Control in Poultry

Lindsay Ann Jones
University of Tennessee - Knoxville, lajones@utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Lindsay Ann Jones entitled "Development of Novel Vaccines for Campylobacter Control in Poultry." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Jun Lin, Major Professor

We have read this thesis and recommend its acceptance:

Irene B. Hanning, Raul A. Almeida

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Development of Novel Vaccines for *Campylobacter* Control in Poultry

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DEDICATION

This thesis is dedicated to my loving husband, Brian, who understood my desire to further my education and helped every step of the way to make it happen, even when that meant sacrificing time spent together or dealing with a cranky wife. Thank you for everything. Also, I dedicate this to my animated and supportive parents and family. Thanks for the lifetime of encouragement, lending your ears, and providing humor when necessary. You are all a reminder of how extremely fortunate I am.

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ABSTRACT

Campylobacter is the leading bacterial cause of human enteritis in developed countries. Human campylobacteriosis is commonly associated with consumption of undercooked, contaminated chicken, a natural host of *Campylobacter*. Thus, control of *Campylobacter* colonization in poultry at the farm level would reduce the risk of human exposure to this pathogen. Vaccination is an attractive intervention measure to mitigate *Campylobacter* in poultry. Our recent studies have demonstrated that the outer membrane proteins CmeC (an essential component of CmeABC multidrug efflux pump) and CfrA (ferric enterobactin receptor) are feasible candidates for immune intervention against *Campylobacter*. By targeting these two promising vaccine candidates, three novel vaccines were developed for different vaccination strategies in this study. To construct DNA vaccines for *in ovo* and intranasal immunization, *cfrA* and *cmeC* genes were cloned into the eukaryotic expression vector pCAGGS; sequencing of the recombinant vectors confirmed the success of cloning. Transfection was also performed to determine the production of CfrA or CmeC in NIH 3T3-L1 and HEK-293 cell lines. To develop effective subunit vaccines for intranasal or oral vaccination, purification of recombinant CfrA (rCfrA) and CmeC (rCmeC) was optimized. Substantial quantities of highly purified rCfrA and rCmeC were produced through nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. The purified rCfrA and rCmeC were further encapsulated into chitosan microsphere. Various encapsulation conditions were explored. To construct the attenuated *Salmonella*-vectored vaccine, *cfrA* and *cmeC* genes were cloned into vector pYA3493 and transferred into *S. enterica* serovar Typhimurium χ8914 [strain 8914], the USDA licensed live attenuated vaccine strain. The oral live

Salmonella vaccines producing CfrA or CmeC (full length or truncated) were successfully constructed; expression of the target protein was confirmed by immunoblotting using specific antiserum. The efficacies of two live vaccines that produce CfrA or CmeC were evaluated using broiler chickens. Specific systemic and intestinal mucosal response was not significantly stimulated upon oral vaccination of chickens with the attenuated *Salmonella* derivatives. Together, three novel *Campylobacter* vaccines were developed in this study, which provides us a solid foundation to further develop and evaluate different vaccination regimens for effective mitigation of *Campylobacter* in poultry in the future.

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CHAPTER ONE: Introduction

Thermophilic *Campylobacter* species including *Campylobacter jejuni* and *Campylobacter coli* are one of the most commonly recognized bacterial causes of foodborne illnesses in the United States and other developed countries (Friedman et al., 2000). As a foodborne pathogen of animal origin, *Campylobacter* is commonly present in livestock and poultry, and contaminated poultry meat is a significant source of infection for human campylobacteriosis (Allos et al., 2001). The importance of *Campylobacte*r in food safety is further indicated by the fact that USDA FSIS recently established performance standards for *Campylobacter* in broiler and turkey slaughter establishments to reduce carcass contamination with this pathogenic organism (Food Safety and Inspection Service, USDA, 2011). Thus, there is an urgent need for enhanced efforts to develop effective intervention strategies that can be applied to control *Campylobacter* in poultry.

It has been well established that prior infection with *C. jejuni* can induce protective immunity against *Campylobacter* infections in poultry, strongly supporting the feasibility for development of vaccines for *Campylobacter* control in poultry (Lin et al., 2009). Thus, vaccination of poultry against *Campylobacter* is regarded as an effective intervention strategy to protect food safety (Lin et al., 2009; Jagusztyn-Krynicka et al., 2009). A successful chicken vaccine should prevent colonization or cause a strong reduction of *Campylobacter* numbers in chickens (> 2 log units) (Rosenquist et al.,

2002). However, there is still no vaccine available to date to control *Campylobacter* infections in poultry; vaccinations of chickens against *C. jejuni* have had only partial success (Lin et al., 2009; Jagusztyn-Krynicka et al., 2009), primarily due to a lack of understanding of pathogenesis mechanisms, the antigenic complexity of this organism, and ineffective vaccination regimen (Lin et al., 2009; Hermans et al, 2011).

The goal of this project is to develop effective, safe, inexpensive, and convenient vaccination strategies that could be practically used in poultry for mitigation of *C. jejuni*. To achieve this goal, in the past 10 years, we have been actively involved in elucidation of immunogenic and protective antigens in *C. jejuni*, a primary and critical step towards the design of protective poultry vaccines. Specifically, we have identified and characterized two surface-exposed proteins, CfrA and CmeC, that play an essential role in *C. jejuni* colonization in the chicken intestine (briefly reviewed in Chapter 1 above). CfrA is a surface-exposed 'gatekeeper' that is essential for *C. jejuni* colonization by mediating ferric enterobactin high affinity iron acquisition (Zeng et al., 2009; Lin et al., 2009). CmeC is an essential outer membrane protein component of CmeABC multidrug efflux that plays a critical role in multidrug resistance and *C. jejuni* colonization (Lin et al., 2002a, 2003; Luo et al., 2003; Akiba et al., 2006; Martinez and Lin 2006; Zeng et al., 2010). The following findings from our previous studies showed that both CmeC and CfrA have significant advantages compared to other immunogenic/protective antigens identified in *C. jejuni* (e.g. flagellin, capsule polysaccharide, MOMP): 1) CfrA and CmeC specific antibodies greatly inhibited the function of corresponding target and significantly reduced growth of *C. jejuni* (Zeng et al., 2009; Lin et al., 2003; Martinez and Lin, 2006;

Zeng et al., 2010); 2) both CfrA and CmeC are prevalent and highly conserved in diverse *C. jejuni* strains with sequence identity of 89% - 98% for CfrA and 97.3% - 100% for CmeC (Zeng et al., 2009, 2008); 3) CfrA and CmeC are highly induced and produced in the intestinal tract (Lin et al., 2002a, 2003; Zeng et al., 2010, 2009); 4) both CfrA and CmeC are immunogenic in poultry and elicit a specific antibody response during *C. jejuni* infection in poultry (Zeng et al., 2009; Lin et al., 2002a, 2003); and 5) inhibition of CmeABC efflux pump by a pump inhibitor increased susceptibility of *C. jejuni* to multiple antimicrobials and reduced *in vivo* colonization of *C. jejuni* in chickens (Martinez and Lin, 2006). Clearly, these comprehensive molecular, immunogenic, functional studies have provided compelling evidence that CmeC and CfrA are promising candidates for developing an effective vaccine against *C. jejuni* in poultry.

To develop effective and practical vaccination strategies for induction of rapid and strong immune response against CfrA and CmeC in poultry, in this project, following specific objectives were pursued:

1. Construct DNA vaccines that will be used for *in ovo* and intranasal vaccination.
2. Develop subunit mucosal vaccines by purification and encapsulation of rCfrA or rCmeC into a chitosan microsphere carrier and adjuvant.
3. Construct oral *Salmonella*-vectored vaccines.
4. Evaluate the immunogenicity and protective efficacy of the live *Salmonella*-vectored vaccines in broilers.

CHAPTER TWO: Review of Literature

2.1- *Campylobacter* as a foodborne threat

Based on The Foodborne Illness Risk Ranking Model (FIRRM) generated by the Emerging Pathogens Institute, *Campylobacter* remains at the top of the most common bacterial pathogen associated with foodborne illness in the United States (Batz et al., 2011). Susceptible populations are focused in industrialized countries such as those in North America and northern Europe due to the infrequency of exposure (Friedman et al, 2000). First recognized as a significant food-borne threat in 1972 (Dekeyser et al, 1972), this pathogen has been actively monitored since. The majority of human infections are caused by *Campylobacter jejuni* (90%) and *C. coli* (10%) (Park, 2002; Lee, 2006). Annually, it is estimated that there are 2 million cases of *Campylobacter* infection (Friedman et al 2000), approximately 840,000 cases reported, over 8,000 resulting in hospitalization, and 76 cases resulting in death (Batz et al, 2011). *Campylobacter* infection within the United States leads to an approximate $1.3 billion a year (Batz et al., 2011) in medical and productivity costs, demonstrating this pathogen's tumultuous impact both clinically and financially. The dose of *Campylobacter* to cause human infection has been reported to be as little as 500-800 colony forming units (CFU) (Robinson, 1981; Black et al., 1988), and the onset of symptoms can occur within days and last up to two weeks (Reviewed by Young et al, 2007). Symptoms of *Campylobacter* enteric disease consist of severe diarrhea, with or without hemorrhagic colitis, fever, and abdominal cramping. Patients infected with antibiotic resistant strains often experience a more severe clinical course (Helms et al., 2005). In severe, but rare cases, commonly associated with antibiotic resistant strains, *Campylobacter* infection can lead to Guillain-

Barre syndrome, classified by flaccid paralysis and the possibility of death (van Gerwe, 2009; 2012).

Human infection is linked to the ingestion of contaminated animal products, cross-contaminated food products, the water or soil that has come in contact with infected animals, or through direct contact with infected animals (Blaser, 1997; Schonberg-Norio et al., 2004). Transmission to humans can also come from infected pets (Evans, 1993), or the presence of flies acting as a vector (Ekdahl et al., 2005). *Campylobacter* is a commensal organism within the gastrointestinal tract of poultry, sheep, goats, pigs, and cattle (Humphrey et al., 2007). While a variety of sources for *Campylobacter* spread exists, investigations of outbreaks and epidemiological studies have pointed to under cooked, contaminated poultry as the main source of human campylobacteriosis (Batz, 2011; Studahl and Andersson, 2000; Frost et al., 2002; Friedman et al., 2004). In Iceland, chicken was frozen prior to 1996, until increased demand created a change to the sale of chilled chicken. Following this change, *Campylobacter* infections within the country peaked in 1999 (Stern et al., 2003). At the same time, in 1999 in Belgium, the presence of dioxins within poultry and eggs resulted in withdrawal in sale. A subsequent 40% drop in human campylobacteriosis was observed (Vellinga and Van Loock, 2002). Moreover, a study in England in which 1,231 patient isolates were analyzed through multi-locus sequence typing (MLST) against 1,145 animal and environmental isolates demonstrated that 97% percent of *Campylobacter*-associated sporadic disease stemmed from poultry or meat sources (Wilson et al., 2008). A more recent study involving MLST in the Netherlands, resulted in a majority (66%) of the 696 cases, linked to poultry (Mughini et al., 2012).

2.2- The need for *Campylobacter* control in commercial broilers

It is estimated that domesticated poultry cause 50-70% of human campylobacteriosis (Allos, 2001). *Campylobacter* is considered a commensal organism within the intestinal tracts of poultry including broilers, layers, turkeys, geese and ducks (Sahin et al., 2002; Yogasundram et al, 1989). Approximately 100% of broilers are colonized with *C. jejuni* at time of slaughter (Jacobs-Reitsma, 1995). Horizontal transmission can occur through a numerous possibility of sources such as fecal contamination of food and water (Shanker et al., 1990), flies (Shane et al., 1995), or other farm animals (van de Giessen et al., 1992). Once a contaminated chicken is present, all chickens within the flock can be colonized within a few days (Shanker et al., 1990). Although the infective dose can be as little as 35 CFU (Stern et al., 1988), *Campylobacter* can colonize in the chicken intestine at levels as high as 10^{10} CFU/ g feces (Shreeve et al., 2000). Chickens remain colonized until slaughter, thereby leading to contamination of poultry meat within the processing plants and onto human consumption (Jacobs-Reitsma, 1995). The likelihood of colonization can vary depending on age (Evans and Sayers, 2000). *Campylobacter* is rarely found in chicks at ages of 2-3 weeks (Evans and Sayers 2000; Stern et al., 2001). This delay can be due to many factors in addition to factors of age and intestinal development because young chicks can be colonized through experimental infection via oral gavage (Stern et al., 1988). The presence of maternal antibodies may also contribute to this lag phase (Sahin et al. 2002).

It has been widely accepted that vertical transmission is rare for *Campylobacter* infection in poultry, which is based on following evidence. First, as mentioned above,

chicks are not colonized with *C. jejuni* within the first 2-3 weeks of life even if the layers were contaminated with *Campylobacter* (Shaker et al., 1986; van de Giessen et al., 1992). Second, broiler flocks are usually colonized with strains differing from parental flocks (van de Giessen et al., 1998; Petersen et al., 2001). In addition, for the eggs from *C. jejuni*-positive layers, only a low percentage of eggs (2 out of 266) were found to have *C. jejuni* on the surface of eggshells (Shanker et al., 1986). When the inner membrane of 167 eggs was experimentally infected with *C. jejuni*, only 2 of the 12 hatched were *C. jejuni* positive (Doyle et al., 1984). Sahin and colleagues (2003) investigated vertical transmission by the experimental infection of 50 eggs. Of the 17 hatched chicks, none of them shed *C. jejuni* for as long as 6 weeks. Additionally, of 500 freshly layed eggs from experimentally infected specific-pathogen free (SPF) layers, only 3 of 65 pooled eggs were positive for *C. jejuni*. No *C. jejuni* was isolated from 800 eggs stored for 7 days at 18°C, or from 95 newly hatched chicks (Sahin et al., 2003).

These studies suggest that vertical transmission of *C. jejuni* into broiler flocks is an unlikely and rare event. Introduction of *C. jejuni* into broiler flocks is predominantly through horizontal transmission. Thus, measures to stop the spread within the broiler flocks, rather than hatcheries, are necessary to decrease *C. jejuni* presence at time of slaughter. Reducing colonization in chickens by 2 log units could potentially reduce the likelihood of human exposure by as much as 33% (Rosenquist et al., 2002).

2.3- *Campylobacter* Colonization Factors

Post ingestion, *Campylobacter* can encounter numerous stressors that could potentially inhibit optimal growth in the intestine (Murphy et al., 2006). The presence of

high-levels of *Campylobacter* in the chicken cecum suggests that *Campylobacter* has evolved effective strategies for successful survival and growth within the harsh environment in the intestine. Several factors play a role in *Campylobacter* colonization within the cecum and these factors are required for chemotaxis, motility, adhesion, temperature regulation, oxidative stress responses, iron regulation, resistance to bile salts and antibiotics (Hermans et al., 2011). It is with these multifactorial processes that *Campylobacter* can enhance its survival within the intestinal environment and flourish, colonizing at high numbers.

C.jejuni is attracted by glycoprotein found within mucin, and L-fucose within mucin and bile (Hugdahl et al., 1988, Hermans et al., 2011). Certain amino acids, such as aspartate, glutamate, serine, proline and cysteine also attract *C. jejuni* (Szymanski and Gaynor, 2012; Hughdal et al., 1988). Chemoattractants, such as these, are sensed by transmembrane methyl-accepting chemotaxis proteins (MCP) (Vegge et al., 2009). Genes such as chemoreceptor transducer-like protein 1 (*tlp1*) as well as *tlp4* and *tlp10* encoding MCPs, have been shown to be rather important for invasion (Vegge et al., 2009). Moreover, *Campylobacter* chemotaxis regulatory gene *cheY*, when mutated, affected colonization levels (Hendrixson et al., 2004). These studies show that factors involved in chemotaxis are also involved in colonization of *C. jejuni* in the intestine.

C. jejuni navigates its way through the intestinal mucous not only through chemotaxis but through the presence of polar flagella on the bacterium's surface. Flagellum filament consists of the protein flagellin, encoded by *flaA* and *flaB*. Chemotactic stimuli modify the expression of these genes (Alm et al. 1993). Moreover, when *flaA* is mutated, colonization decreased (Jones et al., 2004). Besides chemotaxis

and adhesion, upon exposure to intestinal mucus, the flagella secrete Cia, *Campylobacter* invasion antigens, which allows for chicken colonization through *in vitro* cell invasion (Konkel et al., 1999, 2004; Ziprin et al., 2001; Biswas et al., 2007).

Adhesion of *Campylobacter* to epithelial cells can be considered a step in colonization. Some studies have shown that genes *capA,* encoding autotransporter lipoprotein, may or may not be important to colonization (Ashgar et al., 2007; Flanagan et al., 2009). Other adhesins such as FlpA, or fibronectin-like protein A, have been identified in achieving binding to chicken epithelial cells and successful colonization (Flanagan et al., 2009).

Campylobacter has the ability to survive body temperature of the chicken, 42°C, as compared to humans, 37°C (Hermans et al., 2011). Approximately 15 to 20 proteins were found to be differentially expressed when *C. jejuni* was grown at temperatures of 37°C and 42°C (Zhang et al., 2009). All of these proteins were identified as significant antigens and involved in pathogenicity within different hosts (Zhang et al., 2009). Moreover, the heat shock protein, DnaJ, when mutated, reduced colonization in chicks (Konkel et al. 1998; Ziprin et al., 2001).

Campylobacter species are thermophilic, thriving at a temperature range of 37-42°C in microaerophilic conditions (e.g. 10% CO_2, 5% O_2 and 85% N_2); indicating they grow best in environments with reduced oxygen levels (Teufel et al., 2002; Hermans et al., 2011). However, *Campylobacter* has the capacity to resist oxidative stressors arising from either incomplete oxygen reduction from *C. jejuni* itself, or, more importantly from the chicken immune system (Atack et al., 2009). Within the chicken host, *C. jejuni*, must survive cytotoxic nitric oxide (NO) produced by immune cells (Shepherd et al., 2011;

Hermans et al 2011). *Campylobacter* harbors NO-detoxifying mechanisms such as Cgb, *Campylobacter* globin, and a nitrite reductase (Pittman et al., 2005; Smith et al., 2011; reviewed by Hermans et al., 2011). *C. jejuni* converts NO to oxygen through a Cgb-catalyzed dioxygenase pathway (Shepherd et al., 2011; Hermans et al., 2011).

Iron is a necessary cofactor for many enzymes and is required for electron transfer mechanisms. Regulation of iron, especially within the iron-limited environment of the intestinal tract, is needed for colonization (Hermans et al 2011). Mutants in *fur*, *cfrA*, and *ceuE,* all associated with either ferric uptake or ferric enterobactin (FeEnt) binding and uptake, had diminished colonizing effects (Palyada et al., 2004). Inactivation of another FeEnt receptor, CfrB, also abolished colonization of *Campylobacter* in the chicken intestine (Xu et al., 2010). Altogether, iron-acquisition systems are important for *C. jejuni* survival and colonization.

Within the intestinal tract, the presence of bile can be toxic to invading organisms. The CmeABC multi-drug efflux pump is induced in the presence of bile salts and plays a critical role in bile resistance in *Campylobacter* (Lin et al., 2003,2005b). Inactivation of CmeABC efflux pump completely abolished *C. jejuni* colonization in the chicken intestine (Lin et al., 2003,2005b).

It is evident that *Campylobacter* has adapted mechanisms to successfully colonize within the intestinal or cecal environment and withstand such harsh elements as temperature change, oxidative stress, and antimicrobials. While several factors and regulation processes within *Campylobacter* allow for optimal colonization against environmental stressors, many mechanisms are still unknown; however, it is within these

mechanisms that *Campylobacter* can colonize within the chicken cecum without generation of a strong immune response as seen in humans.

2.4- Immune responses to *Campylobacter* infection

In humans, *Campylobacter* infection is considered self-limiting. Symptoms resolve within a week, with systemic IgG, IgA and IgM antibodies peaking from 1-2 weeks after infection, while intestinal secretory IgA (sIgA) peak 16-20 days post infection (Hebrink et al. 1988; Lane et al. 1987). Breast milk containing IgA along with increased antibody titers due to continuous *Campylobacter* exposure; offer some explanation for subclinical symptoms experienced in developing countries (Ruiz et al. 1990; Renom et al. 1992; Blaser et al. 1985). In a volunteer study, re-challenge of previously infected volunteers could not reproduce the disease via ingestion of *C. jejuni* 81-176, suggesting that immunity can be induced to protect *Campylobacter* infections in humans (Black et al, 1988)

While *Campylobacter* generates a strong immune response within humans, the same is not true in poultry. Instead, systemic IgG, IgA and IgM along with intestinal secreted IgA antibodies specific to *C. jejuni* gradually rise 2-3 weeks and 3-4 weeks, respectively, post ingestion and persist for a duration of 8 weeks (Cawthraw et al. 1994). *Campylobacter*-specific antibodies levels coincide with decreased colonization. As mentioned above, chicks are typically not colonized within the first 2 weeks post hatch. Sahin et al (2001) observed that the *Campylobacter* maternal antibodies could also be vertically transferred from infected layer hens to newly hatched chicken. Later Sahin et al (2003) demonstrated that the high-level of *Campylobacter* maternal antibodies in

young chickens delayed *Campylobacter* infection using laboratory challenge experiments. Altogether, this evidence demonstrated the protective nature of *Campylobacter*-specific antibodies and supported feasibility for immunization strategies against *Campylobacter* colonization in poultry.

2.5- *Campylobacter* Vaccine Development

Many *Campylobacter* vaccine studies have been published and vaccinations against *C. jejuni* using chicken and other animal models have had only partial success (Lin, 2009; Jagusztyn-Krynicka et al., 2009). However, there is no vaccine available to date to control *Campylobacter* infections. The ultimate goal of an efficient *Campylobacter* vaccine used in poultry should trigger potent and specific mucosal immune response, have broad spectrum in regards to numerous strains, and be easily applicable and cost effective for the poultry farmer. A successful chicken vaccine is expected to prevent colonization or cause a strong reduction of *Campylobacter* numbers in chickens (> 2 log units) (de Zoete et al., 2007). Following vaccination approaches have been explored for developing effective and safe vaccine against *Campylobacter* in poultry.

Killed whole-cell vaccine. The killed-whole *Campylobacter* cells can initiate an immune response without concern with respect to potential pathogenesis to humans. Vaccination with killed *C. jejuni* whole cells (WCV) has been shown to enhance the mucosal immune responses and partly reduced colonization of *C. jejuni* (Noor et al. 1995; Widders et al., 1996; Rice et al., 1997). There are different routes of administration of this type of vaccine in chickens, which include an *in ovo* route (Noor et

al. 1995), a subcutaneous (s.c.) (Glünder et al., 1998), and an oral route (Rice et al., 1997). Different vaccination routes produced different results. The *in ovo* WCV generated increased serum IgG, IgA, IgM, and bile IgA antibodies. Increased systemic antibodies were stimulated in chickens administered s.c. formalin *C. jejuni* WCV with Freund's adjuvant (Glünder et al. 1998). As for protective efficacy, the oral WCV with *E.coli* heat-labile toxin (LT) vaccine lead to a reduction in *C. jejuni* 1.5 log CFU, while the s.c. WCV reduced colonization for the first 2 weeks only (Rice et al., 1997; Glünder et al. 1998). There was no subsequent challenge in the *in ovo* study; therefore protective efficacy of mucosal antibodies on colonization could not be assessed (Noor et al., 1995). Altogether, *C. jejuni* WCVs may not generate an overall protective immune response (de Zoete, 2007).

Subunit vaccine. Another class of vaccines used for *Campylobacter* colonization is the subunit approach. At the present time, flagellin and other outer membrane proteins of *Campylobacter*, CjaA, have been tested. Poultry vaccination with a portion of the *C. jejuni* FlaA fused to a portion of the heat-labile toxin of *E. coli* (as an adjuvant) generated flagellin-specific antibodies while reducing *Campylobacter* colonization (Khoury and Meinersmann, 1995). Another study (Widders et al., 1996; 1998) included immunization with flagellin alone or in conjunction with heat-killed whole cells through intraperitoneal injection (i.p.) followed by i.p. or oral booster. The concomitant vaccination of flagellin and heat-killed whole cells, with i.p. booster, proved to elicit high systemic IgG and IgM and intestinal IgG antibodies and a 1-2 log CFU reduction in *C. jejuni* colonization. While subunit vaccination with flagellin proteins produced some promising results, the exposed portion of flagellin is subject to modified glycosylation in effort of immune

evasion. Moreover, there exists variation in flagellin across *Campylobacter* strains limiting cross-protection (Power et al., 1994; Logan et al., 2002). One promising subunit vaccine target, CmeC, recently has been tested in broiler chickens (Zeng et al, 2009) and will be detailed in the section below.

Live attenuated Salmonella-vectored vaccine. Attenuated *Salmonella*-based vaccines are another attractive strategy to develop inexpensive and practical oral vaccines for chickens to prevent *Campylobacter* infections (Curtiss et al., 1996). A study using an attenuated *Salmonella* vector vaccine carrying the ABC transporter and lipoprotein, CjaA, reduced colonization by 6 $\log_{10}$. (Wyszynska et al., 2004). Chickens orally vaccinated with a heterologous *Salmonella* Typhimurium Δ*aro*A strain carrying the *cjaA* gene fused to tetanus toxin, elicited CjaA-specific systemic IgY and intestinal IgA antibodies and also reduced *C. jejuni* colonization 1.4 $\log_{10}$ (Buckley et al., 2010). Additionally, Layton and colleagues used *Salmonella*-vectored vaccines carrying Omp18/CjaD, ACE393, and CjaA that produced elevated systemic IgG and intestinal IgA along with reduced *C. jejuni* presence by 4.8 $\log_{10}$ in the ileum as compared to the control group (Layton et al., 2011).

DNA vaccine. DNA vaccination could induce both humoral and cellular immune responses and reduce some of the safety concerns associated with live vaccines (Gurunathan et al., 2000). Chickens intranasally vaccinated with chitosan-DNA vaccine expressing the flagellin gene *flaA,* stimulated *C. jejuni*-specific IgG in the serum and IgA within the intestinal mucosa and reduced *C. jejuni* colonization 2-3 $\log_{10}$ within the cecum (Huang et al., 2010). Chitosan was used as an adjuvant to increase mucosal

absorption (Huang et al., 2010). Overall the study provided chitosan encapsulated DNA as a feasible approach for inducement of an effective immune response against *C. jejuni*.

2.6- *Campylobacter* Antigenic Components

Vaccination studies on many Gram-negative pathogens utilize three particular components of the bacterial cell due to their accessibility to the host's immune system: polysaccharide capsules, lipopolysaccharides (LPS), and outer membrane proteins (OMPs). *Campylobacter* polysaccharide capsules (CPS) have shown to play a role in colonization (Maue et al., 2013; Grant et al., 2005). Moreover, a recent study conducted by Maue and colleagues showed that wild-type *C. jejuni* 81-176 colonized Balb/C mice better than both isogenic mutants lacking CPS (*kpsM* mutant) and those with a CPS lacking sugar *O*-methyl phosphoramidate (Maue et al., 2013). This study also showed that toll-like receptor (TLR) signaling was seen with these CPS mutants as compared wild-type strains and their complements (Maue et al., 2013). While CPS demonstrates a role in colonization and harbors immunoregulatory properties, expression is highly variable among strains due to slip strand mismatch repair, which ultimately, alters the frequency of sugar composition within CPS (Maue et al., 2013). *Campylobacter* possesses lipooligosaccharides (LOS) rather than lipopolysaccharides (LPS) on the cell surface (Jeon et al., 2008). LOS have been associated with *Campylobacter* virulence, such as, for example, molecular mimicry of human gangliosides implicated within Guillian-barre syndrome (Karlyshev et al., 2005). However, much like *Campylobacter* CPS, LOS are exceedingly variable across *Campylobacter* strains (Parkhill et al., 2000) and subjected to phase variations modifications and differences in sugar composition

(Guerry and Szymanski, 2008). Overall, even though much of the *Campylobacter* genome is directed toward carbohydrate synthesis (Parkhill et al., 2000), and CPS and LOS play roles within virulence and pathogenesis, much remains unknown about the mechanisms in their variability as well as their roles and interaction with the host (Guerry and Szymanski, 2008; Maue et al., 2013).

Due to the limitation of the surface-exposed polysaccharide structures for vaccine development as discussed above, *Campylobacter* OMPs are the more promising candidates for vaccine development. The most studied OMP is Fla, the protein involved in motility of flagella. Studies have shown that Fla is required for *Campylobacter* colonization and is immunogenic *in vivo* (Wassenaar et al., 1993; Guerry et al., 1997); however, glycosylation regarding this protein is highly variable. Fla is subjected to phase and antigenic variation, making it an unlikely candidate to confer cross-protection as a vaccine candidate (Caldwell et al., 1985; Logan et al., 1989). Major outer membrane protein (MOMP) is a surface porin, which remains stable under various growth and culture environments (Zhang et al., 2000). Moreover, MOMP may be involved in adherence of *C. jejuni* to host cells depending on its conformational state (Moser et al., 1997; Schroder et al., 1997). While MOMP is a predominant OMP of *Campylobacter*, immunogenic characteristics have yet to be defined (Zhang et al., 2000). An intriguing study using the ABC transporter and OMP CjaA for vaccination via an attenuated *Salmonella* vector reduced colonization by 6 logs, but did not include an empty vector control (Wyszynska et al., 2004). Interestingly, when the same vaccines were repeated in Light Sussex chickens, the colonization levels were reduced by an insignificant 0.75 $\log_{10}$ CFU/g cecal contents versus the control group (Buckley et al., 2010). As mentioned

earlier, more recent results using heterologous CjaA vaccines produced 1.4 log and 4.8 log reductions (Buckley et al., 2010; Layton et al., 2011). Overall, the protective efficacy of this antigen is ambiguous. Moreover, CjaA is localized to the inner membrane of *C. jejuni,* thereby being difficult for CjaA-specific antibodies to access (Wyszynska et al., 2008; Lin, 2009).

Two OMPs, CfrA and CmeC, are promising candidates for developing *Campylobacter* vaccine, which are discussed in sections below.

2.7- CfrA: ferric enterobactin receptor

Iron acquisition systems are critical for bacterial pathogenesis and thus have been proposed as attractive targets for iron-dependent pathogen control (Miethke and Marahiel, 2007). Of these systems, high affinity iron acquisition mediated by siderophore, a small iron chelator, is the most efficient iron scavenging mechanism in Gram-negative bacteria. In *Campylobacter*, the high affinity enterobactin (Ent)-mediated iron scavenging is tightly linked to *Campylobacter* pathogenesis (Miller et al., 2009; Butcher et al., 2010). Thus, FeEnt receptors function as a "gatekeeper" for FeEnt acquisition and have been extensively studied in different organisms (e.g. FepA in *E. coli*) (Klebba, 2003). Although CfrA was identified as an iron-regulated OMP in *C. coli* as early as 1997 (Guerry et al., 1997), its function as a FeEnt receptor was not established until 2004 by using *C. jejuni* NCTC 11168 strain (Palyada et al., 2004). Strikingly, inactivation of the *cfrA* gene alone not only impaired Ent-mediated iron assimilation in *C. jejuni* but also completely abolished colonization of *C. jejuni* in chickens even if the parent strain colonized all chickens (Palyada et al., 2004). This finding indicates that

other iron-uptake system(s) in *C. jejuni* cannot compensate the function of CfrA and CfrA play an essential role in colonization of chickens. Therefore, the surface-exposed CfrA is likely a promising vaccine candidate against *C. jejuni*. To test this, Zeng et al (2009) further examined molecular, antigenic, and functional characteristics of CfrA in *C. jejuni.*

CfrA is induced under iron-restricted conditions and is prevalent in *Campylobacter* strains from various sources and geographic locations (Zeng et al., 2009). Alignment of 15 isolates demonstrated that CfrA is 87-98% identical in amino acid sequence and was expressed in 32 geographically varied *Campylobacter* strains (Zeng et al., 2009). Moreover, the sera from *C. jejuni*-infected chickens can react with CfrA, indicating that CfrA is expressed and immunogenic in chicken hosts (Zeng et al., 2009). Specifically, purified CfrA antibodies may block the function of CfrA and diminish FeEnt-mediated growth promotion under iron-restricted conditions (Zeng et al., 2009).

2.8- CmeC: the OMP of CmeABC multuidrug efflux pump

Recently, we have characterized a unique OMP CmeC, an essential component of the multidrug efflux pump CmeABC that contributes *C. jejuni* resistance to a broad spectrum of antimicrobials and is also essential for *C. jejuni* colonization of the animal intestine by mediating bile resistance (Lin et al., 2002a; 2002b; 2003; 2005; Martinez and Lin, 2006). CmeABC is encoded by the *cmeABC* operon (Lin et al., 2002a). CmeABC shares significant sequence and structural homology with known tri-partite multidrug efflux pumps in other Gram-negative bacteria, and consists of a periplasmic fusion protein (CmeA), an inner membrane efflux transporter belonging to the Resistance-

Nodulation-cell Division superfamily (CmeB), and an outer membrane protein (CmeC)(Lin et al, 2002a). Compared to wild-type 81-176, *cmeABC* mutant showed 2 to 4000 fold decrease in resistance to a range of antibiotics, heavy metals, bile salts, and other antimicrobial agents. Notably, *cmeABC* mutant is hyper-susceptible to bile salts, a group of detergent-like compounds in intestine (Lin et al., 2002a; 2002b; 2003; 2005a; 2005b). PCR and immunoblotting analysis showed that *cmeABC* was broadly distributed in various *C. jejuni* isolates and constitutively expressed in wild-type strains (Lin et al., 2002a).

Notably, inactivation of CmeC alone completely resulted in malfunction of the CmeABC pump and drastically increased susceptibilities of *C. jejuni* to various bile salts, a group of natural antimicrobials present in intestinal tract (Lin et al., 2002a; 2002b; 2003; 2005a; 2005b). Consistent with this finding, when inoculated into chickens, the wild-type strain colonized the birds as early as day 2 post-inoculation with a density as high as 10^7 CFU/g feces; in contrast, the CmeC mutant failed to colonize any of the inoculated chickens throughout the study (Lin et al., 2003). In addition, immunoblotting analysis also demonstrated that CmeC is expressed during *Campylobacter* infection of chickens and elicited a specific antibody response in the host (Lin et al., 2003). Inhibition of CmeABC by the efflux pump inhibitor (EPI), Phe-Arg β-naphthyl-amide dihydrochloride (MC-207,110), increased *C. jejuni* susceptibility to antimicrobials including bile salts (Martinez and Lin, 2006). Consistent with this *in vitro* result, MC-207,110 also reduced *in vivo* colonization of *C. jejuni* in chickens, signifying the importance of CmeABC in *C. jejuni* colonization and the feasibility for controlling *Campylobacter* colonization by targeting CmeC (Lin and Martinez, 2006). Together,

these early findings clearly showed that the CmeABC efflux pump is an attractive target for developing intervention strategies against *C. jejuni*. However, our animal study (Lin and Martinez) indicated that several key issues (e.g. toxicity, in vivo stability, production cost of EPI) challenge the clinical application of EPIs. Thus, alternative ways, such as vaccination, to inhibit the MDR efflux pump should also be explored.

Recently, Zeng et al. (2008) showed the amino acid sequences of CmeC were 98.6% to 100% identical to each other and no "hot spot" of point mutation was observed. This finding provides direct evidence showing that CmeC is a highly conserved OMP and is a promising subunit vaccine candidate. In addition, Zeng et al (2008) also showed CmeC-specific antibodies could specifically inhibit the function of CmeABC efflux pump and increase susceptibility of *C. jejuni* to bile salts. Using purified full-length CmeC, Zeng et al (2010) developed a subunit vaccine and evaluated it in chickens. CmeC doses of 50 and 200 μg were administered subcutaneously or orally, alone or with co-administration of adjuvant modified *E. coli* heat-labile enterotoxin (mLT) with booster immunization (Zeng et al., 2010). Following immunization, serum IgG titers were significantly higher in chickens that subcutaneously received 200 μg CmeC plus mLT than the control; however, neither route of administration stimulation led to stimulation of local secretory IgA (Zeng et al., 2010). Overall, these results indicate that CmeC has great immunogenic potential. While the adjuvant mLT was shown previously to be effective at stimulating an immune response when administered orally to animals (Yuan et al., 2001), the possibility exists that CmeC, mLT, or both components of this subunit vaccine could be degraded within the upper gastrointestinal tract before stimulation of the host immune system could occur (Zeng et al., 2010). To avoid this issue and further

enhance mucosal immunity, encapsulation systems such as chitosan microspheres, as described later, may be a promising adjuvant and carrier (Artursson et al., 1994; Van der Lubben et al., 2001; Arca et al., 2009).

Taken together, these studies clearly show that CmeC is widely conserved, critical in colonization and antimicrobial resistance, and is immunogenic *in vivo*. Overall, CmeC is a promising vaccine candidate.

2.9- The Mucosal Immune System

The approach, delivery route, and adjuvants used for vaccination are important to stimulate protective immunity against mucosal pathogens. Decreasing the quantity of colonization in the intestine of poultry can decrease the probability of human exposure upon consumption. Since *Campylobacter* colonizes the chicken intestine in high numbers, generation of protective immunity in the gut mucosa is an essential goal of vaccination. Mucosal sites contain lymphoid tissue either within the mucosal environment or within the underlying draining lymph nodes because these environments are the typical gateway for pathogen access into the body and are usual sites of high microbial density (Brandtzaeg et al., 1999). Epithelial barriers, such as tight junctions in the gastrointestinal tract, protect mucosal surfaces. These epithelial barriers and associated glands yield innate defenses including antimicrobial peptides and mucins (Neutra et al., 2005). Besides these innate defenses, lymphoid and antigen presenting cells (APCs) are generous in quantity within the intestinal mucosa. Mucosal epithelial cells sense the presence of pathogens through Toll-like receptors (TLRs) that recognize pathogen associated molecular patterns (PAMPs) like flagella or LPS. In response to

PAMP recognition, chemokine and cytokines are stimulated to signal to immune cells underlying the epithelium: dendritic cells (DCs), lymphocytes, and macrophages to generate innate immune responses and adaptive immune responses (Neutra et al., 2005; Kagnoff and Eckmann, 1997). These cells are found within the Gut-Associated Lymphoid Tissue, or GALT, and in Peyer's Patches in the intestinal mucosa. Dendritic cells and M cells from within the follicle-associated epithelium, sample the epithelium surface at these mucosal sites for antigens and present these antigens to T cells (Fujkuyama et al., 2012). Mucosal B cells differentiate into plasma cells, which produce dimeric secretory IgA to combat pathogens and halt invasion through entrapment of antigens in mucus and consequent blocking of pathogens from epithelium adhesion (Lamm et al., 1997). In contrast to other antibody isotopes, sIgA are resistant to protease degradation in the mucosal environments (van Egmond et al., 2001). Secretory IgG is secreted by 5-15% of mucosal plasma cells within the intestine (Brandtzaeg et al., 1999); however, sIgG is subject to protease degradation. IgG, whether mucosal or systemic, has the potential to neutralize pathogens within the mucosa to detain from initiation of systemic infection (Neutra et al., 2006). Mucosal vaccine delivery can induce both systemic antibody in peripheral lymphoid tissue and secretory antibody responses within nasal, oral, small intestine, and large intestine or cecum. Injected vaccines, in contrast, are poor generators of mucosal immunity (Lamm et al., 1997).

2.10- Challenges for developing mucosal vaccines

While the intention for mucosal vaccines is to stimulate mucosal immunity within the environment of pathogen access to the host, some challenges for administration,

similarly faced by these pathogens, exist. The vaccines administered orally, nasally, or directly to the site of the mucosal surface can be diluted by mucosal secretions, degraded by proteases, or are unable to access the epithelium barrier. This implies that large quantities of vaccine are required; however, the exact dose that reaches the mucosa is unable to be determined. Ideally, vaccines would be most effective if they act in similar fashion to invading pathogens. This means that the ideal vaccine would be able to consist of several protein subunits, adhere to epithelial cells, and establish innate and adaptive immunity (Neutra et al., 2006). One vaccine approach to achieve such mimicking is through use of live attenuated pathogen vaccine vectors. Live attenuated bacterial vectors that replicate *in vivo*, such as *Salmonella enterica* serovar Typhimurium, could be very effective for antigen presentation and elicit specific antibody production and T cell responses (Darji et al., 1997).

Protein, peptide and DNA vaccines, including live attenuated vaccines, can abstain from degradation during oral administration through delivery via microsphere encapsulation. Plasmid DNA or protein subunit can be trapped within polymers such as chitosan. Poly (DL-lactide co-glycolide)-encapsulated plasmid DNA has been shown to generate immune responses both systemically and mucosally (Jones et al., 1997). Biodegradable polymers such as these are natural, non-toxic, easy to prepare, and time-released adjuvants to aid in eliciting an immune response and protection of the vaccine.

Another challenge to vaccination lies within timing of administration. Chicks require immune protection at an early age. While macrophages have been detected during embryonic development, the adaptive immune response of the chicken progresses from two weeks of age through 6 weeks of age or time of slaughter. During the first week post

hatching, the quantity of T cells and natural killer cells increases within the intestine. During the second week, most likely as a consequence to colonization of commensal gut microflora, B and T cell populations increase again (Bar-Shira et al., 2003; Muir et al., 2000). The late maturity of the immune system in the chicken can be challenging for vaccination since the generation of antibodies is limited prior to 10 days post-hatch (Bar-Shira et al., 2003; Mast and Goddeeris, 1999). Thus, vaccination strategies may include a booster to further stimulate an optimal immune response.

Finally, besides eliciting an immune response, vaccination for *Campylobacter* in poultry needs to be easy to administer and cost-effective. Oral, intranasal, and *in ovo* vaccination are all routes of administration that can be appropriate for this purpose. Oral and intranasal vaccination is used with many commercial vaccines and can be easily applied to drinking water or through aerosol. *In ovo* vaccination is a new, automatic method that can vaccinate 20,000 to 30,000 eggs per hour. It has been the route of administration for viral diseases such as Newcastle, Marek's, and infectious bursitis (Johnston et al., 1997). Eggs are injected through the shell at 18 days of incubation. While the immune system of chicks post-hatch matures slowly, a study of *in ovo* versus post-hatch vaccination for infectious bursal disease demonstrated that *in ovo* vaccination might elicit a higher innate and adaptive immune response than post-hatch vaccination (Negash et al., 2004). *In ovo* vaccination against bacterial pathogens has not been thoroughly explored as of yet.

CHAPTER THREE: Material and Methods

3.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study, and their sources, are listed in Table 1. *Campylobacter jejuni* NCTC 11168 (JL241) was used for amplification of *cfrA* and *cmeC* genes. JL241 was routinely grown in Mueller Hinton (MH) broth (BD Difco, Sparks, MD) or on MH agar plates under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C. If needed, MH agar was supplemented with C*ampylobacter* Growth and Preston *Campylobacter* Supplements (Oxoid, Bashingstoke, Hampshire, England). *E. coli* χ6097 and *Salmonella enterica* serovar Typhimurium UK-1 χ8914 were grown in Luria-Bertani broth (LB) (BD Difco) broth with shaking (250 rpm) or on LB agar plates containing 50 μg/ml diaminopimelic (DAP) acid at 37°C overnight. When necessary, LB media were supplemented with 50 μg/ml of tetracycline or 50 μg/ml of ampicillin.

3.2. PCR

Primers used in this study and the expected sizes of the products are listed in Table 2, 3, and 4. Each PCR was performed with a 50 μL mixture containing 200 μM deoxynucleoside triphosphates, 200 nM of each primer, 50 ng of JL241 template DNA, 2.5 mM $MgSO_4$, and 5 U *PfuUltra II* high-fidelity DNA Polymerase (Stratagene). The temperature-cycling parameters are typically as follows: 95°C for 5 min for denaturation, 32 cycles of 1 min. at 94°C, 1 min at 58°C, 90 sec at 72°C, and a final extension step of 45 sec at 72°C, though cycling conditions varied according to annealing temperatures of

primers and estimated product sizes. PCR products were further purified with the QIAquick Purification Kit (Qiagen) for cloning procedures or sequencing analysis.

3.3. Sequence analysis of plasmid constructs.

Plasmid DNA was extracted from host cells using Qiagen QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Primer pairs of pYA3493_F/pYA3493_R (Table **X**) and pCAGGS_F/pCAGGS_R (Table **X**) were used to sequence pYA3493 and pCAGGS derivatives, respectively. Sequencing was performed in the Molecular Biology Resource Facility at the University of Tennessee (Knoxville, TN). For recombinant plasmids, proper insertion of *cfrA* or *cmeC* was confirmed by comparing the sequences to those from parent plasmids and the genome of *C. jejuni* NCTC 11168.

3.4. SDS-PAGE and Western Blot analysis

SDS-PAGE and Immunoblotting were performed as described previously with slight modifications (Lin et al., 2002; Zeng et al., 2009). Typically, 5 mL of overnight cultures of *E. coli* or *Salmonella* were centrifuged and resuspended in 50 μL of 1X PBS and 50 μL of 2X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Five to 15 μL of such whole cell lysate suspension or purified protein sample was loaded in each lane and separated by SDS-PAGE with a 12% (w/v) polyacrylamide gel at 80V for 40 minutes followed by 160V for 40 minutes. After SDS-PAGE, proteins in gels were then electrophoretically transferred to nitrocellulose membranes (Bio-Rad) at 90V for 1 hour. The membranes were incubated with blocking buffer (5% Nestle skim milk powder in PBS) overnight at 4°C prior to 1 hour incubation at 25°C with primary antibodies (1:2000 diluted rabbit anti-CfrA sera or 1:1000 diluted

rabbit anti-CmeC sera in blocking buffer). After incubation, the membranes were washed three times with PBS containing 0.05% Tween 20 and incubated with secondary antibody (goat anti-rabbit immunoglobulin G-horseradish peroxidase, diluted 1:2000 for CfrA and 1:1000 for CmeC) for 1 hour at 25°C. After incubation the membranes were washed as described above. The membranes were then developed with the 4CN Membrane Peroxidase Substrate System (KPL, Gaithersburg, MD).

3.5. Construction of DNA Vaccines.

The pCAGGS vector (Fig.1A) is a eukaryotic expression vector containing the chicken β-actin promoter, the CMV immediately early enhancer (CMV-IE), the SV40 origin of replication (SV40 OriC), and the ampicillin resistance cassette (Amp^R) for selection (a kind gift from Dr. Miyazaki, University of Tokyo, Japan) (Niwa et al., 1991). The full-length *cfrA* (2109 bp) and *cmeC* (1472 bp) fragments from *C. jejuni* NCTC 11168 were PCR amplified using primer pairs of pCAGGS_CfrA_F/pCAGGS_CfrA_R and pCAGGS_CmeC_F/ pCAGGS_CmeC_R, respectively (Table 2). All these primers have a *Xho*I site at the 5' end. The PCR product was digested by *Xho*I and ligated into pCAGGS, which previously has been digested with the same enzyme. The ligation mixture was introduced into Top10 cells via electroporation for 4-5 ms at 2.5 kV. Transformants were selected on LB agar plates containing ampicillin. The plasmids from randomly selected transformants were extracted and analyzed by agarose gel electrophoresis. The recombinant plasmids with insertion were further subjected to PCR screening for identification of the recombinant plasmids with correct orientation of specific inserted gene. The identified desired constructs (DNA vaccines) were finally

subjected to sequence analysis to confirm the orientation and integrity of the inserted fragment.

Transfection was then performed to validate the production of CfrA or CmeC by the DNA vaccine in eukaryotic cells. Briefly, 4 μg of recombinant plasmid were transfected into 50-70% confluent NIH-3T3-L1 or HEK-293 cells in a 6-well dish (Corning) using the Lipofectamine 2000 kit (Invitrogen Life Technologies) according to the manufacturer's instructions. Cells transfected with PBS, the pR-M02 plasmid expressing eGFP, and the empty pCAGGS vector served as controls. After 5-6 hours of incubation, Lipofectamine was removed and replaced with complete media (1X DMEM plus Glutamax, 10% heat-inactivated fetal calf serum, 1% Penicillin/Streptomycin [Gibco]). After 24-48 hours incubation at 37°C in 5% CO_2, efficacy of transfection was evaluated by examining the transfection rate of the control pR-M02 plasmid bearing eGFP using fluorescent confocal microscopy, which was performed at the Advanced Microscopy and Imaging Center facility at UTK. In addition, the cells from each well were trypsinized, centrifuged, and resuspended in 100 μL of SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE and Immunoblotting for examining the production of CfrA or CmeC in the transfected cells as described above. Both the 4CN Membrane Peroxidase Substrate System and the Chemiluminescent Substrate kit (KPL, Gaithersburg, MD) were used for immunoblotting. Transfections were performed in duplicate.

3.6. Production of Subunit vaccines

Production of high-purity rCfrA and rCmeC. Production and purification of histidine-tagged rCfrA and rCmeC were performed as described previously with modifications (Zeng et al., 2009, 2010). The *E. coli* constructs for producing full-length, histidine-tagged rCfrA or rCmeC were obtained from our recent studies (Zeng et al., 2009, 2010). The His-tagged rCfrA and rCmeC were purified from the *E. coli* culture using Ni-NTA affinity chromatography. Briefly, approximately 2.5 mL of nickel nitrilotriacetic acid (Ni^{2+}-NTA) agarose resin (Qiagen, Hilden, Germany) was equilibrated with lysis buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 10 mM imidazole with 5 mM of ATP-$Mg2^{+}$ to eliminate GroEL contamination). Bacterial lysate from 1 liter of IPTG-induced culture was mixed with the equilibrated Ni-NTA resin with gentle rocking for 1 hour at 4°C. The mixture was then loaded into a column and flow through was collected. The column was washed with 5 volumes of wash buffer (50 mM sodium phosphate buffer, 300 mM NaCl, 60mM imidazole, 10% glycerol, pH 7.0 supplemented with 2mM β-mercapthanol, 5 mM of ATP, and 5mM of $MgCl_2$). The proteins bound to Ni-NTA were eluted with 5 volumes of elution buffer (50mM sodium phosphate buffer, 300mM NaCl, 300mM imidazole, 10% glycerol, pH 7.0 and 2mM β-mercapthanol). Eluent was collected into 1.5mL tubes. SDS-PAGE was performed to determine the purity and quantity of purified proteins in elution fractions. The rCfrA- and rCmeC-containing fractions were combined and dialyzed against PBS buffer with 0.1% Empigen BB (Sigma) to aid in solubilization of the recombinant proteins. Dialyzed protein was aliquoted into sterile tubes and stored at -80°C. Protein concentration was measured through the bicinchoninic acid (BCA) protein assay kit (Pierce).

Encapsulation of rCfrA and rCmeC in chitosan microspheres (CMs). To best evaluate different methods of chitosan encapsulation, two different procedures were performed. In the first method, chitosan (Sigma, prod. No: 448869, low molecular weight) was completely suspended in 1% of acetic acid solution to produce a 2% stock solution. In one set of chitosan solutions, Pluronic F127 (Sigma) was further added at a final concentration of 1.25%. The chitosan or chitosan/Pluronic F127 was then mixed with purified rCfrA or rCmeC to achieve final chitosan: protein ratio of 3:1 and 1:1, respectively. Subsequently, the mixture was vortexed and adjusted to a pH of ~5 due to the pI values of rCfrA and rCmeC both being around ~5. Samples were incubated at 50°C with continuous shaking at 250 rpm for 15 minutes, followed by overnight incubation with shaking at 250 rpm at 37°C. After incubation, samples were subjected to SEM examination.

The second preparation method of chitosan microspheres (CM) was performed by a procedure as described by Kang et al. (2006) with modifications. Briefly, 0.125 g of chitosan was dissolved in 50 mL 1% acetic acid to make a 0.25% (w/v) solution. To 25 mL of this solution, 1.25% wt. of Pluronic F127 (Sigma) was added and sonicated for 10 minutes. Both chitosan and chitosan/ Pluronic F127 were added dropwise through a needle into 1 mL of 15 % of tripolyphosphate (TPP) (in ddH_2O). The CM-TPPs, as these will be referred to, were separated through centrifugation at 4000 rpm for 15 min. The CM-TPPs were resuspended in 500 µL PBS. The CM-TPPs were adjusted to make a 3:1 concentration ration (CM: protein) and mixed with the purified rCfrA (311 µg/mL original concentration) or rCmeC (639 µg/mL original concentration). The mixture was incubated overnight at 37°C with continuous shaking at 250 rpm. After incubation, 2mL

of each mixture was centrifuged at 3000 rpm for 10 minutes. Supernatant containing unloaded rCfrA or rCmeC was then aliquoted to separate tubes for quantification through the BCA protein assay method. The loaded content was calculated according to the following formula (Kang et al. 2007; Xu et al 2003):

Loading content (%) =

$$\frac{\text{Total amount protein (rCfrA or rCmeC)} - \text{free protein}}{\text{Total protein}} \times 100$$

After incubation, one drop of protein loaded and unloaded control CM-TPPs were placed on observation studs for SEM observation and allowed to air dry for approximately 6 hrs. The samples were visualized using a Leo 1525 field emission SEM or Auriga field emission SEM at the Advanced Microscopy and Imaging Center at the University of Tennessee, Knoxville, TN.

3.7. Construction of *Salmonella*-vectored vaccines

The plasmid pYA3493 (Zekarias et al., 2008) was used for preparation of desired recombinant plasmids that were then transferred to the attenuated *S. enterica* serovar Typhimurium χ8914 (Zekarias et al., 2008) for live vaccine construction. The pYA3493 is an expression plasmid containing the P_{trc} promoter upstream of the β-lactamase signal peptide, and the *asdA* gene for natural selection (see Fig. 1B). The primer pairs of pYA3493_CfrA_F/pYA3493_CfrA_R and pYA3493_CmeC_F/pYA3493_CmeC_R (Table 3) were designed to amplify full-length *cfrA* and *cmeC* genes (with removal of signal peptide) from NCTC 11168 genomic DNA. Specific PCR product was digested with *Eco*RI and *Sal*I for directional cloning of the fragment into the pYA3493 that has

been digested with *Eco*RI and *Sal*I. The ligation mixtures were introduced into competent *E. coli* χ6097 via electroporation at 4-5 ms and 2.5kV; preparation of the competent cells is detailed in the section below. Transformants were selected on LB agar plates. The empty vector, pYA3493 was electroporated into *E. coli* χ6097 to create strain JL1070. After positive identification through plasmid extraction and gel electrophoresis, pYA3493 was then transferred into *S. enterica* serovar Typhimurium *UK-1* χ8914 to create strain JL1059.

Because of failure to obtain desired recombinant plasmids that bear full-length of *cfrA* or *cmeC* gene despite extensive efforts, a panel of alternative primer pairs were designed, which included 1) pYA3493_CfrA_F/pYA3493_CfrA_R (*Eco*RI) and pYA3493_CmeC_F/pYA3493_CmeC_R (*Eco*RI) that contain a single restriction enzyme site (*Eco*RI) for bidirectional cloning (Table X); 2) pYA3493_CfrA_F/CfrA_B1_R, pYA3493_CfrA_F/CfrA_B7_R, CfrA_B3_F/CfrA_B14_R, and CfrA_B14_F/CfrA_C_R (Table 4) for amplifying different truncated *cfrA* fragments. The *Eco*RI and *Pst*I were attached to the 5' end of the primers for directional cloning; and 3) pYA3493_CmeC_F/CmeC_TM2_R, CmeC_TM2_F/CmeC_TM3_R, CmeC_TM3_F/CmeC_C_R (Table X) for amplifying different truncated *cmeC* fragments. The cloning and transformation procedure are the same as described above. Upon identification of the recombinant plasmid of interest in *E. coli* transformant, JL1060 and JL1061 (containing pYA3493_tCfrA and pYA3493_tCmeC, respectively) via sequencing confirmation, the specific plasmid was extracted from the corresponding *E. coli* host and electroporated into competent *S. enterica* serovar Typhimurium *UK-1* χ8914. *Salmonella* strains harboring pYA3493_tCfrA and pYA3493_tCmeC are referred

to as JL1062 and JL1063. Preparation of *Salmonella* competent cells is detailed in the section below. The *Salmonella* transformants were also selected on LB plates. The plasmids from *Salmonella* transformants were further extracted for sequencing analysis (section 3.3). In addition, production of the specific inserted protein (CfrA or CmeC) in both *E. coli* (the intermediate host) and *Salmonella* (the final live vaccine host) was confirmed in by immunoblotting using specific antibodies as described in section 3.4.

To overcome any potentially lethal effect of full-length CfrA or CmeC on *E. coli* host, the plasmid pBR232 (*lacI*^{q}) was transferred to the *E. coli* χ6097 to create strain JL1080 (Table1) in which the promoter activity of pYA3493 was greatly suppressed. In addition, primer pairs were redesigned for directional cloning (Table 3, pYA3493_CfrA_F/pYA3493_CfrA_R for *cfrA* gene and pYA3493_CmeC_F/pYA3493_CmeC_R for *cmeC* gene). The *EcoR*I and *Pst*I digested PCR fragment was ligated into the pYA3493 that has been digested with the same enzymes. Ligation mixture was electroporated into JL1080 (the *E. coli* χ6097 containing plasmid pBR232). Transformants were screened and confirmed as described above. The recombinant plasmids that bear full-length *cfrA* or *cmeC* gene within *E. coli*, JL1109 and JL1110, were transferred into *Salmonella* host strain, creating JL1104 and JL1105, and subsequently validated by sequencing and immunoblotting as described above.

3.8. Competent cell preparation.

A single colony of *E. coli* χ6097 or *S. enterica* serovar Typhimurium χ8914 were inoculated into 50 mL LB broth supplemented with 50 μL of DAP (50mg/mL stock concentration) and incubated overnight at 37°C with constant shaking at 250 rpm.

Following incubations, a 25 ml aliquot of the overnight culture was inoculated into flasks containing 500 mL of pre-warmed LB broth containing DAP. Flasks were incubated at 37°C with agitation at 300 rpm for about 2.5 hours so that the cultures reached an OD_{600} value of 0.35 to 0.4. The flasks were transferred to an ice water bath and swirled to ensure proper and even cooling for 15 minutes. The cultures were then centrifuged at 1000 x g for 20 min at 4°C. Pellets were then resuspended in 500 mL ice-cold Milli-Q water and centrifuged under the same conditions. Pellets were further washed with 250 mL and 10 mL of ice-cold 10% glycerol successively. The pellets were finally resuspended in 1 mL ice-cold GYT media (10% glycerol, 0.125% yeast extract, 0.25% tryptone). The suspension was then diluted with GYT media so that the concentration of cells was equivalent to 2-3 x 10^{10} cells/mL (based on 1.0 OD_{600} = ~2.5 x 10^{8} cells/mL). After diluting, 40 µL of specific competent cells was placed in a 0.2cm electroporation cuvette (Bio Rad) and tested at 4.5 ms and 2.5 kV to ensure no arcing occurred. After testing, microcentrifuge tubes containing 40 µL aliquots of competent cell suspensions were flash frozen (placing them briefly in ethanol at a temperature of -80°C) and stored at -80°C until use.

3.9. Chicken Vaccination Trial

Inoculum preparation. The two vaccine strains, JL1104 (Live-CfrA) and JL1105 (Live-CmeC) (Table 1), were evaluated in this vaccination trial. Three to 5 colonies of each live vaccine strain as well as the vector control JL1059 (Table 1) were randomly picked up from LB agar plate and inoculated into 50 mL LB broth for overnight, static growth in 37°C incubator. Subsequently, 10 mL of the overnight culture was inoculated into 90 mL LB broth and grown statically for about 6 hours to reach an OD_{600} value

between 0.8 and 1.0. Bacteria cultures were then centrifuged and resuspended in PBS to an OD_{600} of 1.0. The inoculums were serially diluted in PBS for CFU enumeration. Chickens were inoculated orally with 200 µL of the respective inoculum.

Chicken immunization and sample collection. One day-old Cornish x Rock (commercial broiler) chicks (n = 80) were obtained from Hubbard Hatchery (Pikeville, TN) and allocated into 4 treatment groups (20 birds per group, 2 cages per group). Upon arrival, cloacal swabs from 5 randomly selected birds from each group were collected for determining the presence of *Salmonella* spp. and *C. jejuni* in the intestine. Specifically, cloacal swabs were placed in 2 mL of PBS containing 1% of gelatin (PBS-G). To examine *Salmonella* contamination, 100 µL of the suspension was spread on MacConkey (BD Difco) plates and incubated overnight at 37°C. The next day, colonies matching the expected phenotype (non-lactose fermenting, gray colonies) of *Salmonella* on MacConkey plates were re-streaked on XLT-4 agar plates (XLT Agar base [BD Difco] and 2.4 mL [per 500 mL] Tergitol [Sigma]) that were incubated for 24-48 h for identifying H_2S-producing colonies. To isolate *Campylobacter*, 100 µL of the suspension was spread on MH agar plates that contain *Campylobacter*-specific selective supplements, followed by incubation at 42°C under microaerophilic condition for 48 hrs.

All chickens were managed in a sanitized wire-floor cage and provided with water and antibiotic-free feed ad libitum. Chicks were maintained at 32°C in the first week and at 25°C thereafter. As shown in Table 5, at 7 days of age, chickens were orally immunized with 200 µL of Live-CfrA (group 3) or Live-CmeC (group 4). Two control groups received either PBS (group 1) or the Live-empty vector (group 2). At 28 days of age, each group was orally challenged with *C. jejuni* NCTC 11168 with dose of

approximately 10^2 CFU/bird; the choice of this dosage is based on the publication by Knudsen et al. (2006) and our previous challenge experience using the same strain (Zeng et al., 2010). After challenge, cloacal swabs were collected every 2-3 days for 10 days. Swabs were placed in 1 mL MH broth and 100 μL were plated on MH agar containing selective supplement for enumeration of *C. jejuni*.

Blood samples were collected via the wing vein of 10 chickens from each group on days 7, 18, 28, and 38 to evaluate systemic IgG and IgA antibodies. Intestinal lavage was taken from each euthanized chick (5 birds per group) on days 18, 28, and 38, and diluted 1:4 in lavage extraction buffer (PBS containing 0.05% Tween 20, 0.05g/mL of EDTA, and cOmplete mini protease inhibitor (Roche, prod. No: 04693159001)), which were used to determine specific mucosal IgA and IgG antibodies.

Spleen, liver, and cecum were also taken to evaluate the presence of inoculated *Salmonella* live vaccine following oral vaccination. Briefly, at days 18, 28, and 38, spleen, liver, and cecum were asceptically collected (5 birds per group), diluted 1:9 in PBS-G, and homogenized using the Stomacher-80 and filter stomacher bags (Fisher). Tissue homogenates (100 μL) were spread on MacConkey plates. Non-lactose fermenting colonies were then isolated on XLT-4 and re-isolated on MacConkey. Pink colonies containing H_2S production on XLT-4 were selected and the identities of the selected colonies were examined by plasmid profile as well as PCR using *cfrA* or *cmeC* specific primers.

ELISA. CfrA-, CmeC-, and *S. enterica* Typhimurium χ8914 membrane-specific antibodies in serum and intestinal lavage samples were measured by indirect ELISA as described previously with modifications (Zeng et al., 2010). Briefly, microtiter plates

(Nunc-Immuno Plate, Thermo Fisher Scientific) were coated with 100 µL of highly purified rCmeC, rCfrA, or *S.* Typhimurium χ8914 membrane protein (as obtained through sonication and ultracentrifugation, see section 3.11) (30ng/well) in coating buffer (1M ammonium acetate and ammonium carbonate, pH 8.2) overnight at room temperature. Plates were washed three times with washing solution (0.05% Tween 20, 1X PBS). Plates were then blocked with blocking buffer (1X PBS, 1% BSA, 0.1% Tween 20) for 1 h at 37°C. Chicken serum and intestinal lavage samples were diluted 1:100 and 1:4 in blocking buffer, respectively, and 100 µL was added, in duplicate, to corresponding wells, followed by 1 h incubation. Next, plates were washed five times with washing solution. To measure systemic IgG, IgA, and mucosal IgG and IgA, secondary anti-chicken IgG and IgA was diluted 1:2000 in blocking buffer and 100 µL was added to each well. After 1 h incubation, plates were washed three times. Plates were developed using the ABTS Peroxidase Substrate Kit (KPL, Gaithersburg, MD) and the reaction was stopped after 10 minutes using 100 µL stopping solution (1X PBS, 1% SDS). Absorbance was measured at OD_{405} nm.

Statistical analysis. Differences in serum and intestinal lavage OD_{405} nm readings among treatment groups were analyzed by least square analysis of covariance with the data at day 7 (day of vaccination) as the covariant; main effects were day of sample collection and treatment. Comparison of OD_{405} nm readings within treatment groups across time was tested by ANOVA. Levels of significance for *P* value were 5% (0.05). All statistical analyses were performed using SAS software (v9.03, SAS Institute Inc., Cary, NC).

3.10. Minimum infective dose determination.

Sixty day-old commercial broilers (Hubbard Hatchery) were assigned into 6 groups (10 birds per group) and maintained in the same environmental conditions as those in the chicken trial described above. By two weeks of age, in addition to the control group that received MH broth, five groups of chickens were orally inoculated with various doses of wild-type *C. jejuni* NCTC 11168 (JL241) (approximately 5, 50, 5 x10^2, 5 x 10^3, and 5 x 10^4 CFU per chicken as determined by colony enumeration of inoculums). After inoculation, cloacal swabs were collected from the chickens every 2-3 days for two weeks and cultured for *Campylobacter*. The minimum infective dose was defined as the lowest dose at which at least one chicken of the inoculated group was colonized within two weeks after inoculation.

3.11. Preparation of membrane fraction. To measure antibody responses against *S. enterica* serovar Typhimurium χ8914, the membranes were isolated from a culture of JL1059, the S. *enterica* serovar Typhimurium χ8914 containing empty vector pYA3493 (Table 1). One liter of overnight culture (37°C, 250 rpm, in LB broth) was subjected to centrifugation at 2500 x g for 30 minutes at 4°C, then washed with PBS. Pellets were resuspended in 20 mL ddH_2O and sonicated for 30 seconds three times with a 1-minute rest period on ice bath. Sonicated culture was then centrifuged at 5,000 x g and the supernatant was further centrifuged at 30,000 x g for 60 minutes. The pellet, representing the membrane fraction, was resuspended in 1 mL ddH_2O and stored at -20°C.

The live *Salmonella* vaccines that express CfrA or CmeC (truncated or full length) were also subjected to membrane fractionation in order to evaluate if the cloned

foreign proteins were localized in the membrane. Membrane samples were evaluated by immunobloting analysis as described previously in an earlier section.

CHAPTER FOUR: Results

4.1. Development of DNA Vaccines

The *Xho*I-digested *cfrA* and *cmeC* PCR fragments were cloned into the vector pCAGGS, which previously had been digested with *Xho*I (Figure 2A). PCR screening identified potentially desired transformants containing recombinant plasmid bearing correct orientation of specific inserted gene, as reflected by the presence specific PCR fragment (Figure 2B and 2C). The recombinant plasmids were further extracted from the selected transformants and were compared to the parental pCAGGS. As shown in Fig. 2D, the recombinant plasmids from the transformants with positive PCR results (pCAGGS_CfrA and pCAGGS_CmeC), displayed a significant shift in size, confirming the *cfrA* and *cmeC* fragments had been successfully cloned into the parent plasmid pCAGGS. The extracted pCAGGS_CfrA and pCAGGS_CmeC plasmids were also sequenced, and no frameshift or mutations in the coding sequences of *cfrA* and *cmeC* were detected, indicating that desired DNA vaccines expressing CfrA or CmeC were successfully constructed.

To confirm the production of CfrA and CmeC by the DNA vaccines in eukaryotic cells, pCAGGS_CfrA and pCAGGS_CmeC were used to transfect NIH-3T3-L1 and HEK-293 cell lines. The control plasmid, pR-M02 encoding eGFP, successfully transfected both NIH-3T3-L1 (Fig. 3A) and HEK-293 (data not shown) cells, as visualized through fluorescent microscopy. However, immunoblotting using specific

antibodies failed to detect CfrA and CmeC from the cells transfected with pCAGGS_CfrA and pCAGGS_CmeC, respectively (Fig. 3B & 3C).

4.2. Development of Subunit Vaccines

Large quantities of high-purity rCfrA and rCmeC are needed for preparation of the subunit vaccine as well as for an ELISA test in this project. After 3 hours of induction with IPTG, the amount of rCfrA and rCmeC were dramatically increased (Fig. 4A & B, lane 2). High purity of rCfrA (Fig. 4A, lane 3) and rCmeC (Fig. 4B, lane 3) were successfully obtained after one-step Ni-NTA chromatography purification. Notably, 5 mM of ATP and Mg^{2+} were added in the lysis buffer, which successfully removed contaminated proteins during purification. From 1 L of IPTG-induced culture, the protein purification procedure yielded approximately 2.5 mg rCfrA and 8 mg rCmeC.

Chitosan encapsulation of rCfrA and rCmeC was performed with two different methods to determine optimal conditions for preparation of the chitosan encapsulated subunit vaccine. The first method entailed mixing chitosan or Pluronic F127-treated chitosan with the purified proteins to create chitosan-protein microspheres. Since chitosan has the tendency to aggregate, Pluronic-F127, a copolymer, was added to keep the aggregation to a minimum. Morphology of the chitosan/Pluronic F127 microsphere (no protein loaded) is shown in Figure 5A. Morphology of chitosan/Pluronic F127-CfrA is pictured in Figure 5B and C. These microspheres are caused by the interaction of chitosan, Pluronic F127, and CfrA. Size of random chitosan/Pluronic F127 microspheres were measured (Fig. 5D), which were in the range of 1 to 7 μm. The chitosan-PBS control, chitosan-CfrA, chitosan-CmeC, and chitosan/Pluronic F127-CmeC are not

pictured as these samples were not diluted and SEM proved difficult to image these microspheres.

The second method of chitosan encapsulation entailed mixing chitosan with tripolyphosphate, TPP, which ionically crosslinks chitosan fibers via electrostatic forces to create a more stable chitosan microsphere (CM) (Desai and Park et al., 2005; Aral and Akbuga, 1998). Once the CM-TPPs or CM/Pluronic F127-TPPs, (to be referred to here) were produced, they were incubated with rCfrA or rCmeC. After incubation, average antigen loading efficiencies of CM-CfrA were 49%, while 55% and 77% for CM-CmeC and CM-Pluronic-F127-CmeC, respectively. Morphology of CM-TPPs with loaded rCfrA or rCmeC were not as expected. While CM-TPPs could be visualized, they appear to have crystallization on the surface (Fig. 6A) or appear desiccated (6B & C). Moreover, visualization was confounded by the visualization of larger crystal formations (Fig 6D), and was suggestive of an improper chitosan: TPP ratio. Since the first method of chitosan microsphere lead to the production of well-formed microspheres, future work will consist of testing the antigen-loading efficiency for comparison to the second method.

4.3. Development of *Salmonella*-vectored vaccines

The full-length *cfrA* and *cmeC* fragments were successfully amplified for directional cloning into the pYA3493 plasmid (Fig. 7A). However, despite extensive efforts for modification of molecular cloning conditions, no *E. coli* transformants containing the desired recombinant plasmids were obtained; all selected transformants were false positives as reflected by the equivalent size of the extracted plasmids to the parental pYA3493 vector (Fig. 7B & C). This finding suggested that expression of the

full-length *cfrA* or *cmeC* gene was lethal to the *E. coli* host. To test this hypothesis, the cloning process was repeated using bidirectional cloning. As expected, PCR analysis showed that the cloned gene (e.g. *cfrA*, Fig. 7D) in all selected transformants was in the reverse orientation within the vector. As an alternative approach, truncated *cfrA* or *cmeC* with correct orientation in pYA3493 were successfully obtained (Fig. 8B) and cloned into the vector (Fig. 8C). Immunoblotting using whole cell lysate demonstrated that the truncated CfrA and CmeC proteins were also produced in their corresponding *E.coli* (JL1060 and JL1061, respectively) or *Salmonella* hosts (JL1062 and JL1063, respectively) with approximate molecular masses of 15.7 kDa and 19.8 kDa, respectively (Fig. 9).

Production of the full-length target proteins has advantages for triggering strong and specific immune response *in vivo*. In addition, it was not clear if production of full-length CfrA or CmeC protein was toxic to *Salmonella*, the final live vaccine strain. Therefore, delicate molecular manipulation was then performed to overcome the challenge by using the original *E. coli* host strain. The pBR232 plasmid was successfully transferred into the previous *E. coli* χ6097 host, creating a new cloning host JL1080 (Table 1). In this new host, due to the presence of the *lacI*^{q} repressor system in pBR232 plasmid, the P_{trc} promoter and downstream antigens were repressed. With the repression from pBR232, the recombinant plasmids bearing full-length *cfrA* or *cmeC* genes were successfully obtained by using this new cloning system (Fig. 10). These recombinant plasmids were then extracted from *E. coli* (strains JL1109 and JL1110, respectively) and successfully transferred into the *Salmonella* live vaccine host, creating a new *Salmonella*-vectored vaccine Live-CfrA (JL1104) and Live-CmeC (JL1105) (Table

1). Immunoblotting using specific antibodies confirmed that both the full-length CfrA (76 kDa) and CmeC (53kDa) were produced in *Salmonella* (Fig. 11). However, numerous bands of lower molecular weight appeared for both CfrA and CmeC within the *Salmonella* host and were suggestive of degradation of these proteins.

4.4. Evaluation of *Salmonella*-vectored vaccines in commercial broiler chickens

S. enterica serovar Typhimurium vaccine strain recovery. *S.* Typhimurium vaccine strains were recovered and from cecum, spleen and liver samples and identified through PCR and plasmid profiling. Table 6 clearly shows the low percentage of *S.* Typhimurium vaccine strains recovered from each group at different time points. The average quantity of *S.* Typhimurium isolated from the cecum and spleen 11 days (day 18) post-immunization was 10^4 CFU/g (range: 6 x 10^2 – 2.8 x 10^4 CFU/g) and 10 CFU/g (range: 10 -1.375 x 10^2 CFU/g) sample, respectively. At day 28 (21 days post oral vaccination), the inoculated *S.* Typhimurium vaccine strains could be isolated from cecum, spleen, and liver samples; however, *Salmonella* cells were still isolated from a low percentage of euthanized chickens (Table 6). Recovery quantity also varied with 1.42 x 10^4 – 2.5 x 10^4 CFU/g of cecal contents, 3 x 10^2 – 1.1 x 10^3 CFU/g of spleen, and 10 CFU/g of liver (1 bird). No *S.* Typhimurium cells were recovered from the cecum, spleen, or liver samples at day 38 (31 d post vaccination).

Systemic and local immune responses. Oral vaccination of chickens with Live-pYA3493 (empty vector), Live-CfrA, and Live-CmeC produced elevated, but not significant, antibody responses in regards to *Salmonella* membrane proteins (Fig 12 & 13). In terms of CfrA- and CmeC-specific antibody responses, there was no significant

enhancement of systemic IgG or IgA or local mucosal IgG or IgA in intestinal lavage samples at different time points post immunization (Fig. 14-17). All groups displayed relatively higher CfrA- or CmeC-systemic IgG prior to immunization, indicating the possibility of maternal antibody presence (Sahin et al., 2001; Sahin et al., 2003).

C. jejuni challenge. For *C. jejuni* challenge, all chickens were orally gavaged with approximately 100 CFU on day 28. Cloacal swabs were used to determine *C. jejuni* colonization every 2-3 days for 10 days. Only one of two cages in both group 2 and group 4 were colonized with an average of 10^6 CFU/g feces from two days post-challenge (day 30) and persisting without change through the end of the experiment (day 38). The other cages of these groups and all of group 1 and 4 were not colonized, as no *C. jejuni* was recovered from cloacal swabbings. Although an amount of 10^2 CFU has previously been reported in successful colonization of the cecum in 2 week-old chickens (Knudson et al., 2006), colonization with this quantity did not produce consistent colonization in this study and the effect of immunization with the *S.* Typhimurium vaccines on *C. jejuni* colonization levels could not be effectively determined.

Minimum infective dose. C. jejuni was not recovered from the cloacal swabs in the chickens that received a dose of 5 CFU/chick or 50 CFU/chick at all time points. However, the chickens that were challenged with 5 x 10^2 CFU/chick or higher doses shed *C. jejuni* 2 days after challenge. The minimum infective dose of NCTC 11168 for a 14-day old chicken was 5 x 10^2 CFU/chicken.

Membrane localization. Full-length and truncated CfrA (76 KDa and 15.7 KDa, respectively) proteins were expressed within the *S.* Typhimurium membrane (Fig. 18).

Figure 19 shows full-length and truncated CmeC was also expressed (53 kDa and 19.8 KDa, respectively) and localized within the *S.* Typhimurium membrane.

CHAPTER FIVE: Conclusion and Discussion

Campylobacter can quickly infect an entire commercial poultry flock and establish itself at high quantities within the cecum of broilers until time of slaughter (Sahin et al., 2002). Horizontal transmission from numerous potential environmental sources is the major route of *Campylobacter* introduction into commercial flocks (Sahin et al., 2002). Intervention strategies at the poultry farm level to reduce *Campylobacter* colonization within poultry creates a challenge, but is necessary to reduce its presence at human consumption (Lin et al., 2009). Three general strategies to reduce *Campylobacter* presence include: 1) Biosecurity measures are reducing environmental exposure, 2) improve host resistance to *Campylobacter* colonization within the cecum (e.g., host genetic selection, vaccination), 3) introduce antimicrobial alternatives to reduce or eliminate *Campylobacter* from the host (e.g., through bacteriophage therapy) (Lin et al., 2009). It is difficult to implement biosecurity measure, such as hygiene, and understand the efficiency of such measures since risks factors, or horizontal transmission, of *Campylobacter* remains ambiguous (Sahin et al., 2002). Bacteriophages are a type of antimicrobial alternative, however, these virus-like particles entail high specificity, the potential transfer for virulence genes, and a complicated bulk production (Lin et al., 2009; Connerton et al., 2008). Bacteriocins, antimicrobial peptides, also fall into the category of antimicrobial alternatives. While some studies have shown a reduction in *Campylobacter* colonization (Stern et al., 2005; 2006; 2008; reviewed by Lin, 2009), many bacteriocins are still under development and much is still unknown about their mechanisms (Lin et al., 2009). The remaining overall strategy, improving host resistance

to *Campylobacter*, includes host genetic selection, competitive exclusion (CE), and vaccination (Lin, 2009). Competitive exclusion products, also known as probiotics, should establish protective gastrointestinal microbiota to prevent *Campylobacter* colonization (Wagner, 2006; reviewed by Lin, 2009). However, success with competitive exclusion products has been limited and acceptance by regulatory agencies could prove challenging (reviewed by Mead, 2000; Lin, 2009). While chicken lines have shown differences in susceptibility (Stern et al., 1990a), much is still unknown about the mechanisms behind *Campylobacter* establishment in chickens (Lin, 2009).

Vaccination is a promising strategy. When *C. jejuni* was incubated with anti-*Campylobacter* immunoglobulin, the dose to establish colonization was increased by 50% (Stern et al., 1990b; de Zoete, 2007). Moreover, chickens administered colonization-attenuated *Campylobacter* strains had unchanged levels of colonization upon challenge with parent strain, as compared to naïve animals (Ziprin et al., 2002; de Zoete et al., 2007). These data demonstrate a protective response of *Campylobacter*-specific antibodies elicited upon colonization and re-colonization and justify how vaccination can be effective for *Campylobacter* control. To date, there is no commercially available vaccine for *Campylobacter* control in poultry (de Zoete et al., 2007; Lin, 2009; Jagusztyn-Krynicka et al., 2009).

The short life span of the commercial broiler, slaughter age of 6 weeks, creates a challenge for induction of an immune response against *Campylobacter* and a subsequent reduction in colonization. The strongest immune response generated by a vaccine is generally at the entry site itself or within the adjacent mucosal epithelium (Lycke, 2012). Since most of the *Campylobacter* load is within the cecum of poultry, it seems feasible

that the generation of a strong immune response from the mucosal environment would be the most effective at targeting colonization. However, mucosal vaccines face several challenges such as dilution and entrapment by mucosal secretions, degradation by proteases, and exclusion by the epithelial barriers (Neutra and Kozlowski, 2006). Moreover, as in our CmeC subunit vaccination trial (Zeng et al., 2010), protein-based subunit vaccines stimulate weak mucosal immune responses when administered orally without optimized vaccination regimen (Neutra and Kozlowski, 2006). Choice of an optimal vaccine adjuvant is essential to "alert" the host's immune system (Neutra and Kozlowski, 2006). Another important requirement in chicken vaccination that needs to be addressed is the ease of mass administration and cost-effectiveness (de Zoete, 2007). Oral and *in ovo* administration routes are the most suitable and often used for this purpose (de Zoete et al., 2007). In order to address this requirement, and challenges faced by mucosal vaccines, we planned to incorporate optimized vaccination strategies using immunogenic CfrA or CmeC, by including: 1) different vaccination routes (*in ovo*, intranasal and oral routes), 2) vaccine formulations (DNA and subunit vaccines), and 3) delivery/adjuvant systems (chitosan microsphere encapsulation and USDA licensed live attenuated *Salmonella enterica* serovar Typhimurium strain).

DNA vaccines can effectively stimulate an immune response in terms of humoral and cell-mediated immunity, even challenging the influence of maternal antibodies. The DNA vaccine approach has been studied in the prevention of numerous pathogens such as hepatitis B4, rabies, papilloma and malaria (Davis et al., 1993; Xiang et al., 1994; Donnelly et al., 1994; Hoffman et al., 1994). As for DNA vaccines in chickens, when injected intramuscularly with plasmid DNA expressing hemagluttin A (HA), chickens

were protected from the lethal influenza virus (Robinson et al., 1993). A heterologous DNA vaccine encoding viral nucleoprotein (NP) also demonstrated protective immunity (Ulmer et al., 1993). Sakaguchi and colleagues injected chickens with the eukaryotic expression vector, pCAGGS expressing the F protein of Newcastle Disease virus; The chickens that received the circularized plasmid did not stimulate a significant immune response, while 2 of 5 chickens injected with the linearized plasmid generate a strong response (Sakaguchi et al., 1996). Huang and colleagues produced a DNA vaccine also using the pCAGGS plasmid, expressing *C. jejuni* protein, FlaA (Huang et al., 2010). In this study, the recombinant plasmid, pCAGGS-*fla*A was encapsulated in a chitosan adjuvant and intranasally administered into White Leghorn chickens one day of age (Huang et al., 2010). Increased *C. jejuni*-specific IgG and mucosal IgA were observed along with a 2-log reduction in colonization within the cecum (Huang et al., 2010). In our study, we also used same plasmid vector pCAGGS for producing pCAGGS_CfrA and pCAGGS_CmeC DNA vaccines. Like the study conducted by Sakaguchi and colleagues, we transfected NIH 3T3 (L1) cells and the highly transfectable HEK-293 cell line (Graham et al., 1977) with these DNA vaccines as well as a control GFP-expressing plasmid. Although the transfection was successful as shown by the transfection of the GFP-expressing plasmid, CfrA and CmeC expression could not be detected by western blot analysis. An alternative approach is needed to confirm the production of CfrA and CmeC in the transfected eukaryotic cells. For example, an immunofluorescent assay using specific antibodies in conjunction with fluorescent (e.g. fluorescein isothiocyanate, FITC) conjugated anti-IgG antibody may make CfrA- or CmeC-producing cells visualized through fluorescent microscopy or through flow cytometry. Upon validation,

efficiency of these DNA vaccines expressing CfrA and CmeC will be evaluated *in ovo* in the future.

Subunit vaccines have been widely used overall (Hansson et al., 2000). Subunit vaccination with an effective immunogenic protein and immune-stimulating adjuvant can trigger systemic and mucosal immunity, especially when administered intranasally or orally. In this study, we optimized a purification procedure to obtain high purity rCfrA and rCmeC for developing subunit vaccines. The key to formulating a subunit vaccine is to select an adjuvant that enhances the delivery of the subunit to the mucosal environment and avoid degradation in the process. The choice of adjuvant that can adhere to epithelial surfaces is essential for enhanced immune responses and can promote admission into cell transport pathways (McNeela et al., 2000; reviewed by Neutra and Kozlowski, 2006). One such adjuvant is chitosan, which was used to encapsulate rCfrA and rCmeC in two different methods in this study. Chitosan is a natural, linear polysaccharide derived from shrimp (Agnihotri et al., 2004). It is bioavailable, biocompatible, cost-effective and has adhesive properties, which could extend retention time in the mucosal environment and create a time-released effect (Artursson et al., 1994; Quan et al., 2008). Furthermore, chitosan microspheres (CM) can reduce vaccine degradation from gastrointestinal proteases and possess the potential to loosen tight junctions between epithelial cells (Thanou et al., 2001). Studies have shown that chitosan has minimal toxicity (Arai et al., 1968). No changes in mucosal cell morphology were seen with intranasal administration of chitosan solution (Illum et al., 1994). A study performed by van der Lubben et al. (2001) using CMs for mucosal vaccine delivery in mice, showed that CMs smaller than 10 μm are suitable for absorption by M cells. In this study, chitosan-protein particles

visualized via SEM were measured to be between 1 μm and 7 μm, being ideal for adsorption. Huang and colleagues' vaccine, pCAGGS-*fla*A, as described earlier, which had a 90% successful chitosan encapsulation efficiency, productively reduced colonization levels of *C. jejuni* (Huang et al., 2010).

While there are some limitations to chitosan, such as aggregation, this can be avoided, as demonstrated by Kang and colleagues (2007), by the addition of Pluronic-F127, a copolymer of polyethylene oxide and polypropylene oxide. The hydrophilic nature of the polyethylene component of Pluronic-F127 is believed to decrease the aggregation of CMs (Kang et al., 2007). Moreover, Pluronic-F127 is water-soluble and has numerous applications in pharmaceuticals (Kang et al., 2009; Wang et al., 1993). Additionally, Pluronic F127 has immune enhancing capabilities and a synergistic relationship with chitosan (Westerink et al., 2002).

In this study, we assessed two different methods of using the chitosan adjuvant with CfrA and CmeC. In the first method, we mixed chitosan or chitosan/Pluronic F127 with rCfrA and rCmeC to create chitosan- or chitosan/Pluronic F127- proteins. These microspheres were created by electrostatic interactions of chitosan, a positively charged polysaccharide (Desai and Park et al., 2005), with the negative charges on the proteins. The strength of interaction in this specific scenario remains unclear. In the future, it will be important to analyze the zeta potential through laser Doppler anemometry (LDA).

In order to evaluate a method that would be able to be mass-produced and efficiently and stably transport the antigenic proteins into the mucosal environments, we tried an alternative method of CM encapsulation with the addition of tripolyphosphate

(TPP) as described by Kang et al (2006). Kang and colleagues used this method in order to evaluate immune stimulating characteristics of vaccine delivery systems in production of a subunit vaccine for the antigen *Bordatella bronchiseptica* dermonecrotoxin (BDD) in swine (Kang et al., 2006). TPP is non-toxic polyanion that interacts with chitosan through electrostatic interactions in acidic medium (Aral and Akbuga, 1998). Specifically, TPP cross-links with chitosan fibers, creating a more stable microsphere (Desai and Park et al., 2005). Due to the potential of aggregation as mentioned before, we prepared chitosan and TPP with and without Pluronic F127, to create CM-TPPs and CM/Pluronic F127-TPPs. Upon antigen loading, at least 49% and as much as 77% of the protein, was loaded into these CMs. This is comparable to the loading efficiency observed previously (Kang et al., 2006). However, SEM visualization to assess morphology proved difficult due to the presence of crystallization. This problem may have arisen due to an improper ratio of chitosan to TPP. The chitosan used by Kang and colleagues had a molecular weight of 10 KDa; while the molecular weight of our chitosan (Sigma, low molecular weight, prod. No: 448869) was 50-190 KDa (Liang et al., 2007). Desai and Park and colleagues discovered in their study of CM-TPP encapsulation of acetaminophen, that as the chitosan concentration increases, the size of the microspheres decreases (Desai and Park et al., 2005). Additionally, chitosan molecular weight can affect antigen loading/ encapsulation capacity (Desai and Park et al., 2005). Moreover, their study demonstrated that as the volume of TPP increases, the swelling capacity of the chitosan microspheres decreases (Desai and Park et al., 2005). Therefore, in the future, we will need to optimize the ratio of chitosan to TPP to aid in the visualization, characterization, and encapsulation efficiency of these microspheres and to avoid

crystallization and aggregate production. With these promising and attractive characteristics of chitosan as an adjuvant, future work will be comprised of intranasal vaccination with these chitosan CfrA- or CmeC-loaded Pluronic-F157/CMs and analysis of both systemic and mucosal immune responses specific to these proteins as well as the effect on *C. jejuni* colonization.

The use of live attenuated mucosal vaccine vectors can generate substantial innate immune responses that bolster adaptive immune responses (Neutra and Kozlowski, 2006). The most commonly used bacterial vectors include attenuated *Salmonella* spp. to carry antigens against viral, bacterial, and parasitic pathogens (Jazayeri et al 2012; Zekarias et al., 2008; Jenikova et al., 2011). *Salmonella* is an ideal organism to invade and colonize effector lymphoid tissues and induce T cells (Kong et al., 2013). In our study, we chose live, attenuated *Salmonella enterica* serovar Typhimurium χ8914 as a vector to carry our immunogenic CfrA and CmeC proteins. Attenuation in this strain was achieved with deletion in *pabA* and *pabB* genes, which encode for 4-amino-4-deoxychorismate synthase required for production of folic acid in *Salmonella;* which the organism is unable to obtain from the environment (Wang et al., 2011). This strain carries a deletion in the *asdA* gene, creating a deficiency in diaminopimelic acid (DAP), a key component of *Salmonella* peptidoglycan. Deficiency in DAP causes lysis of the cell and since DAP is not found within mammalian tissues, the need for DAP is dire. The expression plasmid used to carry CfrA and CmeC, pYA3493, carries the *asdA* gene allowing for retention of the plasmid to be an absolute requirement for the *Salmonella* vaccine vector survival (Curtiss et al., 1989; Galan et al., 1990). The advantage to this complementation system is the absence of drug-resistant gene markers while achieving

selective pressure for retention of the plasmid within the vaccine cells *in vivo*. A study conducted by Ma and colleagues, used a recombinant *S. choleraesuis* vaccine strain carrying the pYA3493 vector expressing p36, p46, p65 and p97R1-Nrdf genes of *Mycosplasma hyopneumonaie.* These recombinant *S. choleraesuis* vaccines, when orally administered, produced significantly higher *Mycoplasma*-specific antibodies as compared to the group intramuscularly injected with the p36, p46, p65-expressing strains (Ma et al., 2011). Finally, a study using the pYA3493 vector expressing *Streptococcus Pneumonaie* protein PspA in a *S.* Typhimurium χ8501 (*Δcrp*, *ΔasdA*), produced significantly (10^4-fold) higher PspA-specific antibodies as compared to the antibody response from a recombinant strain using pYA3496 (including His_6-tagged PspA instead of the β-lactamase signal peptide) (Kang et al., 2003). This study demonstrated that the type II secretion system within pYA3493 is important for secretion of the antigenic protein into the periplasm and mounting a higher immune response as compared to other expression plasmids (Kang et al., 2003). Additionally, our chosen vector strain, *Salmonella enterica* serovar Typhimurium χ8914 is USDA licensed, allowing for a vaccine with proven systemic and mucosal immune enhancement in regards to *Campylobacter* to be approved in an expedied time frame. Zekarias and colleagues used this plasmid and *Salmonella* vector combination previously to generate *S.* Typhimurium vectored vaccines expressing the carboxy-terminal domain of the alpha toxin from *Clostridium perfringens,* PlcC (Zekarias et al., 2007). Upon immunization with these vaccines, IgG and IgA antibody titers were low; however, upon intranasal booster administration with a purified rPlcC protein, serum IgG and bile IgA titers increased. Moreover, intestinal pathology decreased upon *C. perfringens* challenge (Zekarias et al., 2007).

In this study, commercial broilers were vaccinated with the *Salmonella enterica* serovar Typhimurium χ8914 vaccine strain carrying pYA3493_CfrA (Live-CfrA) or pYA3493_CmeC (Live-CmeC) at one week of age. However, *Salmonella* vaccine recovery was limited and ELISA analysis showed systemic and local immune responses were weak and did not vary between experimental groups and the controls. Moreover, CfrA- and CmeC-specific systemic IgG and IgA levels specific to CfrA and CmeC were higher prior to vaccination, suggesting the presence of maternal antibodies. Studies have shown that the stability of the pYA3493 vector is roughly 95% over 50 generations, indicating that antigen synthesis and delivery are not compromised (Xin et al., 2012). Initial construction of our recombinant expression vector demonstrated that overexpression of CfrA and CmeC were likely toxic to the *E.coli* host and required the *lacI*^{q} repression system from within plasmid pBR232. Membrane fractionation of these vaccines showed both CfrA and CmeC are localized in membrane. Therefore, the lack of immunogenicity likely exists within the *Salmonella enterica* serovar Typhimurium χ8914 vector and the metabolic burden that it endures due to overexpression of the antigenic protein. Over-expression of the heterologous protein can lead to hyperattenuation, modified or poorly expressed antigenic proteins, and reduction in viability and colonizing ability, finally leading to poor immunogenicity overall (Galen et al., 2001). This could explain the low recovery rate of our *S.* Typhimurium vaccine strains from orally immunized chickens, indicative of poor colonization efficiency. The pYA3493 plasmid used in this study contains the P_{trc} promoter. The P_{trc} promoter is constitutively expressed in the absence of LacI within *Salmonella*, and can be subjected to LacI repression as seen in combination with the pBR232 plasmid used in this study. This repressor system was

also used with the *Salmonella* vaccine vector and pYA3493 vector. Conceivably, the reasoning behind the hyperattenuation of the *S.* Typhimurium strain in our case was due to the presence of full-length proteins, instead of a portion as was used in the Zekarias study. Additionally, perhaps booster administration with rCfrA or rCmeC would have been needed to see similar results in terms of specific-antibody response.

The attenuated *Salmonella* carrier used in this study has been further modified to relieve the metabolic burden experienced by overexpression of antigenic proteins. Dr. Roy Curtiss III and his team have created strains to incorporate the *araC* P_{BAD} arabinose-promoter, which regulates *lacI* transcription levels when the strain is grown in the presence of arabinose (Wang et al., 2010). Under the *araC* P_{BAD} promoter, P_{trc} is repressed *in vitro* in the presence of arabinose allowing for rapid growth. Once *in vivo* due to the lack of arabinose within the environment, transcription from the *araC* P_{BAD} promoter halts, and the P_{trc} is gradually repressed *in vivo* until it, and downstream antigens, are constitutively expressed (Wang et al., 2010). These recombinant strains are promising for induction of immune responses in regards to antigens that are particularly toxic to the vaccine vector strain. However, a study conducted by Kulkarni and colleagues using a *S.* Typhimurium strain including P_{BAD} and harboring the pYA3493 plasmid expressing either the alpha toxin (AT) or hypothetical protein (HP) of *C. perfringens,* showed low levels of colonization of the recombinant *S.* Typhimurium strain within peripheral tissues, marked by ~30% vaccine strain recovery from chickens (Kulkarni et al., 2010). Kulkarni and colleagues comment on previous issues with toxicity leading to the use of truncated proteins. They also suggest the lack of effective colonization led to a decreased immune response (Kulkarni et al., 2010). Additionally,

different nutritional attenuation strategies in *Salmonella* vaccine vectors have been produced and tested to enhance immunogenicity (Wang et al., 2013). Mutations within nutrition-associated Δ*aroA*, Δ*aroC,* Δ*aroD* (aromatic acids and essential vitamins) along with Δ*cya* and Δ*crp* (elimination of cyclic-AMP synthesis, the latter is arabinose regulated) and lipopolysaccharide (LPS) associated Δ*rfc* (arabinose regulated O-antigen synthesis) have demonstrated immune enhancing properties (Wang et al., 2013). Alternatively, studies using *S. enteritidis ΔaroA* have also proven successful (Layton et al., 2011). Use of a strain without USDA licensure can hinder production and availability of a commercial vaccine.

Future studies with *Salmonella enterica* serovar Typhimurium vaccine vectors expressing CfrA or CmeC may include vaccination of specific pathogen-free (SPF) chicks to eliminate the possibility of maternal antibody presence. Commercial broilers, however, are the best model for evaluation of *Campylobacter* vaccines. Future vaccination trials with commercial broilers may include vaccination at two weeks of age, typically the end of the lag phase of intestinal development and maternal antibody presence; however, on the poultry farm, this would allow more time for potential exposure and less time for generation of an optimal immune response within time of slaughter at 6 weeks. Moreover, as results from the minimum infective dose indicated, a higher dose of *C. jejuni,* specifically 5×10^2 CFU/chick may be needed. In addition, modified *Salmonella* carrier strains will be evaluated for their ability to trigger specific and strong immune response against CfrA or CmeC.

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APPENDIX

Table 1: Bacterial plasmids and strains used in this study

Plasmids or Strains	Description	Source or Reference
Plasmids		
pCAGGS	SV40 *ori*, β-actin promoter, CMV IE, ampR	Dr. Miyazaki (Faculty of Medicine, University of Tokyo, Japan)
pCAGGS-CfrA	2031 bp of full-length *cfrA* in pCAGGS vector	This study
pCAGGS-CmeC	1413 bp of full-length *cmeC* in pCAGGS vector	This study
pR-M02	Control vector (with EGFP) for pReceiver-M02	Genecopoeia
pYA3493	Ptrc, *asd*, pBR*ori, bla* SS; parent vector	Dr. Roy Curtiss III (Biodesign Institute, Arizona State University, Tempe, AZ)
pYA3493_tCfrA	429 bp encoding a 21-143 of *cfrA* protein in pYA3493 vector	This study
pYA3493_tCmeC	540 bp encoding aa 188-368 of *cmeC* protein in pYA3493 vector	This study
pYA3493_CfrA	2031 bp DNA encoding full length *cfrA* in pYA3493 vector	This study
pYA3493_CmeC	1413 bp DNA encoding full length *cmeC* in pYA3493 vector	This study
pBR232	Tc^R , $lacI^q$	Dr. Roy Curtiss III (Biodesign Institute, Arizona State University, Tempe, AZ)
Strains		
JL241	*C. jejuni* NCTC 11168, human isolate	(Gundogdu *et al*. 2007)
JL1102	*E.coli* Top10 cells carrying pCAGGS_CfrA	This study
JL1103	*E. coli* Top10 cells carrying pCAGGS_CmeC	This study
JL243	*E. coli* JM109 containing pCmeC-NHIS	Zeng et al., 2010
JL275	*E.coli* JM109 containing pCFRA-NHIS	Zeng et al., 2009
E. coli K12 χ6097	F- *araD139* Δ(*proAB-lac*) λ-Φ80d*lacZ* Δ*M15 rpsL* Δ*asdA4* Δ(*zhf-2*::Tn*10*) *thi-1*	(Zekarias *et al*., 2009;Nakayama *et al.*, 1998)
JL1080	*E. coli* K12 χ6097 containing pBR232 plasmid	This study (Zekarias *et al*., 2009)
S. enterica serovar Typhimurium χ8914	*ΔpabA1516 ΔpabB232 ΔasdA16*; vaccine vector	(Zekarias *et al*., 2009; Kang *et al.*, 2003)
JL1070	*E. coli* K12 χ6097 containing pYA3493	This study
JL1060	*E. coli* K12 χ6097 containing pYA3493_tCfrA	This study
JL1061	*E. coli* K12 χ6097 containing pYA3493_tCmeC	This study

Table 1: Bacterial plasmids and strains used in this study (continued)		
Strains	**Description**	**Source or Reference**
JL1109	*E. coli* K12 χ6097 containing pYA3493_CfrA	This study
JL1110	*E. coli* K12 χ6097 containing pYA3493_CmeC	This study
JL1062	*S. enterica* serovar Typhimurium χ8914 carrying pYA3493_tCfrA	This study
JL1063	*S. enterica* serovar Typhimurium χ8914 carrying pYA3493_tCmeC	This study
JL1059	*S. enterica* serovar Typhimurium χ8914 carrying empty pYA3493 expression vector	This study
JL1104 (Live-CfrA)	*S. enterica* serovar Typhimurium χ8914 carrying pYA3493_CfrA	This study
JL1105 (Live-CmeC)	*S. enterica* serovar Typhimurium χ8914 carrying pYA3493_CmeC	This study

Table 2: Primers used in construction of the DNA vaccines

Primer	DNA Sequence (5'-3')	Product Size	Target gene
pCAGGS_F	GAGCCTCTGCTAACCATGTTC	N/A	The sequence upstream and downstream of multiple cloning site
pCAGGS_R	TTTTGGCAGAGGGAAAAAGA		
pCAGGS_CfrA_F	CCGCTCGAGATGAAAAAAATATGTCTATCAGTTTGC	2091 bp	Full-length *cfrA*
pCAGGS_CfrA_R	CCGCTCGAGTTAAAAGTTACCATTGATAGAAATATACATTC		
pCAGGS_CmeC_F	CCGCTCGAGATGAATAAAATAATTTCAATTAGTGCTATAGC	1479 bp	Full-length *cmeC*
pCAGGS_CmeC_R	CCGCTCGAGCTATTCTCTAAAAGACATATCTAAATTTTTTGA		

Table 3: Primers used in construction of full-length CfrA and CmeC *Salmonella* vectored vaccines

Primer	DNA Sequence (5'-3')	Product Size	Target gene
pYA3493_F pYA3493_R	CAATTAATCCGGCTCGT CGCTTCTGCGTTCTGATTTA	N/A	The sequence upstream and downstream of multiple cloning site
pYA3493_CfrA_F (*Eco*RI) pYA3493_CfrA_R (*Sal*I)	CCG**GAATTC**CAAAATGTAGAACTAGA TAGCTCAATCG AC**GCGTCG**ACTTAAAAGTTACCATTG ATAGAAA TATACATTC	2025 bp	Full-length *cfrA* without signal peptide
pYA3493_CmeC_F (*Eco*RI) pYA3493_CmeC_R (*Sal*I)	CCG**GAATTC**CCAAATTTAAATATTCC CGAAGC AC**GCGTCG**ACCTATTCTCTAAAAGAC ATATCTA AATTTTTTGA	1419 bp	Full-length c*meC* without signal peptide
pYA3493_CfrA_F (*Eco*RI) pYA3493_CfrA_R (*Eco*RI)	CCG**GAATTC**CAAAATGTAGAACTAGA TAGCTCAATCG CCG**GAATTC**TTAAAAGTTACCATTGA TAGAAATATACATTC	2025 bp	Full-length *cfrA* without signal peptide
pYA3493_CmeC_F (*Eco*RI) pYA3493_CmeC_R (*Eco*RI)	CCG**GAATTC**CCAAATTTAAATATTCC CGAAGC CCG**GAATTC**CTATTCTCTAAAAGACA TATCTAAATTTTTTGA	1419 bp	Full-length *cmeC* without signal peptide
pYA3493_CfrA_F (*Eco*RI) pYA3493_CfrA_R (*Pst*I)	CCG**GAATTC**CAAAATGTAGAACTAGA TAGCTCAATCG AAA**CTGCAG**TTATAAAGATGTCTCTT CTTTTAAATCAGGA	2025 bp	Full-length *cfrA* without signal peptidc
pYA3493_CmeC_F (*Eco*RI) pYA3493_CmeC_R (*Pst*I)	CCG**GAATTC**CCAAATTTAAATATTCC CGAAGC AAA**CTGCAG**TTACTTGGCTAAATTTA CATTTTGGTAAA	1419 bp	Full-length *cmeC* without signal peptide

Table 4: Primers used in construction of truncated CfrA and CmeC *Salmonella* vectored vaccines

Primer	DNA Sequence (5'-3')	Product Size	Target product
pYA3493_CfrA_F	CCGGAATTCCAAAATGTAGAACTAGATAGCTCAATCG	429 bp	*cfrA* after signal peptide to first beta loop
CfrA_B1_R	AAACTGCAGTTACCATTTATCACTTACTTTTTTGGTAATG		
pYA3493_CfrA_F	CCGGAATTCCAAAATGTAGAACTAGATAGCTCAATCG	870 bp	*cfrA* plug domain and loops 4-6
CfrA_B7_R	AAA**CTGCAG**TTAATTTTCATAAACACCCTCATGACTT		
CfrA_B3_F	CCG**GAATTC**TTGATGAATGACAAATTGGGT	870 bp	Middle region of *cfrA*
CfrA_B14_R	AAA**CTGCAG**TTATAAAGATGTCTCTTCTTTTAAATCAGGA		
CfrA_B14_F	CCG**GAATTC**GAGACATCTTTAAACTATGAAATAGCAGC	657 bp	Amplify the C-terminal region of *cfrA*
CfrA_C_R	AAA**CTGCAG**TTAAAAGTTACCATTGATAGAAATATACATTC		
pYA3493_CmeC_F	CCGGAATTCGCTTATGAAAATGAAAATGCTCTT	516 bp	Through the 2nd transmembrane region of *cmeC*
CmeC_TM2_R	AAACTGCAGTTACTTGGCTAAATTTACATTTTGGTAAA		
CmeC_TM2_F	CGG**GAATTC**GCTTATGAAAATGAAAATGCTCTT	540 bp	2nd transmembrane portion through 3rd transmembrane of *cmeC*
CmeC_TM3_R	AAA**CTGCAG**TTACTTGGCTAAATTTACATTTTGGTAAA		
CmeC_TM3_F	CCG**GAATTC**AATTTAGCCAAGCTTAATAAAGATGAA	390 bp	C-terminal region of *cmeC*
CmeC_C_R	AAA**CTGCAG**CTATTCTCTAAAAGACATACTAAATTTTTTGA		

Table 5: Evaluation of the live *Salmonella*-vectored vaccines in chickens.

Group	Number of Chickens	Immunization at 7d	Sample collection
1	20	200 μL PBS	**Blood**: 7d, 18d, 28d, 38d; **Intestinal lavage**: 18d, 28d, 38d; **Spleen**: 18d, 28d, 38d; **Liver**: 18d, 28d, 38d
2	20	200 μL vector control (χ8914/pYA3493, JL1059)	
3	20	200 μL Live_CfrA (JL1104)	
4	20	200 μL Live_CmeC (JL1105)	

Table 6: Recovery of *S.* Typhimurium vaccine strains from euthanized chickens at different time points.

Sampling site/ Group	Number of positive chickens (5/group/time point)		
	18 d	28 d	38 d
Cecum			
Group 2	2	0	0
Group 3	1	2	0
Group 4	0	0	0
Spleen			
Group 2	1	0	0
Group 3	2	1	0
Group 4	0	1	0
Liver			
Group 2	0	0	0
Group 3	0	1	0
Group 4	0	0	0

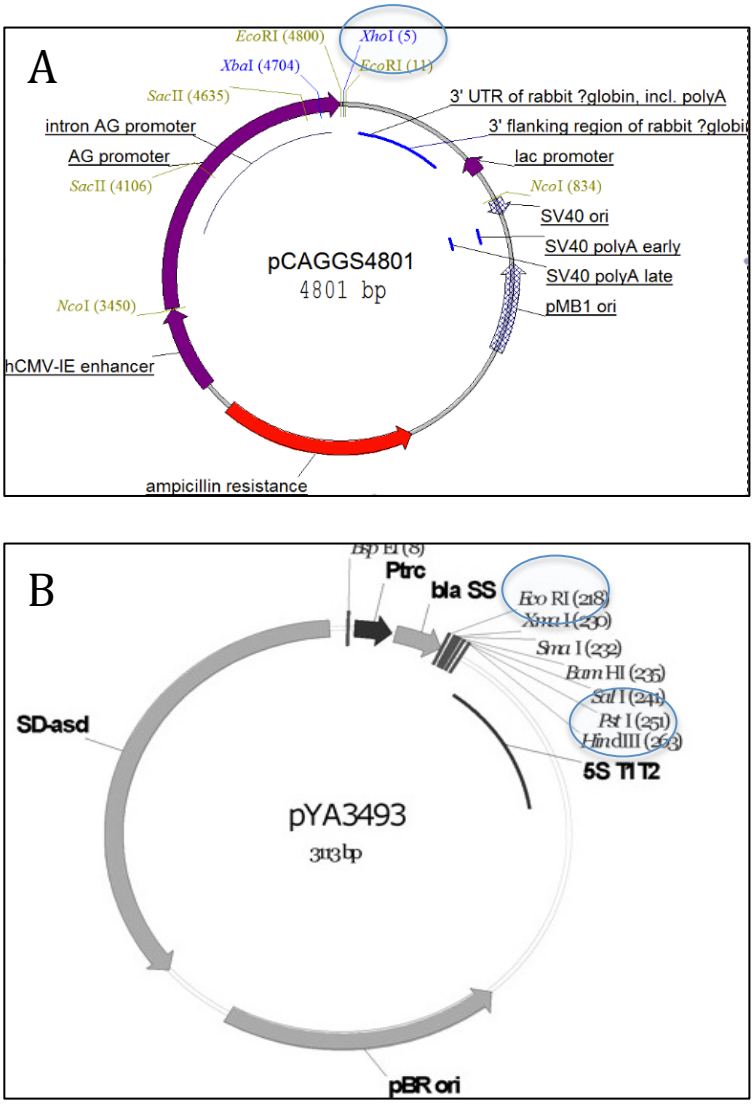

Figure 1-Plasmid vectors used in this study. (A) *DNA vaccine parental vector.* Eukaryotic expression vector, pCAGGS contains chicken β-actin promoter, SV40 origin of replication, the CMV-immediate early enhancer (CMV-IE) and the ampicillin resistance cassette. (B) *Salmonella-vectored vaccine parental vector.* Expression vector pYA3493 harbors the P_{trc} promoter upstream of the beta-lactamase signal peptide and also contains the *asdA* gene for selection. Restriction sites used in the final cloning procedures are encircled.

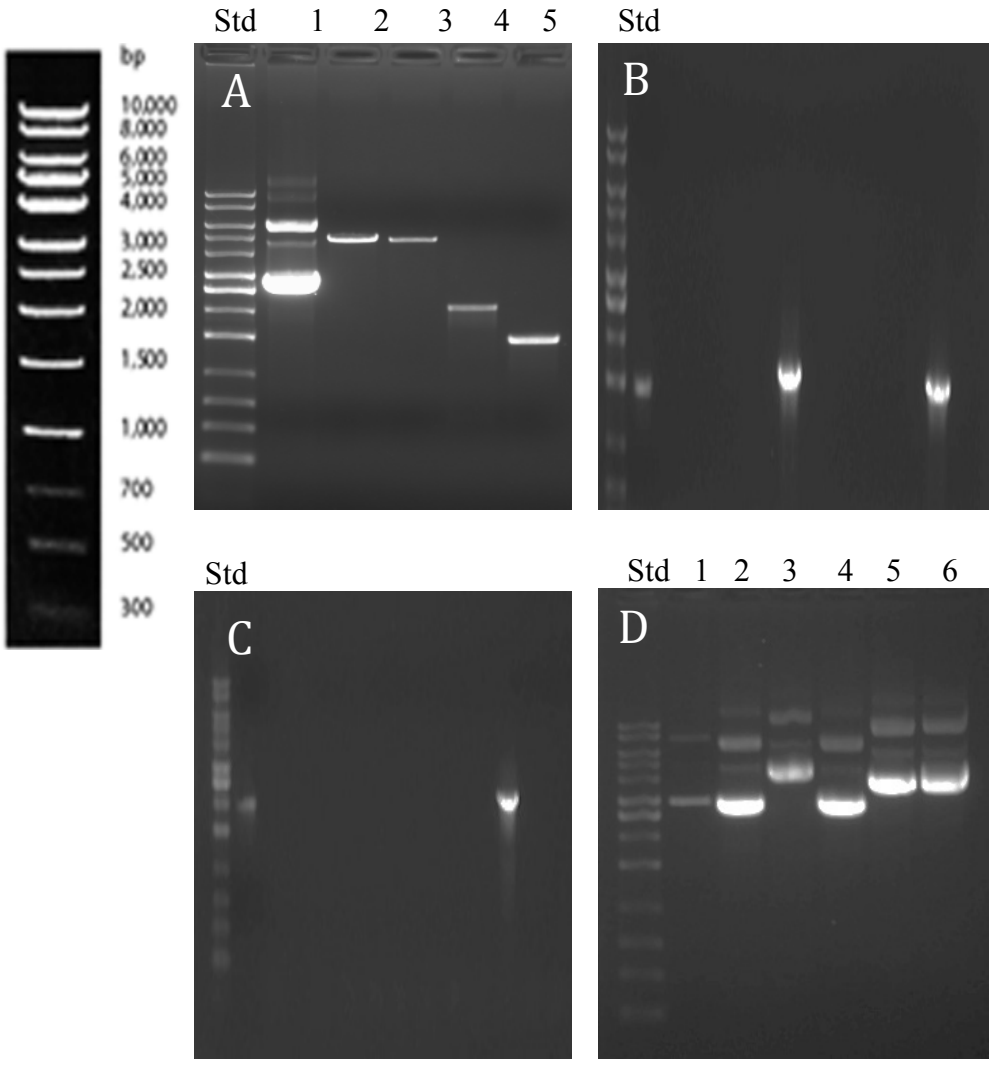

Figure 2- Construction of DNA Vaccines. (A). *Molecular Cloning.* Std is a 1kb molecular marker (Phenix, as pictured on the left), lane 1 is the parental, undigested pCAGGS, lane 2 is *Xho*I digested pCAGGS, lane 3 is *Xho*I digested and dephosphorylated pCAGGS, lane 4 is *Xho*I digested CfrA PCR product, lane 5 is *Xho*I digested CmeC PCR product. (B) *Screening for transformants expressing CmeC.* PCR screening for identification of the transformants bearing pCAGGS_CmeC with correct orientation. Presence of the positive PCR band (~1500 bp) indicates the desired construct with correct orientation of inserted gene. (C). *Screening for transformants expressing CfrA.* PCR screening for identification of the transformants bearing pCAGGS_CfrA with correct orientation of inserted *cfrA* gene. (D). *Profile of extracted plasmids.* Lane 1: pCAGGS; Lane 2: plasmid from a false positive transformant for pCAGGS_CfrA (negative PCR in panel C), Lane 3: pCAGGS_CfrA (positive PCR in panel C); Lane 4: plasmid from a false positive transformant for pCAGGS-CmeC (negative PCR in panel B); and Lanes 6 & 7: pCAGGS_CmeC (positive PCR in panel B).

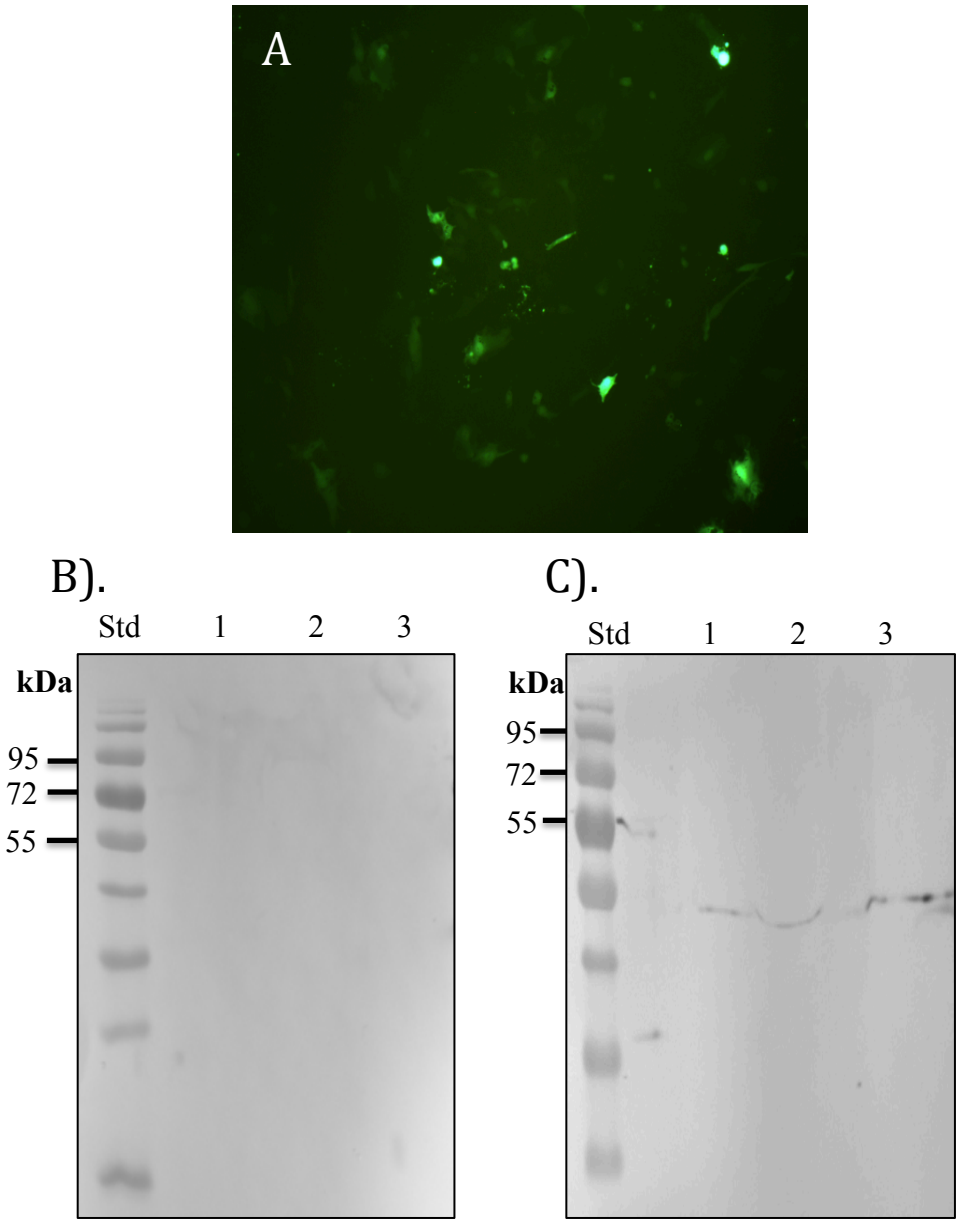

Figure 3. Expression of CfrA and CmeC from the corresponding DNA vaccine in transfected eukaryotic cells. (A). *Fluorescent microscopy of control transfected cells.* NIH 3T3-L1 cells transfected with eGFP expressing plasmid. (B). *Immunoblot analysis of CfrA expression by pCAGGS_CfrA in HEK-293 cells.* Lane 1:HEK-293 cells (negative control); Lane 2: the HEK-293 cells transfected with pCAGGS (negative control); Lane 3: the HEK-293 cells transfected with pCAGGS_CfrA. (C). *Immunoblot analysis of CmeC expression by pCAGGS_CmeC.* Lane 1:HEK-293 cells; Lane 2: the HEK-293 cells transfected with pCAGGS; Lane 3: the pCAGGS_CmeC transfected HEK-293 cells.

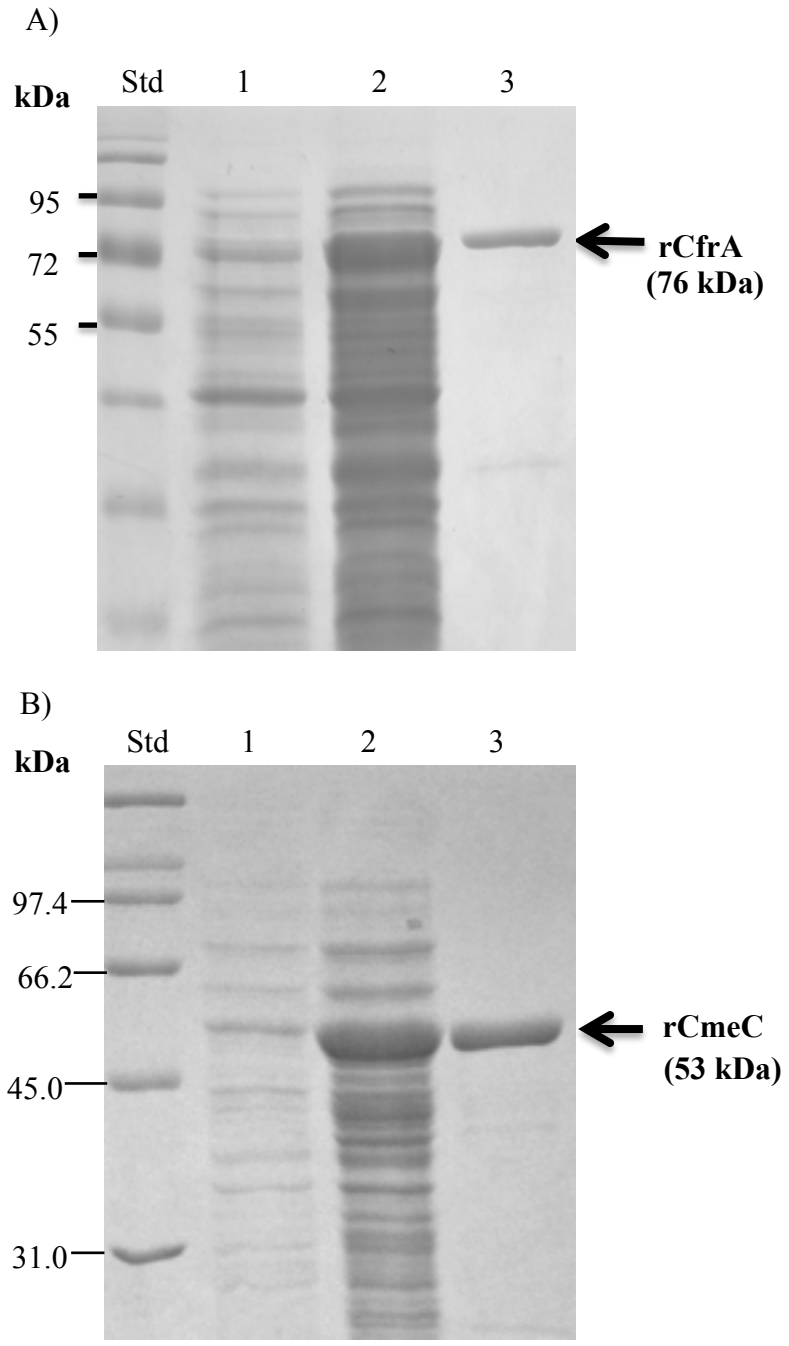

Figure 4- SDS-PAGE analysis of purified recombinant proteins (A**).** *Production of rCfrA*. Lane 1: whole cell lysate of non-induced *E. coli* (JL275); Lane 2: whole cell lysate of IPTG-induced *E. coli*; Lane 3: rCfrA purified by Ni-NTA affinity chromatography. (B). *Production of rCmeC.* Lane 1: whole cell lysate of noninduced *E. coli* (JL243); Lane 2, whole cell lysate of induced *E. coli*; Lane 3: rCmeC purified by Ni-NTA affinity chromatography.

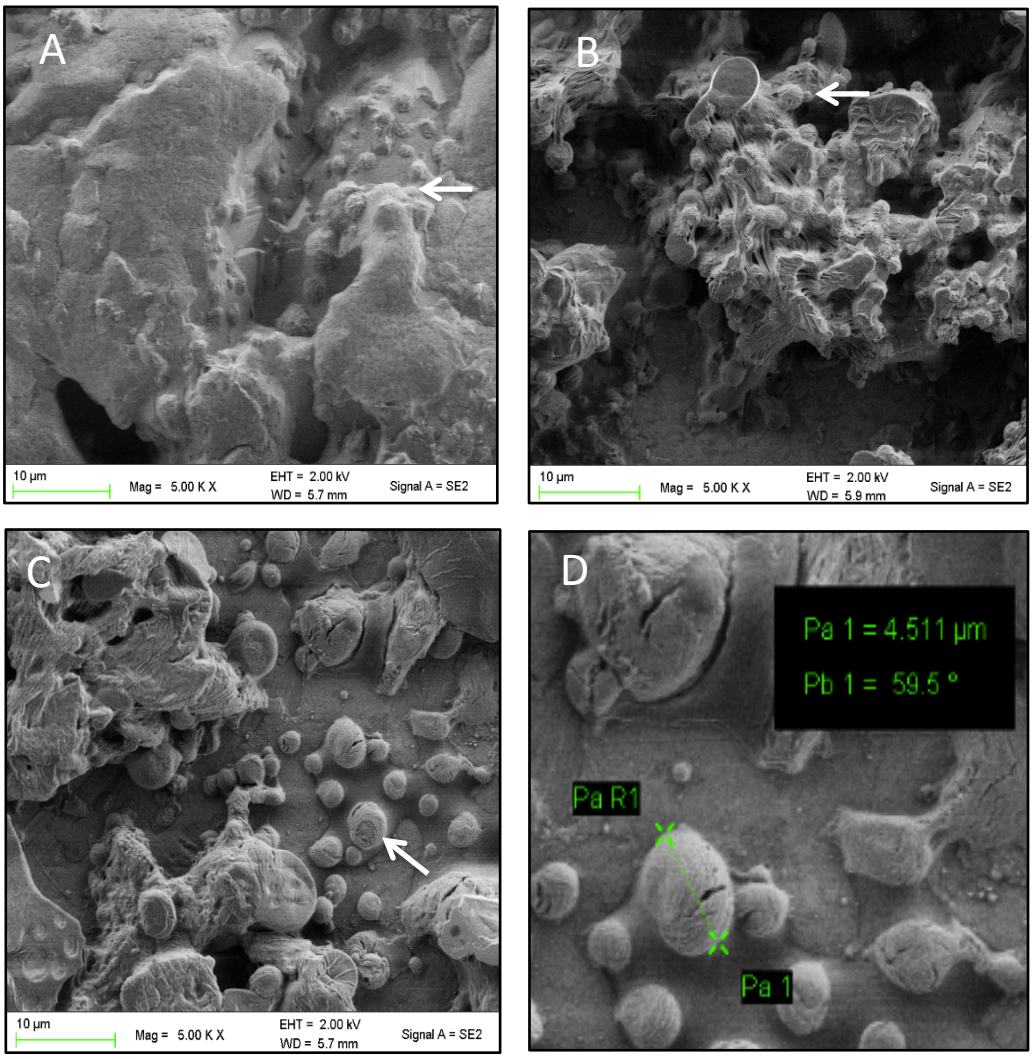

Figure 5- SEM of Chitosan/Pluronic-F127-protein. (A). Chitosan/Pluronic-F127-PBS control. (B).1:1 Chitosan/Pluronic-F127-CfrA (C). 3:1 Chitosan/Pluronic-F127-CfrA. (D). measurement of Chitosan-CfrA microsphere. Magnification at 5,000X (panels A-C) or 10,000X (panel D).

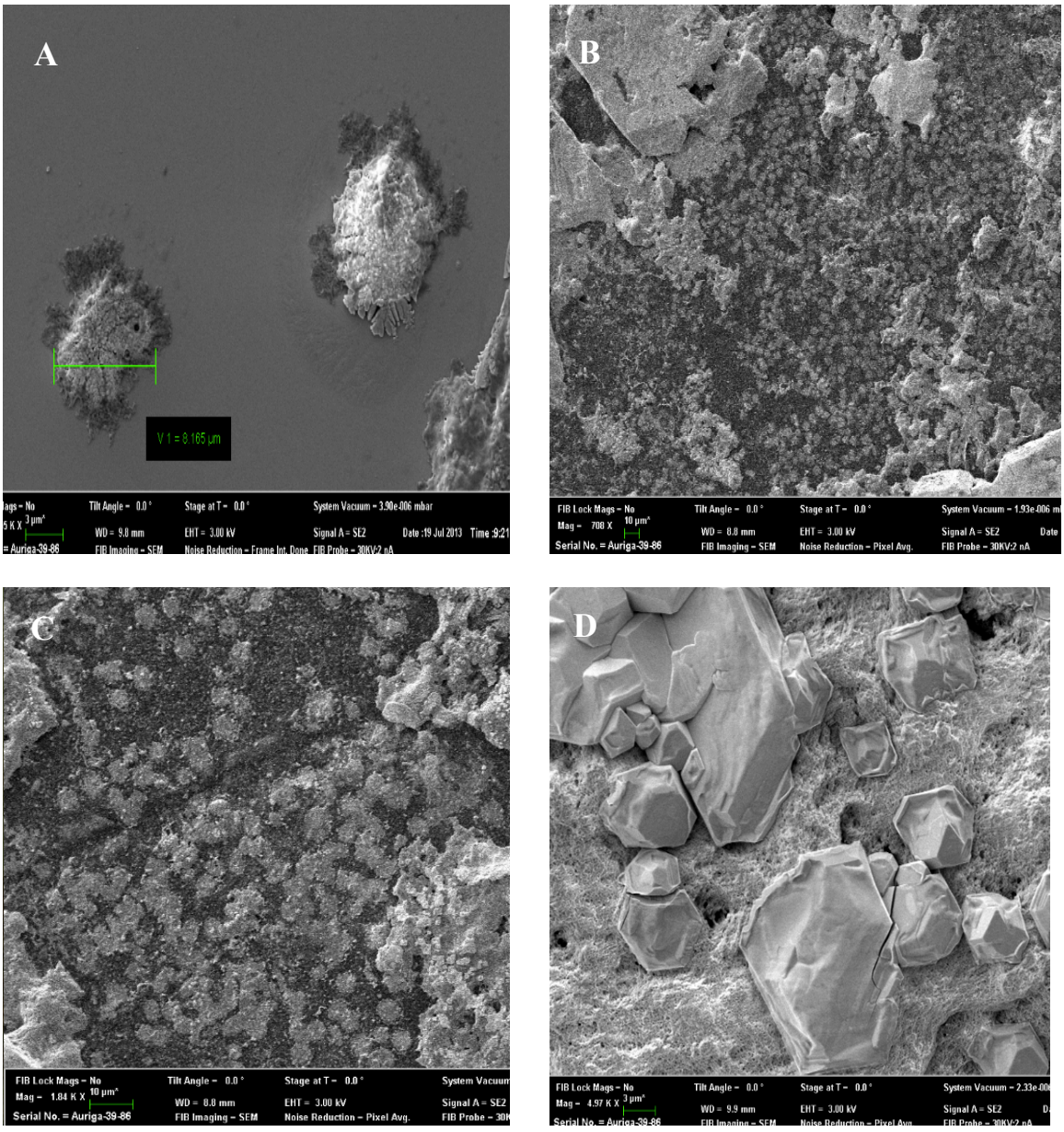

Figure 6- SEM of chitosan or chitosan/ Pluronic F127 with tripolyphosphate (TPP). **(**A) CM-TPP showing crystallization of the surface, Magnification at 5,000X. (B),(C) The CM/Pluronic F127-TPP were shown with magnification at 700X (Panel B) and 2,000X (Panel C). (D) Surface of CM/Pluronic F127-TPP showing crystallization, Magnification at 5,000X

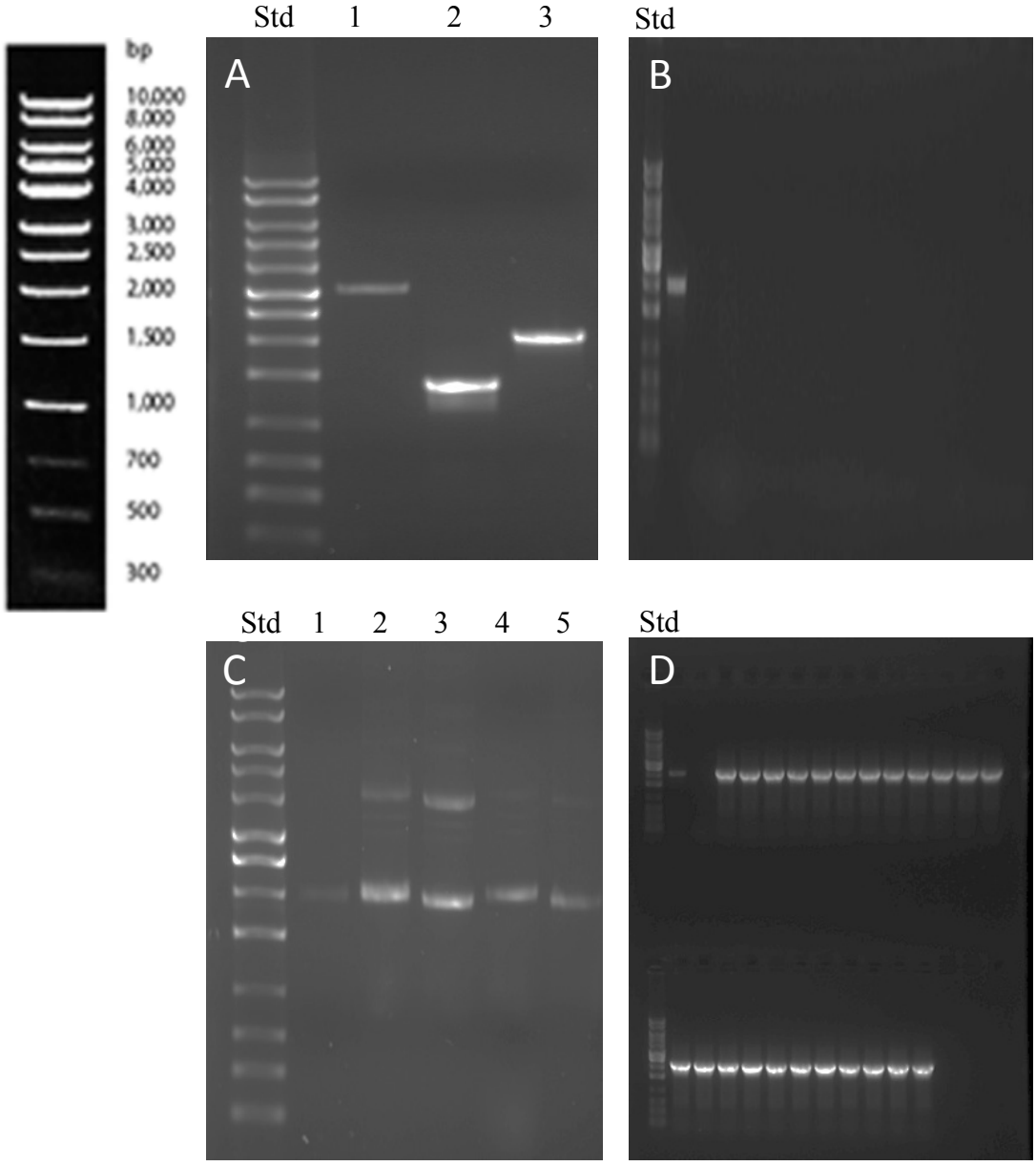

Figure 7- Construction of *S.* Typhimurium vaccines expressing full-length CfrA or CmeC. (A) *Molecular cloning.* Lane 1: *Eco*RI/*Sal*I digested pYA3493; Lane 2: digested *cmeC*; Lane 3: digested *cfrA*. (B) *Screening for transformants.* Lane 1: original amplified *cfrA* PCR product control; remaining lanes are negative for *cfrA* insert. (C) *Profile of extracted plasmids.* Lane 1: parental pYA3493; Lanes 2-5: screened plasmids showing same size (negative). (D) PCR screening of birdirectional cloning. The representative figure here shows *cfrA* was inserted only in the reverse orientation in all selected tranformants. When using specific PCR primers for desired forward orientation, all were negative (figure not shown).

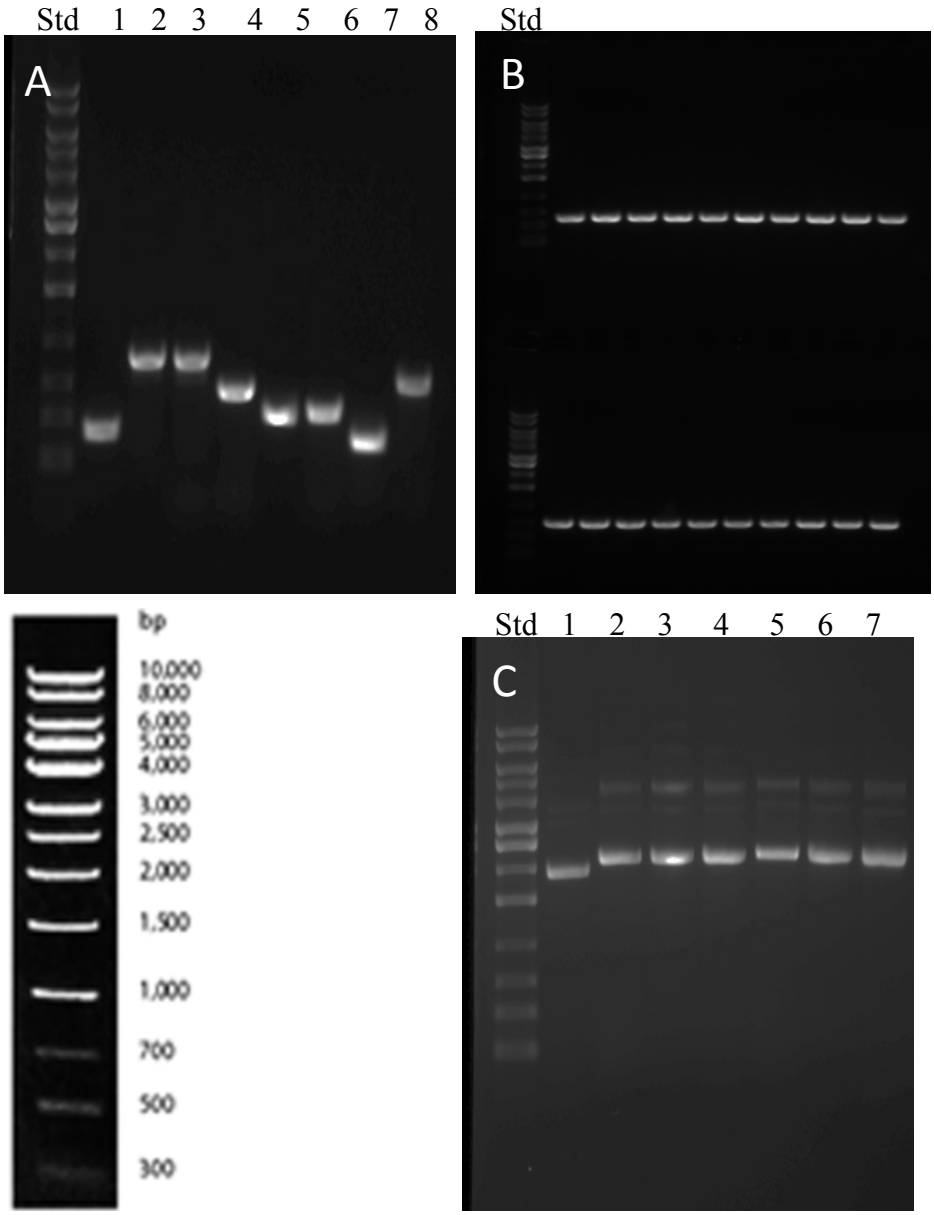

Figure 8- Construction of *S.* Typhimurium vaccines expressing truncated proteins. **(**A) *PCR amplification*. Truncated CfrA and truncated CmeC products. (B) *PCR screening of transformants*. All randomly selected colonies contain insert. (C) *Profile of extracted plasmids.* The parental plasmid pYA3493 (Lane 1) with the plasmid bearing truncated *cfrA* (Lane 2-4) and truncated *cmeC* (Lanes 5-8).

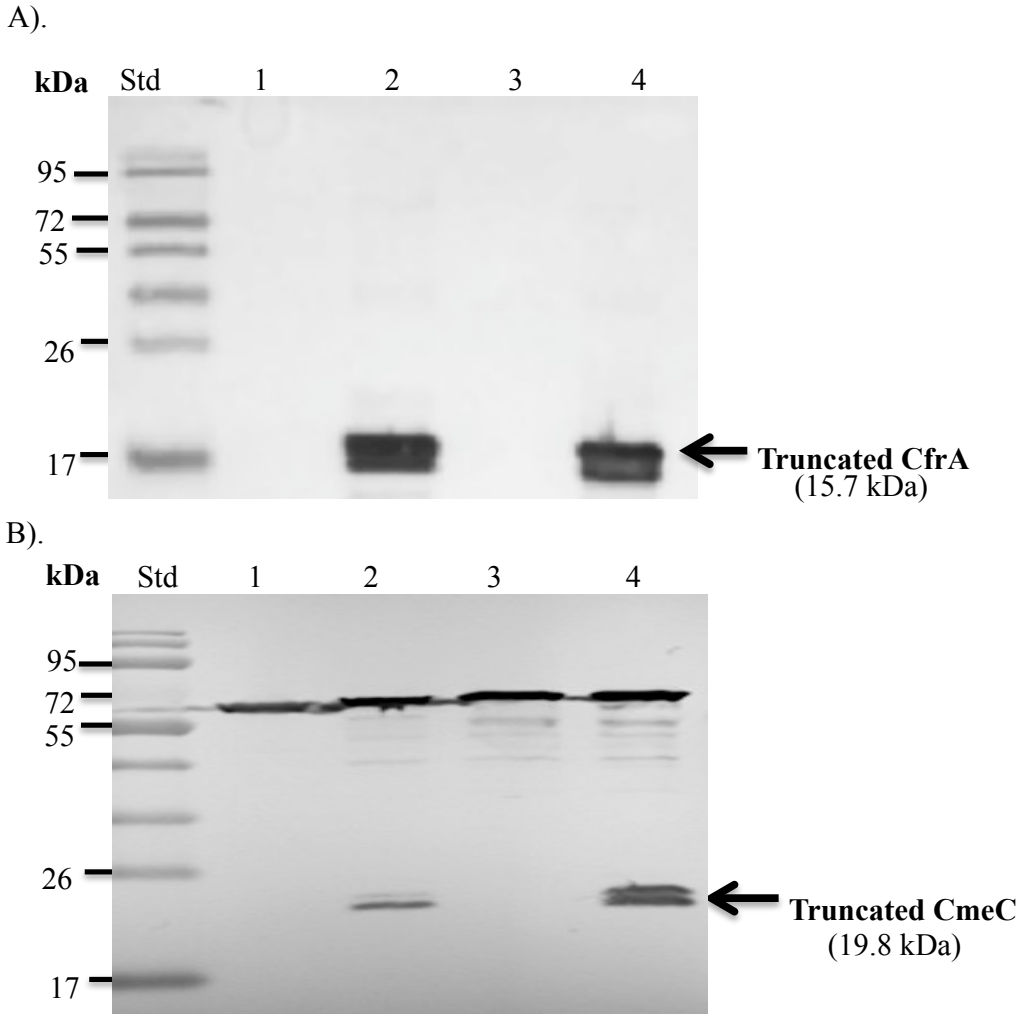

Figure 9 -Expression of truncated CfrA and CmeC in the *E. coli* and *S.* Typhimurium constructs. (A) *Immunoblot analysis of truncated CfrA.* Lanes 1 & 3: empty vector pYA3493 in *E. coli* and *S. enterica* serovar Typhimurium, respectively; Lanes 2 & 4: pYA3493_tCfrA within *E. coli* and *S.* Typhimurium (JL1062), respectively. (B) *Immunoblot analysis of truncated CmeC*. Lanes 1 & 3: empty vector in *E.coli* and *S.* Typhimurium, respectively; Lanes 2 & 4: pYA3493_tCmeC within *E.coli* and *S.* Typhimurium (JL1063), respectively.

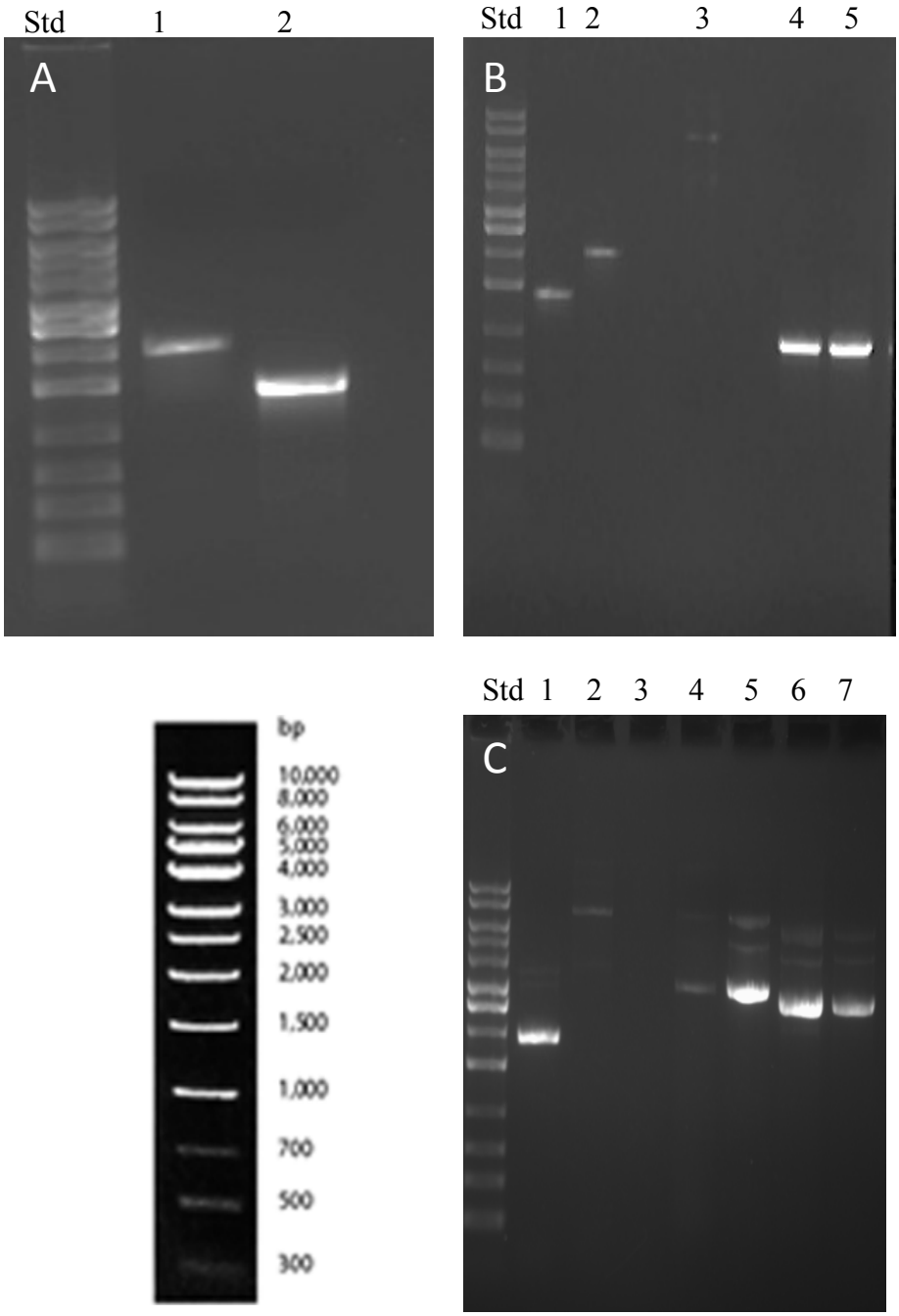

Figure 10- Construction of *S.* Typhimurium vaccines expressing full-length CfrA or CmeC with optimized molecular cloning strategy. (A) *Amplified PCR products with EcoRI and PstI restriction sites.* Lane 1: amplified and treated *cfrA*; Lane 2: amplified and treated *cmeC* (B) *Molecular cloning,* Lane 1:digested *cmeC*; Lane 2: *cfrA;* Lane 3: pBR232; Lanes 4 & 5: pYA3493. (C) *Profile of extracted plasmids.* Lane 1: parental pYA3493; Lane 2:pBR232; Lanes 4 & 5: plasmids extracted from transformed pYA3493_CfrA colonies; Lanes 6 & 7: plasmids extracted from transformed pYA3493_CmeC colonies; Lane 3: no sample loaded.

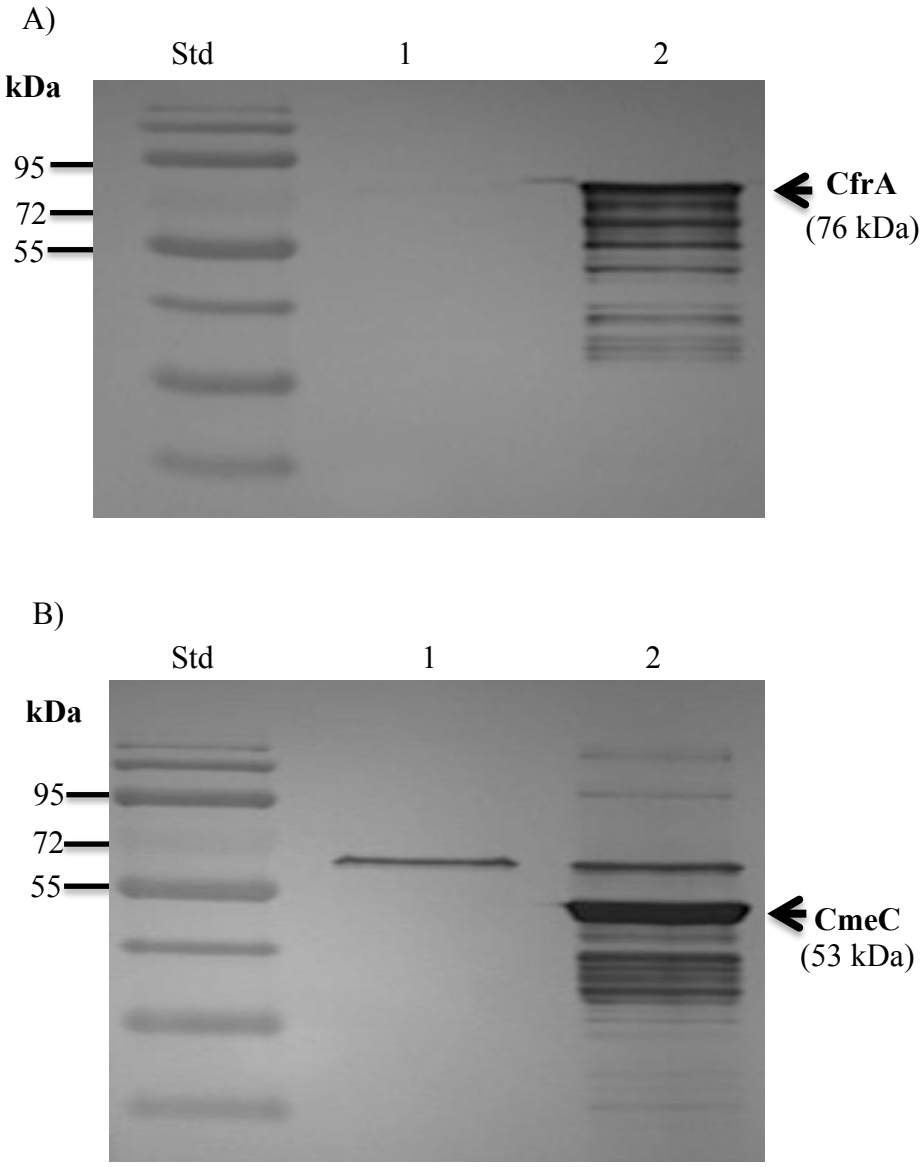

Figure 11-Expression of full-length CfrA and CmeC in the *S.* Typhimurium vaccine **(**A) *Immunoblot analysis for full-length CfrA production*. Lane 1: the host starin containing empty vector pYA3493; Lane 2: the host strain containing pYA3493_CfrA (JL1104). (B) *Immunoblot analysis for full-length CmeC production.* Lane 1: the host strain containing empty vector pYA3493; Lane 2: the host strain containing pYA3493_CmeC (JL1105).

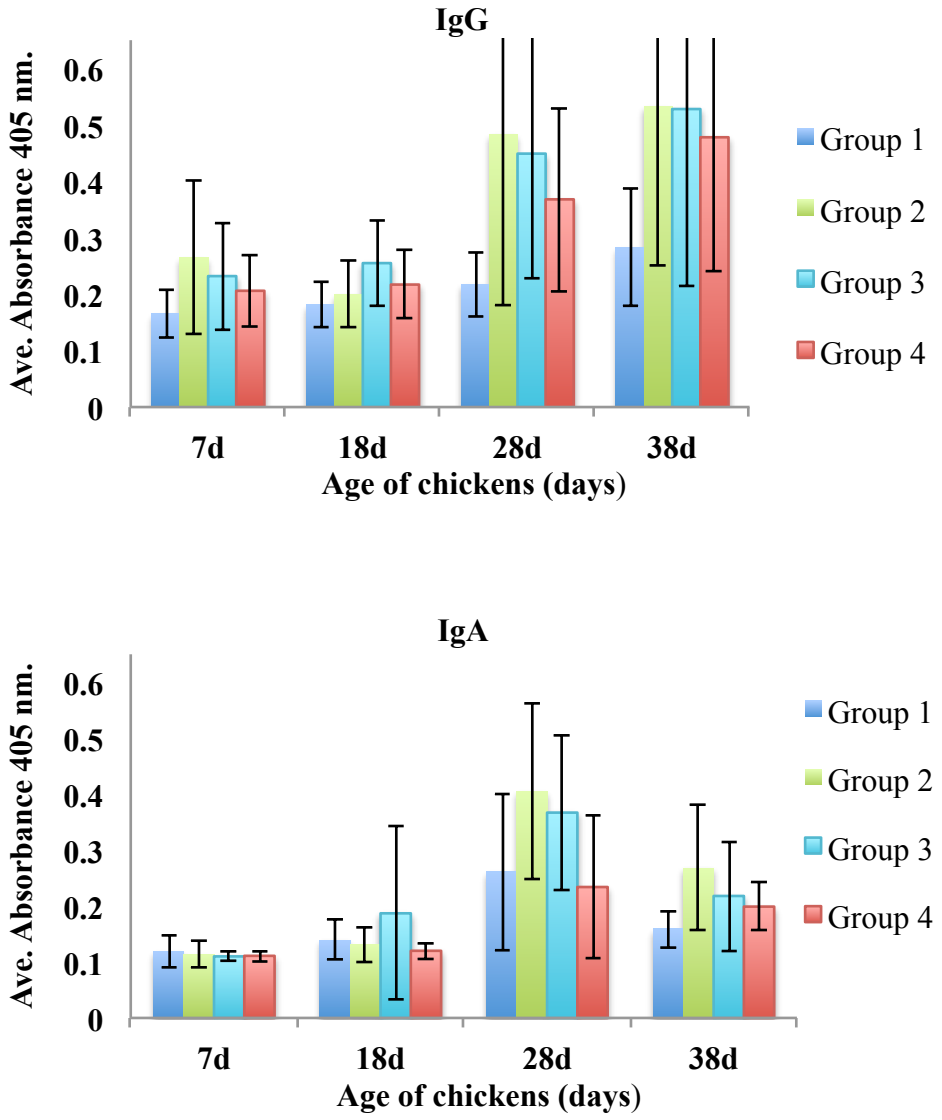

Figure 12- Systemic Immune Responses to *S.* Typhimurium. Indirect ELISA analysis of systemic antibody level to the *S. enterica* serovar Typhimurium strain membrane. Serum was collected prior to oral vaccination (7 d), 18 d, 28 d, and 38 d. Group 1:PBS control; Group 2: the empty vector control; Group 3:Live-CfrA; Group 4: Live-CmeC. Error bars represent standard deviation.

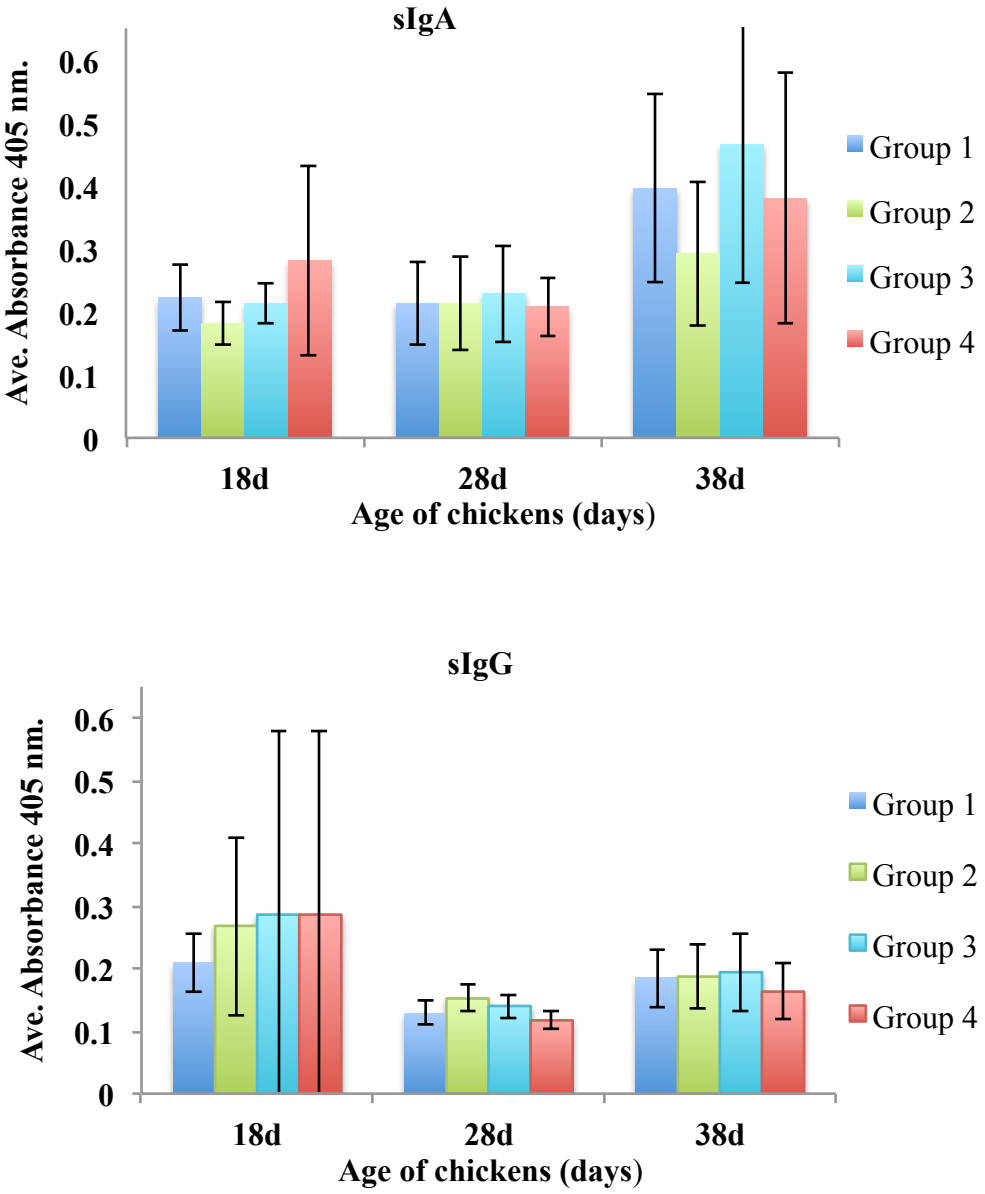

Figure 13. Mucosal immune response to *S.* Typhimurium. Indirect ELISA analysis of mucosal antibody level to the *S. enterica* serovar Typhimurium strain membrane. Intestinal lavage was collected at 18 d, 28 d, and 38 d. Group 1: PBS; Group 2: the empty vector control; Group 3:Live-CfrA; Group 4: Live-CmeC. Error bars represent standard deviation.

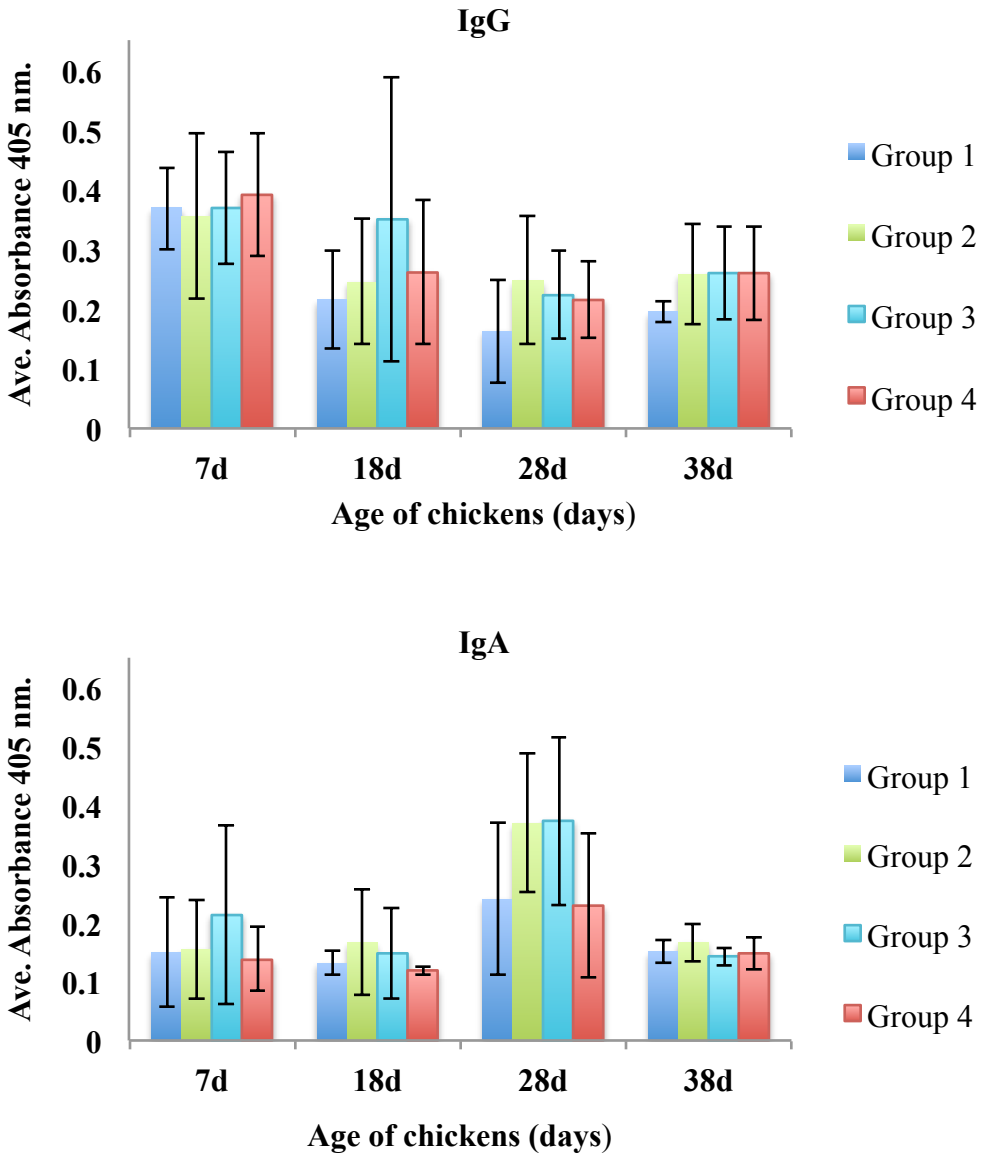

Figure 14- Systemic Immune response specific to CfrA. Indirect ELISA analysis of systemic antibody level to the CfrA. Serum was collected prior to vaccination (7 d), 18 d, 28 d, and 38 d. Group 1: PBS; Group 2: pYA3493, the empty vector control; Group 3:Live-CfrA; Group 4: Live-CmeC. Error bars represent standard deviation.

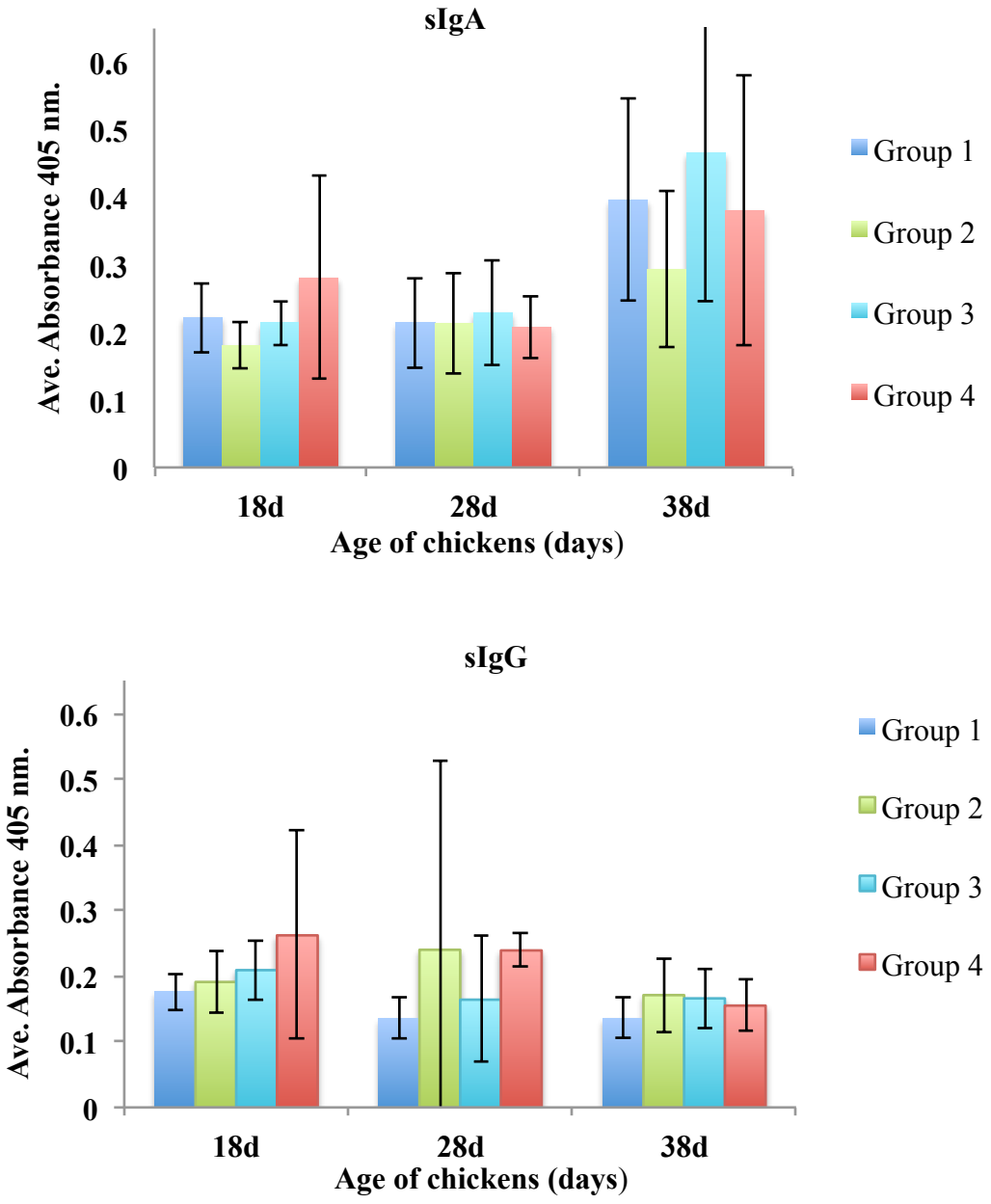

Figure 15- Mucosal immune response specific to CfrA. Indirect ELISA analysis of mucosal antibody level to the CfrA. Intestinal lavage was collected at 18 d, 28 d, and 38 d. Group 1: PBS; Group 2: the empty vector control; Group 3:Live-CfrA; Group 4: Live-CmeC. Error bars represent standard deviation.

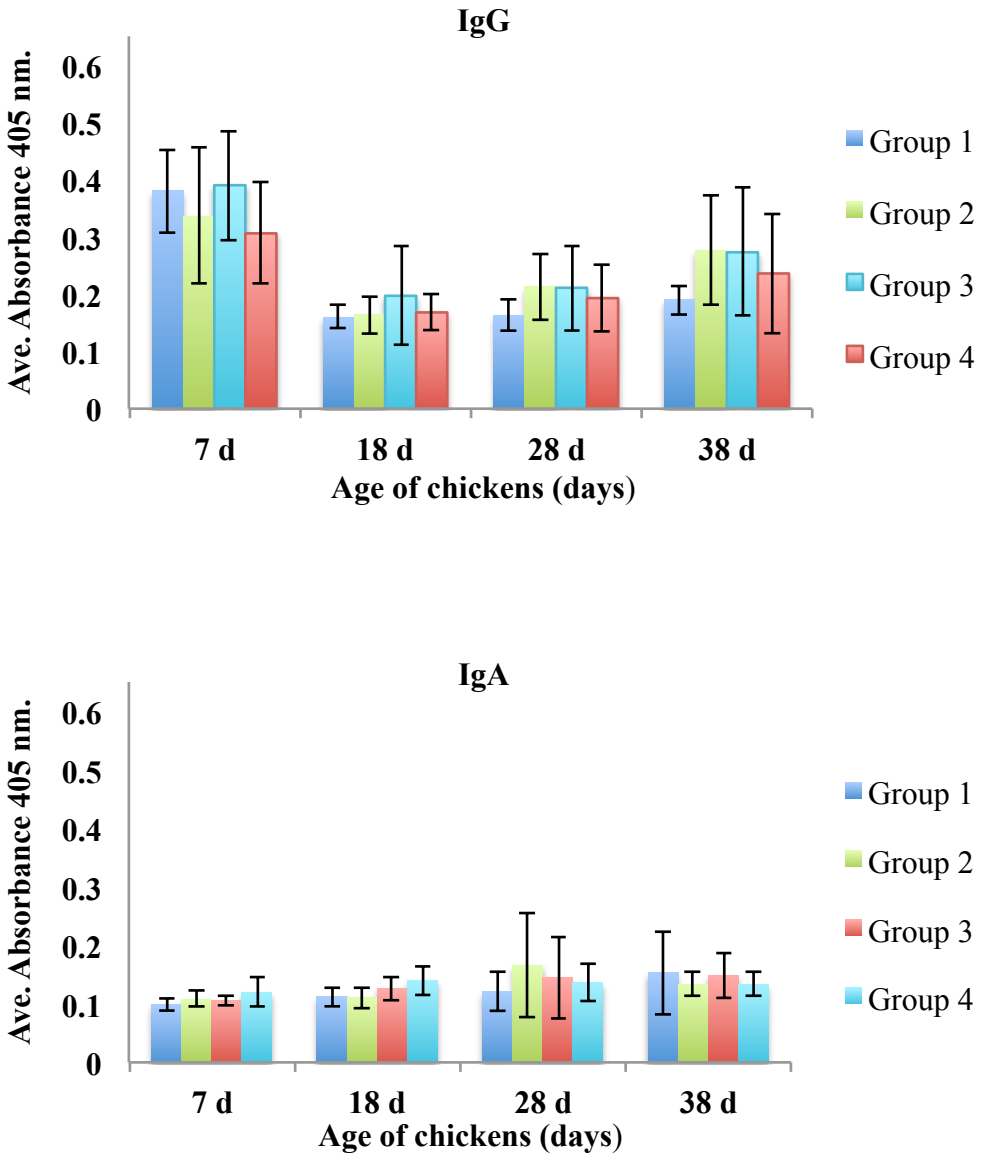

Figure 16- Systemic immune response specific to CmeC. Indirect ELISA analysis of systemic antibodies to the CmeC. Serum was collected prior to vaccination (7 d), 18 d, 28 d, and 38 d. Group 1: PBS; Group 2: the empty vector control; Group 3:Live-CfrA; Group 4: Live-CmeC. Error bars represent standard deviation.

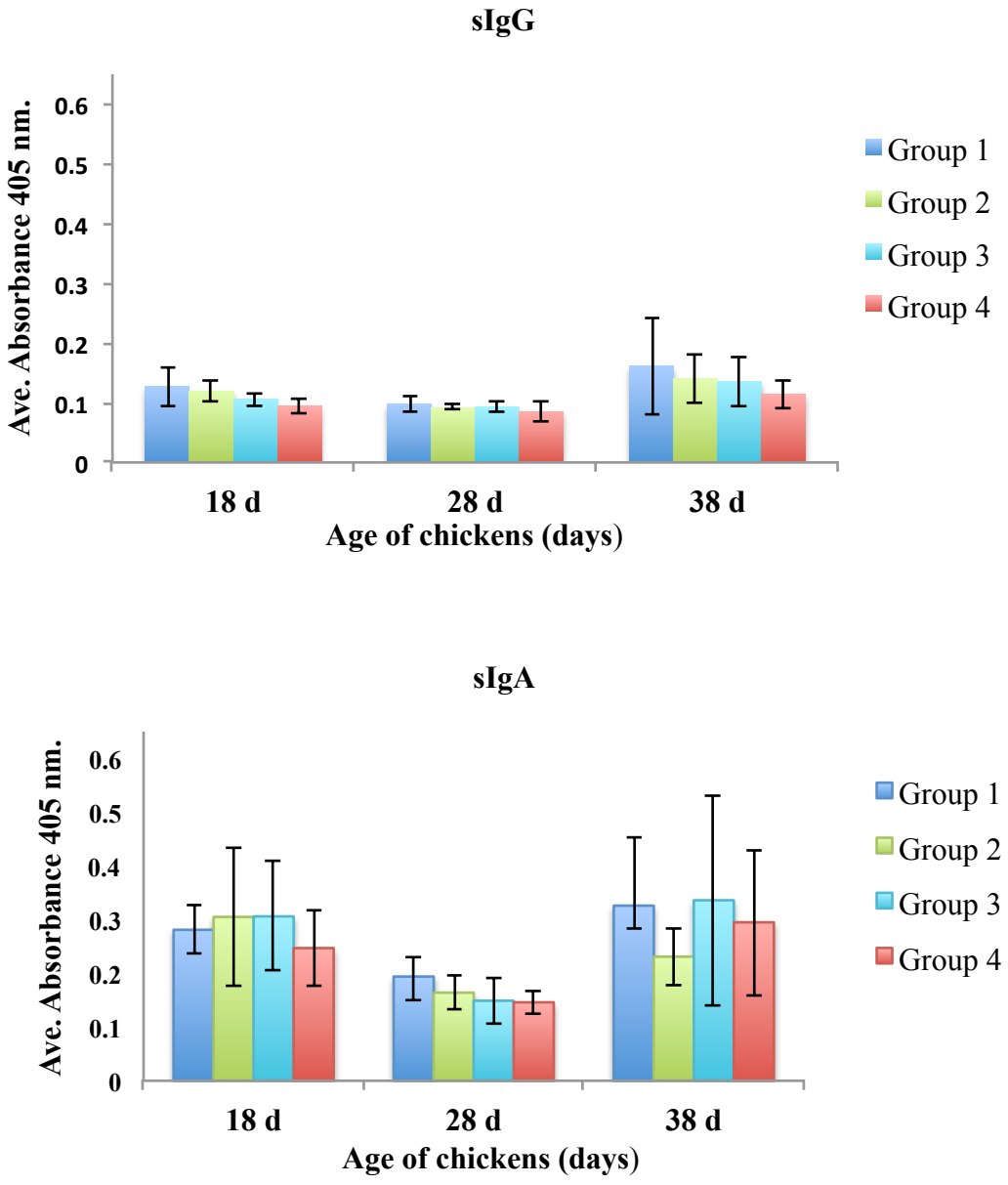

Figure 17- Mucosal immune response Specific to CmeC. Indirect ELISA analysis of mucosal antibody levelto the CfrA. Intestinal lavage was collected at 18 d, 28 d, and 38 d. Group 1: PBS; Group 2: the empty vector control; Group 3:Live-CfrA; Group 4: Live-CmeC. Error bars represent standard deviation.

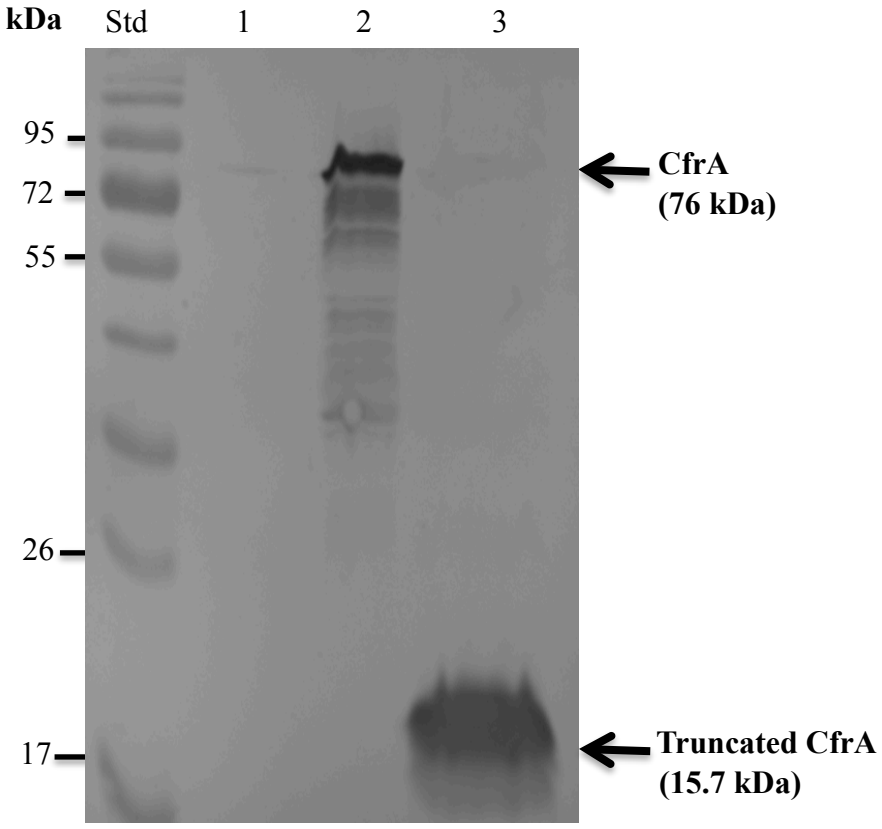

Figure 18- Immunoblotting demonstrating membrane localization of CfrA in *Salmonella*-vectored vaccines. Membrane fractions were extracted and subjected to immunoblotting using CfrA-specific antibodies. Lane 1: the host strain containing pYA3493 (negative control); Lane 2: the host strain containing pYA3493_CfrA (Live-CfrA, full-length CfrA); Lane 3: the host strain containing pYA3493_tCfrA (truncated CfrA).

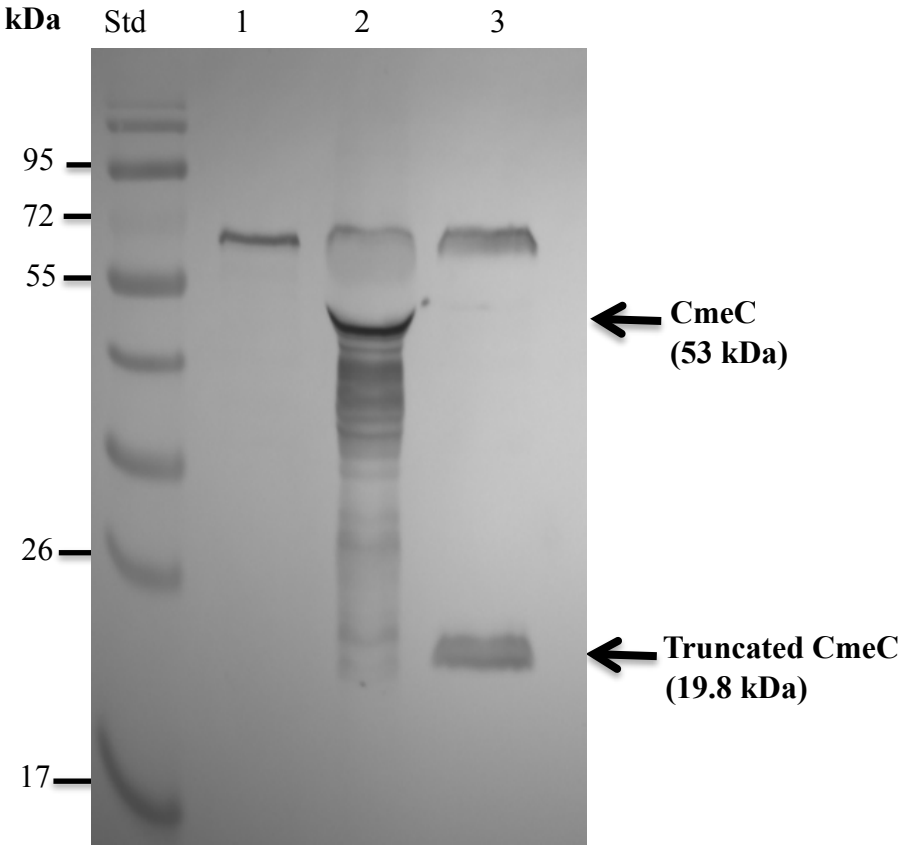

Figure 19- Immunoblotting demonstrating membrane localization of CmeC in *Salmonella*-vectored vaccines. *S.* Typhimurium carrying pYA3493_CmeC (Live-CmeC) or pYA3493_tCmeC. Lane 1: the host strain containing pYA3493 (negative control); Lane 2: the host strain containing pYA3493_CmeC (Live-CmeC, full-length CmeC); Lane 3: the host strain containing pYA3493_tCmeC (truncated CmeC).

VITA

Lindsay Ann Jones was born on April 24, 1982 and grew up in Clinton, CT until graduating from The Morgan School in 2000. In 2004, she received a B.S. in Pathobiology from The University of Connecticut in Storrs, CT. In 2005, she began employment as a Microbiology Medical Technologist in the Department of Pathology at the Massachusetts General Hospital in Boston, MA and in 2006, started attending classes at Harvard Extension School in Cambridge, MA. In 2009, she began HIV research under Dr. Eric S. Rosenberg in the Department of Medicine at the Massachusetts General Hospital. She married Dr. Brian J. Adams in July of 2011. In August of that year, she enrolled in the Masters Program at the University of Tennessee in Knoxville, TN.