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Development and Evaluation of Novel Vaccination Strategies for Campylobacter Control in Poultry

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I am submitting herewith a thesis written by Xiang Liu entitled "Development and Evaluation of Novel Vaccination Strategies 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.

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We have read this thesis and recommend its acceptance:

Michael O. Smith, Irene Hanning

Accepted for the Council:

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

**Development and Evaluation of Novel Vaccination Strategies for *Campylobacter*
Control in Poultry**

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Xiang Liu
August 2015

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ABSTRACT

Chicken is the primary natural host of *Campylobacter*, the leading bacterial cause of human enteritis in the US and other developed countries. Thus, mitigation of *Campylobacter* in chicken using innovative approaches, such as vaccination, will have a significant impact on food safety and public health. Our previous studies have demonstrated that the two outer membrane proteins, CmeC (the essential component of the CmeABC multi-drug efflux pump) and CfrA (a ferric enterobactin receptor), are feasible candidates for immune intervention against *Campylobacter*. DNA vaccine has appeared to offer various advantages for poultry, particularly when combined with *in ovo* vaccination. Chitosan-encapsulated subunit vaccines have also been demonstrated to induce both systemic and mucosal immune response via intranasal vaccination. To further develop effective vaccines to mitigate *Campylobacter* in poultry, two vaccination strategies that may have potential for mass vaccination on poultry farms were developed and evaluated in this project. To develop effective DNA vaccines for *in ovo* vaccination, *cmeC* or *cfrA* genes were cloned into eukaryotic expression vector pCAGGS with introduction of Kozak sequence to further enhance the production level of inserted genes in eukaryotic cells. Large quantities of DNA vaccines were prepared and used for two independent *in ovo* vaccination trials to evaluate the immune response and protective efficacy of the validated DNA vaccines. However, *in ovo* injection of the DNA vaccines at 18th day of embryonation, regardless using neutral lipid-protected vectors or not, failed to trigger significant immune response in broilers. To develop chitosan encapsulated subunit vaccines for intranasal vaccination, the conditions for preparation of

nanoparticles using chitosan were optimized. In addition to the pCAGGS-CmeC and pCAGGS-CfrA DNA vaccines, large quantities of recombinant CmeC and CfrA proteins were purified and used for preparing chitosan encapsulated subunit vaccines. A chicken experiment (6 treatment groups with 20 chickens per group) was performed to evaluate immune response and protective efficacy of intranasal immunization with four chitosan encapsulated subunit vaccines. Nevertheless, the intranasal subunit vaccines failed to induce immune response and protection against *Campylobacter* in chickens.

Key Words: *Campylobacter*, poultry, DNA vaccine, CmeC, CfrA, *in ovo* vaccination, intranasal, chitosan

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CHAPTER 1: Review of Literature

1.1. General features of *Campylobacter*

Campylobacter, a genus of Gram-negative microaerophilic bacteria, was first observed in the colons of infants who died of ‘cholera infantum’ in 1886 (Samie et al., 2007) and then successfully isolated from human feces in 1972 (Dekeyser et al., 1972). Ever since the discovery, *Campylobacter* has been recognized as an important human enteric pathogen worldwide. According to the Foodborne Illness Risk Ranking Model (FIRRM) generated by the Emerging Pathogens Institute, *Campylobacter* remains the most common foodborne pathogen causing the greatest burden to the public health in the United States (Batz et al., 2011; Batz et al., 2014). Among a large and diverse group of species, *C. jejuni* causes most of the cases, while *C. coli* causes 1–25% of the *Campylobacter*-related diseases (Kaakoush et al., 2015; Man, 2011; Sahin et al., 2015). The susceptible populations of *Campylobacter* were reported to be concentrated in the United States and European countries, likely due to infrequent exposure to this pathogen (Friedman, 2000). The seasonality of campylobacteriosis has also been observed with a peak during the summer months, which might be related to the increase of flies and other vectors (Gözl et al., 2014; Nichols, 2005).

The majority of human infections are caused by the consumption of undercooked, contaminated animal products, such as meat and milk, especially chicken meat (Gözl et al., 2014). Nonetheless, people can also be infected by consumption of contaminated water, contact of animals and other environmental sources (Gözl et al., 2014). The typical

clinical symptom of campylobacteriosis is an acute diarrhea, often accompanied with abdominal cramping, headache and fever (Blaser, 1997). Considered a self-limiting disease, the latent period of campylobacteriosis is usually 2–5 days while the disease can last for up to 2 weeks (Young et al., 2007). Nevertheless, the symptom may still last for several weeks and medical intervention is required for 10% of reported cases (Lee and Newell, 2006). *C. jejuni* infection is also associated with Guillain-Barre' syndrome (GBS), a life-threatening autoimmune disease which leads to peripheral nervous damage with a mortality of 2-7% and bad prognosis (Mawla et al., 2014).

In 2013, FoodNet identified 6,621 cases of infection, 1,010 hospitalizations, and 12 deaths caused by *C. jejuni* (Crim et al., 2014). However, it has been widely considered that *C. jejuni* leads to 400-500 million cases annually (Ruiz-Palacios, 2007). About 11% of the population acquires the infection each year in the US (Ruiz-Palacios, 2007). Moreover, *C. jejuni* has been estimated to cause 200 deaths per year in the United States (Ruiz-Palacios, 2007). The annual cost correlated to this disease is estimated to be between \$1.2 to 4 billion per year in the U.S (Batz et al., 2014; Frenzen, 2008).

One study showed that a mere 400 colony forming units (CFU) of *C. jejuni* can result in human illness (Black et al., 1988). Serum IgA, IgG, and IgM antibodies can be detected in people who are infected by *Campylobacter* in one week (Herbrink et al., 1988), while intestinal secretory IgA (sIgA) can be detected 2-3 weeks post infection (Lane et al., 1987). In a study in 1988, the volunteers who have been infected with *C.jejuni* were re-challenged with *Campylobacter* but the typical campylobacteriosis symptoms failed to appear in these volunteers (Black et al., 1988), indicating that humans can be protected against *Campylobacter* through immunity.

1.2. Epidemiology of *Campylobacter* in poultry

The *Campylobacter*-poultry combination is the leading pathogen-food combination responsible for the food-related human illness, according to FIRRM (Batz et al., 2011). Molecular epidemiologic studies have suggested that chicken is the primary source of human campylobacteriosis worldwide (Agunos et al., 2014; Sheppard et al., 2009; Wilson et al., 2008). Meanwhile, poultry is one of the world's most important animal protein resources, due to the higher feeding efficiency and faster growth rate compared to pork and beef (Smil, 2002). It is expected that chicken will become the largest meat product worldwide in 2020s, as a result of changing food preferences, increasing household income and population growth (OECD/FAO, 2012).

Campylobacter is considered a commensal organism within the intestinal tracts of poultry (Sahin et al., 2002). Once the first bird in a flock becomes colonized, infection spreads to the entire flock in just a few days (Gözl et al., 2014; Katsma et al., 2005). This rapid spread of *Campylobacter* throughout the flock is likely a result of fecal-oral transmission, compounded by communal water and feed (Lee and Newell, 2006). Based on the visualization of the confocal scanning laser microscopy, *C. jejuni* can also survive in feather follicles and the pores on chicken skin at a depth of 20–30 μm upon contacting its poultry host, which provides *C. jejuni* with a microenvironment with little exposure to oxygen, appropriate humidity, and temperature to survive stress conditions (Chantarapanont et al., 2003). Colonization of *C. jejuni* can persist for the lifetime of the broilers, consequently leading to carcass contamination at the slaughter facility. Although *Campylobacter* can be isolated from most intestinal sites of broiler chickens, it is mainly found in the cecal and cloacal crypts where it does not adhere to epithelial cells but is

found in the mucous layer (Lee and Newell, 2006). Broiler chicken can carry *C. jejuni* as high as 10^6 to 10^{10} CFU per gram of feces (Lee and Newell, 2006; Rice et al., 1997). The detection of *C. jejuni* in tissues other than the intestinal tract, such as the spleen, lung, heart and liver, suggests that this pathogen can translocate intestinal epithelial cells and become systemic (Knudsen et al., 2006).

Epidemiological simulation has shown that the reduction of *C. jejuni* contamination in poultry carcasses by two log units can result in a reduction in the incidence of human infection by 30-fold (Rosenquist et al., 2003). This further indicates that on-farm control of *C. jejuni* would make significant impact on the reduction of campylobacteriosis in human.

1.3. Chicken host immune response to *Campylobacter* infections

For decades, *Campylobacter* has been considered to be commensal to chicken, as no clear phenotypic difference between infected birds and normal birds can be observed (Shane, 2000; Van de Giessen et al., 1992). Nevertheless, recent research has shown that there was a prolonged inflammatory response as well as gut mucosa damage and diarrhea in some breeds of chicken (Humphrey et al., 2014). Awad et al. (2014) also showed that *C. jejuni* infection can affect the structure of chicken intestinal epithelium. These may infer that *C. jejuni* cannot be considered as a commensal bacterium; *C. jejuni* may cause infection in chickens.

Many studies have reported that *C. jejuni* infection could lead to both mucosal and systemic immune response in chickens (Cawthraw et al., 1994; de Zoete et al., 2007;

Widders et al., 1998). Cawthraw and colleagues (1994) have shown that *C. jejuni*-specific serum IgG, IgM and IgA rose 2 weeks after infection with a peak at 5-7 weeks after infection, and then declined. Intestinal mucosal IgA against *C. jejuni* also rose 3-4 weeks after challenge (Cawthraw et al., 1994). Furthermore, the elevated levels of *C. jejuni* - specific antibodies are associated with the reduction of *C. jejuni* colonization level, suggesting an important role of the humoral immunity in controlling *C. jejuni* infection in chicken (Lin, 2009). *C. jejuni*-specific maternal antibodies can also be vertically transferred from layers to the offspring (Sahin et al., 2001), which contributes to the delay of *Campylobacter* infection in young chickens during the first 2 weeks after hatching (Sahin et al., 2003). Together, these findings indicate that *C. jejuni*-specific antibodies may reduce *Campylobacter* colonization in birds, providing us a strong rationale to utilize immune intervention to reduce *C. jejuni* load in poultry as investigated in this project. *C. jejuni* has been demonstrated to be recognized by Toll-like receptor 4 (TLR4) as well as TLR21 in chickens, which then leads to the innate immune responses in the intestinal tract that cause an influx of inflammatory cells, such as heterophils, a functional equivalent of neutrophil (de Zoete et al., 2010; Meade et al., 2009; Smith et al., 2008).

1.4. Immune intervention to reduce *Campylobacter* load in poultry

Multiple approaches have been developed to control *Campylobacter* on the broiler farm level. Those approaches include 1) reduction of environmental exposure, e.g. biosecurity measures; 2) antimicrobial alternatives, such as bacteriophage therapy and bacteriocin treatment; and 3) increase of host resistance, such as host genetics selection

(Lin, 2009). However, many of these proposed intervention strategies still have significant disadvantages and are still not practically available (Lin, 2009). An early study by Stern et al (1990) showed the *C. jejuni* dose needed for chicken colonization increased by 50% if chickens were pre-incubated with *Campylobacter*-specific immunoglobulins. This suggests that immune intervention, either vaccination or passive immunity, can be an effective strategy to reduce *Campylobacter* colonization in chickens. This hypothesis was also supported by evidence described in the above section.

An effective chicken *Campylobacter* vaccine should meet the following standards:

1) the vaccine should prevent colonization or reduce bacteria numbers for more than 2 log units in chicken; 2) the vaccine must induce a quick immune response as chicken usually contact with *Campylobacter* a couple of days post-hatch; 3) immunity should be cross-protective against different *Campylobacter* isolates; 4) the vaccine should be cost-effective and easy to deliver; and 5) the vaccines should be safe for both chicken and human (de Zoete et al., 2007). Various vaccines have been developed and evaluated for protection against *Campylobacter* in avians, including live attenuated vaccines, killed whole-cell vaccines (WCV), live attenuated *Salmonella*-vectored vaccines, and subunit vaccines.

Killed WCV have been the most widely studied for various pathogens and have been demonstrated to be effective in controlling intestinal pathogens (Pace et al., 1998). In chickens, several WCV against *Campylobacter* have also been tested. In one study, birds were administered with 10^9 formalin-killed *C. jejuni* through oral route multiple times within 16 days before challenge. The cecal *C. jejuni* level in the vaccine groups were ~ 1.5 log units lower than the control (Rice et al., 1997). However, in another

formalin-killed *C. jejuni* trial, no serum or mucosal immunoglobulins could be detected and the mean cecal colonization level was only 1 log unit lower than the control (Cawthraw et al., 1998).

Outer membrane proteins (OMPs) are attractive antigens for immune intervention in Gram-negative bacteria (Lin et al., 2002a) and various recombinant subunit vaccines as well as live attenuated *Salmonella*-vectored vaccines expressing *Campylobacter* OMPs were exploited. These vaccines include the flagellum vaccines (Meeusen et al., 2007; Widders et al., 1996; Widders et al., 1998), a CmeC subunit vaccine (Zeng et al., 2010), a CadF-FlaA-FlpA fusion vaccine (Neal-McKinney et al., 2014), an attenuated *Salmonella*-vectored CjaA vaccine (Wyszyńska et al., 2004), an attenuated *Salmonella*-vectored Peb1 vaccine (Sizemore et al., 2006), and a live *Salmonella* vaccines expressing Cj0113 (Omp18/CjaD), Cj0982c (CjaA), and Cj0420 (ACE393) (Layton et al., 2011).

As the major component of the flagellum, Fla is one of the best-studied antigenic proteins in *Campylobacter*. In two flagellin vaccine trials, birds were vaccinated with flagellin alone or in combination with heat-killed bacteria intraperitoneally (IP), followed by an IP or oral booster. The vaccine with the flagellin/whole-cell combination administered IP/IP provided higher serum and mucosal antibody levels and resulted in a 10-100 folds reduction of cecal CFU level after challenge (Widders et al., 1996; Widders et al., 1998). Another flagellum vaccine developed by Meeusen also induced partial protection (2007). The reason for the partial success might be that flagellin is not surface exposed in the flagellar structure and it might be modified by glycosylation which leads to antigenic variation (Doig et al., 1996; Widders et al., 1998). In the CmeC subunit vaccination trial, the vaccine triggered systemic immune responses while the intestinal

secretory IgA response was not significantly stimulated (Zeng et al., 2010). Therefore, this CmeC vaccine did not confer protection of broilers against *C. jejuni* colonization with that specific vaccination regimen. The CadF, FlaA, and FlpA peptides vaccine has been reported to reduce cecal *C. jejuni* colonization by three log units after challenge recently (Neal-McKinney et al., 2014).

Attenuated *Salmonella*-based vaccine is another attractive approach for *C. jejuni* control in poultry. In one study, an attenuated *Salmonella* CjaA vaccine triggered *C. jejuni*-specific serum IgG and intestinal IgA and reduced wild type *C. jejuni* infection in the chicken cecum (Wyszyńska et al., 2004). However, the attenuated *Salmonella* strain expressing Peb1 failed to induce protection against *C. jejuni* in chicken (Sizemore et al., 2006). Recently, Layton and colleagues (2011) developed live *Salmonella* vaccines expressing Cj0113 (Omp18/CjaD), Cj0982c (CjaA), or Cj0420 (ACE393) and administered them to chickens orally on the first day post-hatching. All three candidates induced serum and intestinal mucosal immune responses and decreased *C. jejuni* recovery from the ileum, with the best response (4.8 log unit reduction) from the Cj0113 group.

Passive immunization has also been studied to reduce *C. jejuni* colonization in broilers. In a study by Al-Adwani et al. (2013), specific-pathogen-free laying hens were hyper-immunized with one of the five *C. jejuni* colonization-associated proteins or CAPs (CadF, FlaA, MOMP, FlpA, or CmeC) and egg-yolk-derived antibodies (IgY) were then obtained from egg-yolk powder (EYP). Indirect enzyme-linked immunosorbent assays (ELISA) showed that *C. jejuni* CAP-specific IgY levels were significantly ($P < 0.05$) higher in both serum and EYP obtained from immunized hens as compared with the non-

immunized hens. The study also showed that CadF-, MOMP-, and CmeC-specific IgY greatly reduced *C. jejuni* colonization in the chicken hepatocellular carcinoma cells. In a follow-up study, laying hens were immunized with seven *C. jejuni* CAPs (CadF, FlaA, MOMP, FlpA, CmeC, Peb1A, and JlpA) respectively or as a cocktail containing equal parts of each EYP. Nevertheless, no significant differences in the cecal colonization of *C. jejuni* were observed between the treated chickens and the control (Paul et al., 2014). In another study, laying hens were immunized with either a whole-cell lysate or the hydrophobic protein fraction of *C. jejuni* (Hermans et al., 2014). Results showed that preventive administration of hyper-immune egg yolk significantly reduced *C. jejuni* level in chickens about four log units. Western blot analysis in combination with mass spectrometry also revealed that the immunodominant antigens (AtpA, EF-Tu, GroEL, CtpA et al.) are highly conserved and were involved in a variety of cell functions of *C. jejuni*.

In summary, these studies suggest that vaccination and passive immunization are partially successful in reducing *C. jejuni* colonization in poultry. However, the findings from some trials need to be confirmed and the vaccination regimens need to be further optimized to enhance local mucosal immune responses for effective protection against *C. jejuni* colonization in the chicken intestine.

1.5. DNA vaccine and *in ovo* vaccination

In 1990, the plasmid DNA encoding luciferase was reported to transfect muscle cells *in vivo* after an intramuscular injection (Wolff et al., 1990). The luciferase activity was present in the muscle for over 2 months. This finding led to the development of the

first DNA vaccine reported in *Science* in 1993 (Ulmer et al.). Since then, various DNA vaccines have been developed against infectious diseases, cancer, autoimmunity and allergies in human and animals (Liu, 2011; Restifo and Rosenberg, 1999). The principle of DNA vaccine is straightforward: the gene encoding foreign antigen is cloned into an appropriate eukaryotic expression plasmid that can be replicated in a bacterial host; then the purified recombinant plasmid can be directly used as a vaccine with or without adjuvant (Shah et al., 2014). DNA vaccination has a variety of advantages, including the ability to induce both cellular and humoral immune response, lack of risk for infection, long-term persistence of immunogen and the ability to be easily manufactured by standard molecular biology techniques (Restifo and Rosenberg, 1999; Wahren and Liu, 2014). DNA vaccines are also more stable for storage and shipping because of the structural and chemical characteristics when compared to traditional vaccines (Shah et al., 2014).

Many veterinary DNA vaccines have been licensed, including an equine vaccine against West Nile Virus, a fish vaccine against infectious hematopoietic necrosis virus, a pig vaccine expressing Growth Hormone Releasing Hormone, and a therapeutic canine vaccine for melanoma (Redding and Weiner, 2009; Wahren and Liu, 2014). In chickens, several DNA vaccines also have been developed, most of which are viral vaccines. A recombinant plasmid encoding the VP2 gene fragment of Infectious Bursal Disease Virus was tested in chickens through the intramuscular route and the pVP2 vaccine group developed a higher titre of anti-VP2 antibodies than the control (Pradhan et al., 2014). Moreover, splenocytes from the vaccine group showed a significantly higher proliferation to the whole viral and recombinant antigen, which implies that the DNA vaccine elicited

both B and T cell responses. In another trial, the DNA vaccine encoding chicken infectious anaemia virus (CIAV) VP1/VP2 genes were co-administered with truncated chicken high mobility group box 1 (HMGB1 Δ C) as adjuvant in chicken with a booster and a strong immunity for CIAV was observed (Sawant et al., 2015). Recently, scientists have also studied different DNA vaccines against coccidiosis in poultry. In one study by Shah et al. (2011), five different DNA vaccines were tested in chickens intramuscularly. The result indicates that vaccination groups greatly ($P < 0.05$) alleviated intestinal lesions, body weight loss and oocyst count. Similarly, in another DNA vaccination study by Xu and colleagues (2008), the vaccinated chickens also showed significant ($P < 0.05$) lower weight gain loss and higher oocyst decrease ratio, imparting partial protection against homologous challenge.

DNA vaccines against *Campylobacter* have also been developed recently (Huang et al., 2010; Liu et al., 2014). Intranasal vaccination of chickens with a DNA vaccine expressing the flagellin gene *flaA* induced *C. jejuni*-specific serum IgG and intestinal IgA and reduced *C. jejuni* colonization by 2-3 log units within the cecum (Huang et al., 2010). In a mice study for evaluating a DNA vaccine expressing *C. jejuni* PEB1 permease, the stimulation index of lymphocytes, serum IgG, IL-4, IFN- γ , and intestinal IgA were significantly higher in the mice immunized with DNA vaccines with a protein boost via the intranasal route (Huang et al., 2010; Liu et al., 2014).

In ovo delivery, an attractive vaccination route for chickens (de Zoete et al., 2007), offers various advantages when combined with DNA vaccine (Haygreen et al., 2005). In 1997, a plasmid encoding β -galactosidase delivered into the breast muscle via *in ovo* route achieved successful gene transfer and expression, which showed the potential for

the development of *in ovo* DNA vaccines (Johnston et al.). The immune system of chickens is well developed by day 18 of inoculation, indicating the feasibility for stimulating immune response via *in ovo* vaccination (Ricks et al., 1999). In addition, *in ovo* vaccination is a fully automatic method to vaccinate massive numbers of eggs (20,000 to 30,000 per hour), and has been applied to various vaccines for viral, bacterial and protozoal diseases in broilers, without compromising embryo viability (Johnston et al., 1997; Ricks et al., 1999). This method also reduces eggs handling, improves hatchery manageability, and reduces cost and labor (Johnston et al., 1997). To date, many poultry vaccines have been approved by the USDA for *in ovo* administration (Johnston et al., 1997).

1.6. Chitosan and its application in subunit vaccine development

Chitosan, a family of natural linear polysaccharides consisting of β -(1-4)-linked glucosamine and N-acetylglucosamine, is commercially obtained by partial deacetylation of α -chitin produced from the exoskeletons of crustacea or the cell walls of fungi (Kang et al., 2009; Smith et al., 2014). Chitosan is positively charged and soluble in an acidic solution with a charge density depending on pH and the degree of deacetylation. Based on its chemical properties, chitosan, particularly chitosan salts, has been widely applied in drug and vaccine delivery systems for the controlled release of subunit vaccines due to the ease of preparation, bioavailability, biocompatibility, and low toxicity (Alpar et al., 2005; Illum et al., 2001; Kang et al., 2006). In addition, chitosan can stimulate immune responses and serve as an appropriate adjuvant for the subunit vaccine (Kang et al., 2006). In clinical use, chitosan is often chemically modified through their

composition with hyaluronan (Lim et al., 2001) or Pluronic F127[®] (F127) (Kang et al., 2009) to enhance the bioavailability and membrane permeability. It has been demonstrated that the positively charged chitosan can form tight junctions with the negatively charged mucin layer and facilitate the paracellular transport of hydrophilic macromolecules through the nasal route (Ilium, 1998; Kang et al., 2009). Moreover, due to its positive charge in a weak acidic environment, chitosan associates easily to the negatively charged DNA, which enhances the possibility of a chitosan-encapsulated DNA vaccine (Roy et al., 1999).

Nasal delivery is an attractive vaccination route because it can elicit both systemic and mucosal immune responses (Jabbal-Gill et al., 2012; Smith et al., 2014) and avoid destruction of the drugs or vaccines in harsh the gastrointestinal environment (Costantino et al., 2007). In particular, nasal delivery can protect the vaccines from enzymatic degradation because of relatively low enzymatic activity in the nasal cavities (Sarkar, 1992), enabling a low dose of vaccine to be used for triggering the desired immune response. It has been demonstrated that the dose for intranasal vaccination can be reduced four-fold compared with the oral route without affecting the efficacy of the vaccine (Rudin et al., 1998).

A number of human vaccines coadministered with chitosan have been published. A chitosan-encapsulated inactivated mutant diphtheria toxoid vaccine was reported to generate significantly stronger neutralizing antitoxin serum antibodies as well as Th2 type of cell responses (McNeela et al., 2004; Mills et al., 2003). In another vaccination trial of chitosan-encapsulated vaccines for *Neisseria meningitidis* serogroup C polysaccharide, the mean titre of serum bactericidal antibody (SBA) rose 24-fold after

two nasal immunizations comparable to intraperitoneal immunization (Huo et al., 2005). In a study for tetanus toxoid (TT) vaccine, mice were immunized intraperitoneally with TT in the presence of chitosan/ F127 and boosted intranasally with the same vaccine, leading to a significant enhancement in the systemic anti-TT antibody level (Westerink et al., 2001).

In chicken, the Newcastle disease virus (NDV) F gene plasmid (pFDNA)-CS/PLGA-NPs encapsulated with chitosan-coated poly lacticco-glycolic acid nanoparticles induced stronger cellular, humoral, and mucosal immune responses compared to the plasmid DNA vaccine alone (Zhao et al., 2014). Moreover, research by Huang et al. (2010) mentioned above also demonstrated that the chitosan-encapsulated DNA vaccine is a feasible approach to induce an effective immune response against *C. jejuni* in chickens.

CHAPTER 2: Introduction

Campylobacter is the leading bacterial cause of foodborne human illnesses in the US (Batz et al., 2011). This pathogenic organism causes watery diarrhea, fever, and abdominal cramping in patients and is associated with Guillain-Barre Syndrome, an acute flaccid muscular paralysis that may result in respiratory muscle compromise and death (Blaser, 1997; Lee and Newell, 2006; Mawla et al., 2014). There are more than 2 million estimated cases of campylobacteriosis every year in the US (Friedman, 2000); over 8,000 of which result in hospitalization, and 76 of which result in death. Therefore, it was estimated that the annual medical and productivity costs resulting from *Campylobacter* infection were more than 1 billion dollars in costs in the US (Batz et al., 2011). Poultry is considered the major reservoir of *Campylobacter* and therefore the main source of human campylobacteriosis (Sahin et al., 2002). On-farm control of *Campylobacter* in poultry would reduce the risk of human campylobacteriosis and have a significant impact on food safety and public health. Of various approaches, vaccination appears to be a promising strategy to reduce *Campylobacter* load in poultry (Lin, 2009). However, to date, there is still no vaccine available to control *Campylobacter* infections in poultry primarily due to a lack of understanding of pathogenicity, the antigenic complexity of this organism, and the challenges to induce strong mucosal immune response for *Campylobacter* vaccines (Jagusztyn-Krynicka et al., 2009; Lin, 2009).

Previous studies have shown that prior infection with *C. jejuni* could induce protective immunity against *Campylobacter* colonization and shedding level in poultry, strongly supporting the feasibility of developing vaccines for *Campylobacter* control in

poultry (reviewed in Chapter 1 above). A successful chicken vaccine is expected to prevent colonization or cause a strong reduction of *Campylobacter* numbers in chickens of more than 2 log units (de Zoete et al., 2007). To achieve the goal of developing novel, safe, and inexpensive vaccination strategies that could be conveniently and practically used to control *C. jejuni* in the chicken industry, our laboratory has made significant progress in the past years to identify conserved protective antigens in *C. jejuni*, a paramount and critical step towards the design of effective vaccines against *Campylobacter*. Specifically, our previous studies have discovered two attractive candidates, CmeC and CfrA, for developing *Campylobacter* vaccines (Jones, 2013; Lin et al., 2005; Lin et al., 2002b; Lin et al., 2003; Zeng et al., 2009, 2010). CmeC, the outer membrane component of the CmeABC multi-drug efflux pump, is highly conserved across *C. jejuni* strains (Zeng et al., 2010). More important, CmeC, which is significantly induced by intestinal bile salts, plays an essential role in bile resistance and consequently plays a critical role in *C. jejuni* colonization in the intestinal environment (Lin et al., 2003). Another candidate, CfrA, is an outer membrane protein associated with iron acquisition in the intestinal tract and is also essential for *C. jejuni* colonization in the chicken intestine (Palyada et al., 2004). Our laboratory has shown that CfrA is also prevalent and highly conserved in *C. jejuni* strains and its expression is also induced in the intestine (Zeng et al., 2009). This evidence indicated that CmeC and CfrA are feasible vaccine candidates for developing effective vaccines against *C. jejuni* in poultry.

Recently, by targeting CmeC and CfrA, development of various vaccines (e.g. DNA vaccine, *Salmonella*-vectored vaccine) for different vaccination strategies have been initiated and explored in our laboratory (Jones, 2013). This preliminary work

provides us a solid foundation to further develop and evaluate different vaccination regimens for effective mitigation of *Campylobacter* in poultry as described in this project.

Three specific objectives were pursued in this project:

1. Optimize DNA vaccines and evaluate immune response and protective efficacy of *in ovo* DNA vaccination to control *Campylobacter* colonization in poultry.
2. Optimize and prepare large quantities of chitosan-encapsulated subunit vaccines (CmeC/CfrA-based DNA vaccines as well as purified recombinant proteins).
3. Evaluate immune response and protective efficacy of intranasal vaccination with chitosan-encapsulated subunit vaccines in broilers.

CHAPTER 3: Materials and Methods

3.1. Bacterial strains and their growth conditions

All the bacterial strains and plasmids used in this project, as well as their sources, are listed in Table 1. The *E. coli* strains JL894, JL1185 and JL1118 were used to produce pCAGGS, pCmeC-K, and pCfrA-K, respectively. The *E. coli* strains JL243 and JL275 were used for the purification of His-tagged recombinant protein CmeC and CfrA, respectively. The *E. coli* strains were grown on Luria-Bertani (LB, BD Difco) plates containing 50 µg/mL of ampicillin or in LB broth containing 50 µg/mL of ampicillin with shaking (250 rpm) at 37°C overnight.

The standard *C. jejuni* strain NCTC 11168 (JL241) was cultured on in Mueller Hinton (MH) broth (BD Difco, Sparks, MD) or on MH agar (BD Difco) plates at 42°C under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂), which were generated using nitrogen and carbon dioxide gas packs (Airgas®, PA) in an Heracell™ 150i Tri-Gas Incubator (Thermo Scientific). MH agar plates supplemented with *Campylobacter* Growth and Preston *Campylobacter* Supplements (Oxoid, Basingstoke, Hampshire, England) were used for selective growth of *Campylobacter* from cloacal swabs for the chicken trials.

3.2. Modification and validation of constructed DNA vaccines

Full length fragments of *cmeC* and *cfrA* with the Kozak sequence were PCR amplified from *C. jejuni* NCTC 11168 with primer pairs of

pCAGGS_CmeC_F2/pCAGGS_CmeC_R and pCAGGS_CfrA_F2/pCAGGS_CfrA_R, respectively (Table 2). The truncated fragments of *cmeC* and *cfrA* with the Kozak sequence were PCR amplified from *C. jejuni* NCTC 11168 with primer pairs of pCAGGS_CmeC_TM2_F/ pCAGGS_CmeC_TM3_R and pCAGGS_CfrA_F2/pCAGGS_CfrA_B1_R, respectively (Table 2). Those amplified *cmeC* and *cfrA* fragments were digested with *XhoI* and ligated into the *XhoI*-digested expression vector pCAGGS. The ligation mixture was introduced into *E. coli* Top10 cells via electroporation for 4-5 ms at 2.5 kV. Transformants were selected on LB agar plates containing 50 µg/mL of ampicillin and the plasmids were extracted from randomly selected transformants and analyzed by agarose gel electrophoresis. The identified constructs with insertion of desired gene fragments (pCmeC-K, ptCmeC-K, pCfrA-K, and ptCfrA-K) were finally subjected to sequence analysis to confirm the orientation and integrity of the inserted fragment and the presence of the Kozak sequence adjacent to the start codon of cloned gene. The primer pCAGGS_F and pCAGGS_R (Table 2) were used for sequencing.

To validate the production of target *cmeC* or *cfrA* gene in eukaryotic cells, transfection was subsequently performed using the modified DNA vaccine vectors and HEK-293 cells. Approximately 4 µg of the specific vectors were transfected into 50-70% confluent 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 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% CO₂, cells from each well were trypsinized, centrifuged, and resuspended in 100 µL of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2.5% SDS, 25% glycerol, 125 mM Tris-Cl [pH 6.8], 0.01% bromophenol blue and 100 mM dithiothreitol). The samples were subjected to SDS-PAGE and immunoblotting analyses as detailed below.

3.3. SDS-PAGE and Immunoblotting

Five µL of the above 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 25 minutes followed by 160V for 40 minutes by electrophoresis. Following SDS-PAGE, proteins in gels were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) in transfer buffer at 90V for 1 hour. The membrane was then incubated in blocking buffer (5% Nestle skim milk powder in PBS) for 1 hour at room temperature with shaking followed by overnight incubation at 4°C. Then the membrane was incubated with primary antibodies (1:1000 diluted rabbit anti-rCmeC or -CfrA sera in blocking buffer) for one hour at room temperature. Following incubation, the membrane was washed with wash buffer (PBS containing 0.05% Tween 20) for three times. Next, the washed membrane was incubated with a secondary antibody (goat anti-rabbit IgG-horseradish peroxidase, diluted 1:5000) for one hour at room temperature and subsequently washed as described above. The SuperSignal[®] West Dura Extended Duration Substrate (Thermo Scientific) was used to develop the nitrocellulose membrane.

3.4. Mass production of the DNA vaccines

The control plasmid pCAGGS and the two modified DNA vaccine vectors that produced full-length target protein (pCmeC-K and pCfrA-K) were extracted from the *E. coli* JL894, JL1185, and JL1118 culture using a QIAGEN Plasmid Maxi Kit (Qiagen, Hilden, Germany). Briefly, a single bacterial colony was inoculated into 200 mL of LB medium with 50 µg/mL ampicillin and grown at 37°C for 12–16 h with vigorous shaking (approx. 300 rpm). The bacteria cells were harvested by centrifugation at 6000 × g for 15 min at 4°C. The bacterial pellet was resuspended in 10 mL Buffer P1. Buffer P2 was added and the mixture was incubated at room temperature for 5 min. Chilled Buffer P3 was then added to enhance precipitation of proteins and the mixture was incubated on ice for 20 min. The supernatant containing plasmid DNA was finally obtained by centrifuge at 20,000 × g twice at 4°C. Meanwhile, the QIAGEN-tip 500 was equilibrated with Buffer QBT. The supernatant was then applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The column was washed by 2 × 30 ml Buffer QC and the DNA was subsequently eluted by 15 mL Buffer QF. The eluted plasmids were precipitated by adding 10.5 mL (0.7 volumes) isopropanol and washed by 70% ethanol. DNA pellets were air dried for one hour and redissolved in sterile water. DNA concentration was determined by both UV spectrophotometry at 260 nm as well as agarose gel analysis.

3.5. Mass production of high-purity rCmeC and rCfrA

The *E. coli* constructs for producing N-terminal Histidine-tagged rCmeC (JL243) and rCfrA (JL275) were obtained from our recent studies (Zeng et al., 2009, 2010). The full-length Histidine-tagged rCmeC and rCfrA were purified from *E. coli* culture using

Ni²⁺-NTA affinity chromatography as described previously with modifications (Zeng et al., 2009, 2010). In brief, the JL243 or JL275 strain was inoculated into one liter LB with 50 µg/ml ampicillin and the production of the proteins was induced in the log phase cells (OD₆₀₀= 0.5) by IPTG for 3 hours. The bacterial cells were obtained by centrifugation and subsequently lysed for protein purification. Three mL Ni²⁺-NTA agarose resin (Qiagen, Hilden, Germany) were equilibrated with lysis buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl and 10 mM imidazole). The lysate was mixed with the equilibrated Ni-NTA resin on a rotator for 2 hours at 4°C. The lysate-resin mixture was then loaded into a 15 mL plastic column and the flow through was collected. The column was washed with 10 mL of wash buffer (50 mM sodium phosphate, 300 mM NaCl, 60mM imidazole, 10% glycerol, pH 7.0 supplemented with 2mM β-mercapthanol, 5 mM of ATP, and 5mM of MgCl₂) four times. The rCmeC or rCfrA bound to Ni-NTA were eluted with 10 volumes of elution buffer (50mM sodium phosphate, 300mM NaCl and 300mM imidazole, pH 7.0) to ten 1.5 mL microcentrifuge tubes. The samples were analyzed by SDS-PAGE to determine the quantity and purity. The rCmeC or rCfrA elution with high quantity and purity were further dialyzed against PBS buffer and then stored at -80°C for the preparation of subunit vaccines. The concentration of the recombinant proteins was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce).

3.6. Preparation of chitosan-DNA and chitosan-protein nanoparticles

The chitosan/pCmeC-K and chitosan/pCfrA-K nanoparticles were prepared using the method described previously with slight modifications (Huang et al., 2010). Briefly,

equal volume of chitosan (Sigma, prod. No: 448869, low molecular weight) solution (200 µg/mL in 5mM sodium acetate buffer, pH 5.5) and specific DNA vaccine vector (100 µg/mL in 5mM sodium sulfate solution) were both preheated to 50°C and then mixed and incubated at 25°C for two hours. The corresponding chitosan microsphere (CM) nanoparticles containing pCemC-K and pCfrA-K henceforth are called CM-pCmeC and CM-pCfrA, respectively.

The chitosan/rCfrA and chitosan/rCmeC were synthesized by the procedure described by Kang et al. (2006) with modification. First, a 0.25% chitosan solution (2% aqueous acetic acid) with 1.25% Pluronic F127 (Sigma) was prepared by continuous stirring. Pluronic-F127 is a hydrophilic copolymer of polyethylene oxide and polypropylene oxide which is able to decrease the aggregation of CMs (Kang et al., 2007). Twenty-five mL of the chitosan/F127 solution was then extruded dropwise through a needle into one mL of 15% tripolyphosphate (TPP) which can crosslink chitosan fibers through electrostatic forces to stabilize CMs (Aral and Akbuğa, 1998; Desai and Park, 2005). CMs were obtained by sonicating the chitosan/F127-TPP mixture followed by centrifugation. The homogenous chitosan/protein solution were obtained by mixing CMs with recombinant proteins in a 3:1 concentration ration (CM: protein) and incubated overnight at 37°C with continuous shaking at 250 rpm. The corresponding CM nanoparticles with rCmeC and rCfrA henceforth are called CM-CmeC and CM-CfrA, respectively.

To test the association efficiency of encapsulation, the chitosan-plasmid or chitosan-protein complexes were centrifuged and the supernatant was collected for

measuring the concentration of unloaded DNA or protein. The association efficiency was calculated as follows:

Association efficiency (%)

$$= \frac{\text{Total amount DNA (pCfrA-K/pCmeC-K) – unloaded DNA}}{\text{Total DNA}} \times 100(\%)$$

Or

Association efficiency (%)

$$= \frac{\text{Total amount protein (CfrA/CmeC)– unloaded protein}}{\text{Total protein}} \times 100(\%)$$

The freshly prepared chitosan-DNA or chitosan-protein particles were also morphologically examined under a transmission electron microscopy (TEM, Zeiss Libra 200 MC) or a scanning electron microscopy (SEM, Zeiss Auriga) at the Advanced Microscopy and Imaging Center at the University of Tennessee, Knoxville, TN.

3.7. *in ovo* DNA vaccination experiments

Preliminary trial

A pilot experiment was conducted out to determine the hatchability of eggs following *in ovo* injection. At day 18 of embryonation, thirty eggs were randomly divided into two groups, with 20 eggs in the treatment group and the remaining in the control group that were not subject to *in ovo* injection. For the treatment group, each of 20 embryonated eggs was injected with 100 µL of sterile ddH₂O on approximately day 18 of incubation. The 23 gauge needle with 1 inch in length was used for injection and

transparent scotch tape was used to seal the injection site. The treated eggs were put back into the incubator together with the eggs in the control group for an additional three days of incubation until chicks were hatched.

in ovo DNA Vaccination Trial 1

In the first chicken trial (Table 3), 70 embryonated eggs were obtained from Pilgrim's Pride Corporation (Chattanooga, TN) and incubated for 18 days in an incubator and candled to select fertile eggs. At day 18, all the eggs were randomly divided into four treatment groups (17-18 eggs per group) and injected with 100 μ L of sterile water (Group 1), pCAGGS empty plasmid (Group 2), 50 μ g pCmeC-K in 100 μ L sterile water (Group 3) or 50 μ g pCfrA-K in 100 μ L sterile water (Group 4) into the amniotic fluid as described in other publications (Ding et al., 2005; Ding et al., 2004; Guo et al., 2008). After hatch, there were 16, 15, 17 and 12 birds in Group 1, 2, 3 and 4 respectively. The hatchability of each group were 89%, 83%, 94% and 67%, respectively. All chicks were kept in clean wire-floor cages and provided with water and antibiotic-free feed *ad libitum*. The room temperature was maintained at 32°C in the first week and at 25°C afterwards. At day 14 post-hatch, all the chickens were challenged orally with *C. jejuni* NCTC 11168 with a dose of 10⁴ CFU per chicken. Cloacal swabs from each bird were collected every 2~3 days from day 14 to day 28 post-hatch and suspended in 100 μ L of MH broth. The samples were then spread on MH plates with a dilution of 1:1, 1:100 and 1:10⁴ and incubated at 42°C under microaerophilic condition for 48 hours for *C. jejuni* CFU enumeration. Blood samples were also collected via wing vein from each chicken at day

14, 21 and 28 post-hatching and analyzed by ELISA for CmeC- and CfrA-specific IgG and IgA as described below.

in ovo DNA Vaccination Trial 2

The design of the second trial is shown in Table 4. There were several significant modifications of this trial when compared to Trial 1 above. First, the DNA vaccines were specifically emulsified with equal volume of neutral lipid (incomplete Freund's adjuvant, MP Biomedicals) prior to *in ovo* injection. The neutral lipid is expected to protect DNA vectors against degradation by DNase in the amniotic fluid (Oshop et al., 2003). Second, rCmeC and rCfrA proteins (50 µg per egg) were included as controls to determine whether these protein antigens could induce an immune response. Third, blunt-ended needles (Lab Std. LL Pipetting Needle 20RW×1.5", Lab Express Management) were used for *in ovo* injection in this trial to minimize potential damage to the embryos. Finally, the challenge date was delayed from 14 day post-hatching (Trial 1) to 21 day post-hatching in order to provide chickens enough time for immune response and antibody production. At day 18 of embryonation, eggs were randomly divided into 6 treatment groups with 15-17 eggs per group and treated as described in Table 3. After hatch, there were 14 birds in sterile water group, 11 birds in pCAGGS/IFA group, 13 birds in pCfrA-K/IFA group, 14 birds in pCmeC-K/IFA group, 14 birds in CfrA/IFA group and 9 birds in CmeC/IFA group. The hatchability of each group were 93%, 73%, 76%, 82%, 82% and 60%, respectively. Chicken management, *C. jejuni* challenge, blood and cloacal sampling were the same as Trial 1. In this trial, intestinal samples were also collected at the last day and suspended in lavage extraction buffer (PBS containing 0.05% Tween 20,

0.05g/mL of EDTA, and complete mini protease inhibitor [Roche, prod. No: 04693159001]), by a ratio of 1:10 (1g sample: 10mL lavage extraction buffer) and were used for determining specific mucosal immune response using ELISA as detailed below. The procedure for chicken management and *C. jejuni* challenge and cloacal swabbing are the same as those described for Trial 1.

3.8. Intranasal immunization with chitosan encapsulated subunit vaccines (Trial 3)

This trial was to comprehensively evaluate the immune response and protective efficacy of intranasal immunization of broiler chickens with four chitosan encapsulated subunit vaccines (CM-pCmeC, CM-pCfrA, CM-CmeC, and CM-CfrA). A total of 120 one-day-old broilers were obtained from the Hubbard Hatchery and assigned into six groups with 20 chicks per group (Table 5). Chickens were managed in sanitized wire-floor cages at 32°C in the first week and at 25°C thereafter. Clean water and antibiotic-free feed (prepared by Johnson Animal Research and Teaching Unit) were provided. For the primary immunization at 7 days of age, the CM-CmeC or CM-CfrA was inoculated intranasally with a dose of 100 µg per bird (100 µL per nostril for both nostrils), CM-pCmeC or CM-pCfrA was inoculated intranasally with a dose of 50 µg per bird (100 µL per nostril for both nostrils). The PBS or CM was inoculated as control. The same vaccination regimen was performed at day 21 as a booster immunization. Chickens were challenged with 10⁴ CFU of *C. jejuni* NCTC 11168 at day 35. Serum samples were gained from the chickens in each group at day 7, 21, 35 and 44 and used for ELISA assay. On day 21 and 44, intestinal lavages were collected from 5 representative chickens in

each group for monitoring mucosal IgA against CmeC and CfrA. Fecal swabs were also collected every 2~3 days for two weeks after *C. jejuni* challenging.

3.9. Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay was used to analyze serum and intestinal immunoglobulins against CmeC and CfrA in this study. In general, microtiter plates (Nunc-Immuno Plate, Thermo Fisher Scientific) were coated with 100 μ L high-purity rCmeC or rCfrA per well with an optimal concentration of 300 ng/mL in coating buffer (0.01 M Ammonium acetate/ammonium carbonate, pH= 8.2) and incubated at room temperature for approximately 18 hours. Plates were washed with 200 μ L washing solution (0.5% Tween 20 in PBS) using a plate washer and blotted dry. Plates were then coated with 100 μ L of blocking buffer (PBS with 1% BSA and 0.1% Tween) to each well and incubated at 37°C for one hour, and washed again. Serum samples diluted 1:100 or intestinal samples diluted 1:50 in blocking buffer were added to each well and the plates were incubated at 37°C for one hour followed by washing of the plates. A 96-well plate was coated with 100 μ L of anti-chicken IgG/IgA conjugated AP antibody (KPL) diluted 1:2000 in blocking buffer. After incubation at 37°C for one hour, plates were washed and ABTS peroxidase substrate (KPL) was added to each well. For serum IgG assays, plates were incubated at room temperature for 10 minutes. For serum and intestinal IgA assays, plates were incubated for 20 minutes. Stopping solution (1% SDS) was added to each plate prior to read of absorbance under OD_{405nm} by a plate reader.

Statistical analysis was performed using SAS software (v9.4, SAS Institute Inc., Cary, NC). Specifically, differences in serum or intestinal sample OD_{405 nm} readings

among different groups were analyzed using least squares analysis of covariance with date as the covariant; main effects were date and treatment. Comparison of OD_{405nm} readings within all the groups across time was tested by analysis of variance (ANOVA). Levels of significance for *P*-value were 5% (0.05).

CHAPTER 4: Results

4.1. Modification and production of DNA vaccines

The DNA vaccines containing CmeC gene (pCAGGS_CmeC) and CfrA gene (pCAGGS_CfrA) have been successfully constructed in previous studies (Jones, 2013). Despite that no frameshift or mutation in the coding sequences of *cmeC* and *cfrA* were detected, immunoblotting using specific antibodies failed to detect CmeC and CfrA from the cells transfected with pCAGGS_CmeC and pCAGGS_CfrA, respectively (Jones, 2013), indicating that the production of cloned bacterial gene is low, likely due to the lack of Kozak consensus sequence that plays a major role in the initiation of the translation process. To address this issue, in this project, the previously constructed DNA vaccine vectors, pCAGGS_CmeC and pCAGGS_CfrA, were modified by introducing Kozak consensus sequence (gccRccATGG) immediately upstream of the cloned gene. Two CmeC DNA vaccines, pCmeC-K and ptCmeC-K, were successfully constructed and were expected to produce a full-length and truncated CmeC, respectively (Table 1). Two CfrA DNA vaccines, pCfrA-K and ptCfrA-K, were also successfully constructed and were expected to produce a full-length and truncated CfrA, respectively (Table 1). Figure 1 shows the electrophoresis pattern of the four modified DNA vaccine vectors as well as parent vector pCAGGS.

To confirm the expression efficacy of the newly constructed DNA vaccine vectors in eukaryotic cells, SDS-PAGE and immunoblotting were subsequently performed to determine the production of rCmeC and rCfrA in the transfected HEK-293 cells. As shown in Figures 2A and 2B, both truncated and full length target proteins (rCmeC and

rCfrA) were detected, which clearly demonstrated that introduction of the Kozak sequence plays a critical role in success expression of bacterial genes in eukaryotic cells.

After the production efficacy of the DNA vaccines was confirmed, large quantities of high-purity pCmeC-K, pCfrA-K and pCAGGS were extracted from *E. coli* culture using QIAGEN Plasmid Maxi Kit for preparation of DNA vaccines. The yields of high-purity pCmeC-K, pCfrA-K and pCAGGS were 250~400 µg, 100~150 µg and 300~500 µg per 200 mL *E. coli* culture respectively, based on spectrophotometry at 260 nm. Overall, approximately 11 mg of pCmeC-K, 8 mg of pCfrA-K and 5 mg of control pCAGGS vectors were obtained in this project.

Large amounts of recombinant proteins rCmeC and rCfrA were produced from JL243 and JL275 strains using Ni²⁺-NTA affinity chromatography matrix for preparation of subunit vaccines and ELISA tests in this project. After 3 hours of induction with IPTG, the concentrations of N-terminal Histidine-tagged rCmeC and rCfrA were greatly increased (Figures 3A and 3B). Figures 4A and 4B show that both rCmeC and rCfrA were successfully obtained after one-step Ni²⁺-NTA chromatography purification. Every liter of IPTG-induced culture yielded ~5 mg of rCmeC or ~3 mg of rCfrA. In total, 30 mg of rCmeC and 20 mg of rCfrA were obtained in this study.

4.2. Preparation of chitosan-DNA and chitosan-protein nanoparticles

Uniform particles were obtained by coacervation between chitosan and DNA. The association efficiency of CM-pCmeC and CM-pCfrA was 50% and 70%, respectively. The morphology of the CM-pCmeC is shown in Figure 4. TEM observation confirmed

that freshly synthesized CM-pCmeC complexes were 50~100nm in size and nearly spherical.

For CM-protein encapsulation, Pluronic F127 was added to the chitosan solution to decrease the aggregation of CMs and tripolyphosphate (TPP) was used to enhance the stability of CM. After the CM/Pluronic F127-TPP was prepared, it was incubated with rCmeC or rCfrA overnight at 37°C. The average association efficiencies of CM-CmeC and CM-CfrA were both 50%. Morphology of the CM-CmeC was shown in Figures 5B and 5C. Based on SEM images, the nanoparticles appeared to aggregate. Furthermore, large amounts of crystallization were observed on the surface of both CM control and CM-CmeC, likely due to the use of PBS as diluent.

4.3. *in ovo* DNA vaccination experiments

In the *in ovo* injection pilot experiment, the hatchability of eggs in the treatment group was 95% (19 eggs hatched and 1 died) which is the same as that in control group (9 eggs hatched out of 10), suggesting that *in ovo* injection did not affect the hatchability of the eggs.

After demonstration of hatchability, the pCmeC-K and pCfrA-K DNA vaccines were used in Trial 1 for *in ovo* vaccination at day 18 of embryonation. As shown in Figure 6, the pCmeC-K and pCfrA-K vaccines failed to enhance serum IgG titre in chickens compared to the two negative controls at different days post-immunization. Chickens in ddH₂O control had higher level of CfrA-specific antibodies at day 21 and day 28, which was likely caused by individual variations in this group. Two days after

challenge, all chickens were colonized by NCTC 11168 and the colonization level peaked at 7 days post-challenge, with an average shedding level of $\sim 10^7$ CFU/g feces (Figure 7). However, no significantly ($P > 0.05$) lower colonization of *C. jejuni* was observed in the two vaccination groups.

In Trial 2, the *in ovo* vaccination strategy was modified by adding two positive controls (rCmeC and rCfrA), emulsifying and protect DNA vaccines with neutral lipid and delaying the challenge date from day 14 to day 21 post-hatching. Based on Figures 8 and 9, systemic and mucosal immunoglobulins specific for *C. jejuni* increased in all groups after challenge, indicating immune responses against *Campylobacter* in all birds. CmeC- and CfrA- specific systemic IgG and IgA were significantly ($P < 0.05$) elevated 7 days after infection of NCTC 11168 in all groups, indicating that immunity for *C. jejuni* was successfully induced (Figures 8 and 9). Furthermore, birds in the pCfrA-K vaccine group had significantly ($P < 0.05$) higher levels of serum CfrA-specific IgG, while birds in the CfrA protein group showed significantly ($P < 0.05$) higher levels of serum IgA for CfrA. ELISA analysis failed to show a significant ($P > 0.05$) difference in intestinal mucosal IgA titre in different groups (Figure 10). However, mucosal IgA levels in the two DNA vaccine groups (pCmeC-K and pCfrA-K) were moderately elevated compared to the other groups. Consistent with the patterns of immune responses, challenge of chickens with NCTC 11168 at day 21 post-hatching did not show a significant difference of colonization among groups (Figure 11). All chickens were colonized by *C. jejuni* at day 2 post-challenge. Shedding levels peaked at day 4 with an average of 10^8 CFU/g feces 4 days after challenge. During the chicken trial, we observed that the birds grew

much slower than normal. Furthermore, the chickens developed curled-toes and could not stand very well.

4.5. Intranasal immunization of chitosan encapsulated subunit vaccines in chickens

Trial 3 was carried out to determine the efficacy of the chitosan-encapsulated subunit vaccines (CM-pCmeC, CM-pCfrA, CM-CmeC and CM-CfrA) via the intranasal route. One hundred and twenty chickens were immunized at the age of 7 days with a booster of the same vaccines at day 21. After 14 days, all the chickens were challenged with NCTC 11168.

Intranasal vaccines failed to enhance serum IgG and IgA levels at different days post-immunization (Figures 12 and 13), although serum IgG and IgA levels were higher in all groups 9 days post-challenge. Consistently, level of IgA in intestinal lavages were not significantly ($P > 0.05$) different among groups (Figure 14). As shown in Figure 15, no significant difference was observed on shedding levels of *C. jejuni* between the vaccine groups and the controls ($P > 0.05$).

In this trial, we observed that chickens in all groups displayed poor growth performance and grew slowly. In the beginning, the toes were flexed and chickens tended to stand on their hocks. Later on, the toes were completely curled downward and inward and complete weakness of legs was present in many chickens. A large number of chickens died within the first three weeks of the trial. At the last stage of the trial, we had only four chickens left in the PBS control group and three in the CM-CmeC vaccine group. In the other four groups (CM, CM-pCmeC, CM-CfrA and CM-pCfrA), we had 8~9 chickens left in each group. The reduction of sample size definitely greatly

undermined the power of the statistical analysis. After the symptoms occurred, veterinarians from the College of Veterinary Medicine at University of Tennessee collected blood samples from two sick chickens to investigate whether the birds were infected by pathogens. The infectious disease testing (Avian Encephalomyelitis, Avian Influenza, Newcastle Disease and Marek's Disease) were all negative. After discussions, we found feed might be the cause of the problem and checked the formulation for feed from the farm. Based on the formulation, vitamin/mineral combined mix product should be added to the feed. Nevertheless, when we checked the records of feed processing, we found only a new mineral mix (942 Poultry Trace Mineral premix NB-8608, Nutra Blend) with a high level of Selenium (Se) was added using the old formulation. No vitamin mix was added in the feed. Based on a Selenium level test by the veterinarians, serum Se levels of the sick control chickens were elevated at 0.22 ppm, while normal is 0.085-0.150. The lack of vitamin mix and high titre of Se in the chicken feed caused the poor growth rate and high death rate. To solve the problem, we changed the premix feed to the AN Chick Starter/Groper complete feed (Tennessee Farmers Cooperative) and the birds recuperated their health and stopped dying gradually.

CHAPTER 5: Discussion

DNA vaccine, the so-called ‘third generation vaccine,’ is a safe and stable technology that can induce both humoral and cell mediated immunity. Ever since the first DNA vaccine was developed in humans in 1993 (Ulmer et al., 1993), a variety of DNA vaccines have been tested and licensed, including those designed to prevent and control cancer, allergies, and infectious diseases (Liu, 2011; Wahren and Liu, 2014). In chicken, various DNA vaccines have been developed, most of which were against viruses (Pradhan et al., 2014; Sawant et al., 2015). Furthermore, a number of DNA vaccines against coccidiosis in poultry have also been reported (Shah et al., 2014).

The Kozak sequence is the sequence adjacent to the translational start site (AUG) on eukaryote mRNA molecules, which can be recognized by the ribosome. The Kozak sequence varies in different species and different mRNA molecules. The amount of synthesized protein is often dependent on the specific Kozak sequence, which determines the affinity between the eukaryotic ribosome and mRNA. The consensus sequence of Kozak sequences in vertebrates is gccRccATGG (Kozak, 1987), where upper case letters denote a high level of conservation while lower case letters denote a relatively low level conservation. In our previous study (Jones, 2013), the pCAGGS vector (Hitoshi et al., 1991), a eukaryotic expression vector containing the chicken β -actin promoter, the CMV immediately early enhancer (CMV-IE) and the SV40 origin of replication (SV40 OriC), was used for the construction of DNA vaccines pCAGGS_CmeC and pCAGGS_CfrA. Although research suggested that pCAGGS has a high expression efficiency and is widely applied for expression of viral antigens in animals (Bu et al., 2003a; Bu et al.,

2003b; Hitoshi et al., 1991), the DNA vaccine vectors did not display the desired expression level of target *Campylobacter* genes in eukaryotic cells in our previous study (Jones, 2013), likely due to the lack of the Kozak sequence of the cloned bacterial genes. In this study, two CmeC DNA vaccines (pCmeC-K and ptCmeC-K) and two CfrA DNA vaccines (pCfrA-K and ptCfrA-K) were successfully constructed by the introduction of the Kozak consensus sequence to the upstream of the *cmeC* or *cfrA* genes. The production of these newly-constructed DNA vaccines in eukaryotic cells was confirmed by immunoblotting analysis in this study. This modification was also successfully used in other DNA vaccine construction with purpose of expressing bacterial genes. In one study, *Brucella abortus* lumazine synthase gene including the Kozak consensus sequence was cloned in pcDNA3 plasmid (Velikovskiy et al., 2002). Expression of the cloned gene was confirmed *in vitro* by transient transfection of COS-7 cells and the vaccine was demonstrated to elicit high levels of protection against smooth and rough species of *Brucella* in mice via the intramuscular route. In another study by Cassataro et al. (2005), a pCIOmp31 DNA vaccine vector containing the Kozak sequence was also demonstrated to express the Omp31 gene and induce a good immune response against *B. melitensis* and *B. ovis* for BALB/c mice intramuscularly. Similarly, the plasmids expressing *wapA*, *il-5* or *ctb* gene were constructed by incorporating the Kozak sequence into all the genes in a vaccination study of *Streptococcus mutans* in mice (Han and Dao, 2007). The expression of the transfected genes was also assayed by immunoblotting. Although the recombinant plasmids without the Kozak sequence were not set as controls in these three studies, the results indicated that it is critically important to consider the modification of the Kozak

sequence for success expression of cloned prokaryotic gene, when constructing DNA vaccine for expressing bacterial gene target.

The full-length DNA vaccines (pCmeC-K and pCfrA-K) were evaluated in two *in ovo* vaccination trials. However, neither of the two DNA vaccines were able to successfully induce an efficient immune response and good protection in broiler chickens. There are many factors that can determine the success of a DNA vaccination strategy. The dosage of the DNA vaccine is believed to be a critical factor (Leitner et al., 1999). Normally, a higher dose of the vaccine triggers a stronger immune response, although they are not linearly correlated. An appropriate amount of DNA may effectively induce an immune response regardless of the body size of the animal (Cox et al., 1993; Davis et al., 1996). For *in ovo* vaccination, injection of 60 µg DNA could lead to an 80% expression rate in chicken embryos, significantly higher than the expression rates of 45% for 30 µg plasmids and 50% for 100 µg plasmids (Oshop et al., 2003). In an intranasal DNA vaccination mice trial, Wang and colleagues (2004) have shown that the administration of 20 µg of liposome-emulsified DNA vaccine in one nostril followed by the administration of 40 µg of the same DNA vaccine in both nostrils resulted in higher systemic IgG and IgA titres. In another intranasal vaccination trial, 10 µg HIV DNA vaccine also led to significantly higher antibody titre in mice compared to the controls (Okada et al., 1997). In conclusion, 50 µg of DNA vaccines should be an appropriate dose to induce good immunity and protection against *Campylobacter* for chickens via *in ovo* or intranasal routes.

However, *in ovo* vaccination of rCmeC and rCfrA proteins failed to stimulate high levels of systemic and intestinal mucosal immune response in Trial 2. This finding

raises a concern for triggering sufficient immune response via *in ovo* vaccination in our system. Since the first *in ovo* delivery of a Marek's disease vaccine in the 1982 (Sharma and Burmester, 1982), a variety of exogenous materials have been administered into eggs during incubation, including vaccines, vitamins, amino acids, and drugs (Gore and Qureshi, 1997; Kadam et al., 2013). As a result, the *in ovo* regimen should be a straightforward process for vaccine delivery. To optimize *in ovo* vaccination, attention has been placed on several factors including the embryonic stage of development, the target site and the protocol for injection. It has been proposed that the immune system of chicken has been well developed at day 18 of embryo development (Ricks et al., 1999) and this is the best timing for *in ovo* injection (Salahi et al., 2011). Study also showed that pre-hatching chicks naturally consume the amniotic fluids until hatching (Nace, 1961). As for the needles used for *in ovo* vaccination, 18-25 gauge needles at the depth of one inch are widely used to inoculate materials into the amniotic cavity (Barjesteh et al., 2015; Ge et al., 2014; Makanya et al., 2015; Salahi et al., 2011). For our chicken Trials 1 and 2, we are confident that the vaccines were injected successfully into the amniotic fluid and taken up by the bird embryos.

Intranasal vaccination stimulates immunity in the nasopharynx-associated lymphoid tissue (NALT) and vaccination via intranasal route has been demonstrated to induce systemic immunity as well as mucosal immune responses in other mucosa sites, such as gastric mucosa, respiratory tracts and genital tracts (Brandtzaeg, 2011; Jabbal-Gill, 2010; Pasetti et al., 2011; Tribble et al., 2010). Moreover, intranasal vaccination requires a lower antigen and adjuvant doses compared with oral vaccination (Lycke, 2012). Intranasal vaccines can be protective only if they were delivered with components

that enable the effective uptake of the antigens across the mucosal membrane. Chitosan, which has both mucoadhesive and adjuvant properties, has been found to enhance the recognition of antigens by the innate immune system and the induction of mucosal immune responses (Lycke, 2012; Van der Lubben et al., 2001). In chickens, a chitosan-coated F gene plasmid DNA vaccine triggered stronger cellular, humoral, and mucosal immune responses against Newcastle disease virus (NDV) compared to the plasmid DNA vaccine alone when administered via the intranasal route (Zhao et al., 2014). Moreover, a chitosan-encapsulated *flaA* vaccine induced *C. jejuni*-specific serum IgG and intestinal IgA in chickens (Huang et al., 2010). It also reduced *C. jejuni* colonization by 2-3 log in the cecum (Huang et al., 2010). All of the above results showed that chitosan-encapsulated vaccines can induce excellent protection against *Campylobacter* when inoculated intranasally. However, there are some limitations for chitosan encapsulation such as aggregation (Kang et al., 2007). To address this concern, when preparing the chitosan-protein nanoparticles, Pluronic-F127, a hydrophilic copolymer was included to decrease the aggregation of CMs. Furthermore, Pluronic-F127 has also been shown to enable immune response and function as a synergist with chitosan (Westerink et al., 2001). To date, significant challenges still exist for the development of effective mucosal vaccines. Since the vaccines are given directly on mucosal surfaces, they face the same hurdles as pathogens do: 1) they are diluted and entrapped by mucosal secretions; 2) the vaccines might be excluded by epithelial barriers and 3) recombinant protein vaccines might be degraded by proteases while DNA vaccines might be cleared by nucleases (Neutra and Kozlowski, 2006).

As reviewed by Zoete and colleagues (2007), an effective vaccine should be not only cost-effective, but also easy to produce and administer in larger quantities. The yields of recombinant CmeC and CfrA that were purified using the one-step Ni²⁺-NTA chromatography purification were ~5 mg/L culture and ~3 mg/L culture, respectively. This yield level is still low from mass production standpoint. In addition, the cost of the protein production using Ni²⁺-NTA chromatography is still high at this stage. Preparation of the proteins is also lengthy using the methods and systems described in this project. As a result, the recombinant strains and production procedures still need to be modified and optimized in the future. The yields of the plasmids are also very low (250~400 µg/200mL culture for pCmeC-K and 100~150 µg/200 mL culture for pCfrA-K). In addition, DNA extraction using QIAGEN Plasmid Maxi Kit is expensive. In the study of DNA vaccination against chicken IBDV (Pradhan et al. (2014), the yield of DNA vaccine vector pVAXVP₂₅₂₋₄₁₇ was only 0.4 mg/1 L culture using endotoxin-free plasmid gigaprep kit (Qiagen, Hilden, Germany). In order to improve the yields of the DNA vaccines and decrease the expenses, it is very important to develop and choose high-copy expression plasmid.

The on-site prepared chicken feeds used for our vaccination trials did not contain the required vitamin mix. Vitamins are organic compounds and vital nutrients that an organism requires in limited amounts but cannot synthesize in sufficient quantities (Lieberman and Bruning, 1997). Vitamins must be obtained through the diet. As a result, vitamin deficiencies have a variety of negative influences on host such as chickens. The chickens with vitamin A avitaminosis develop clinical signs such as lack of growth, ruffled feathers, weakness and ophthalmia (Aydelotte, 1963; Elvehjem and Neu, 1932).

Vitamin A deficiency can also significantly depress the host immune response and lower resistance to some poultry diseases by compromising the mucosal epithelial barriers and impairing Th2-mediated systemic or mucosal antibody responses to antigens in parasitic, viral and bacterial infections (Carman et al., 1992; Chun et al., 1992; Gangopadhyay et al., 1996; Ross and Hammerling, 1994; Sklan et al., 1994; Stephensen, 2001; Wiedermann et al., 1993). The clinical signs of riboflavin (vitamin B-2) deficiency in chickens include retarded growth rate, curled toe paralysis and reluctance to move (Jortner et al., 1987; Wyatt et al., 1973), which were also observed for the chickens in our vaccination trials. Moreover, a study by Pinkerton and Bessey (1939) has shown that riboflavin deficiency greatly lowered the resistance to endemic typhus and resulted in a fatal disease in rats. Vitamin E also plays an important role in normal immunity development as well as production and function of antibodies in different animals (Erf et al., 1998; Finch and Turner, 1996). Marsh et al. (1986) has reported that, deficiency of vitamin E significantly impaired bursal growth and reduced the lymphocytes in the bursa in Single Comb White Leghorn chickens. For our vaccination trial, we also found that the selenium level in the chicken feed was high. Selenium is an important trace nutrient possessing immune-stimulating properties (Hatfield et al., 2011). However, excess Se in the diet is toxic and impairs immune functions. Green and Albers (1997) reported that mallards which died from more than 20 mg/kg of selenomethionine showed lymphocytic necrosis and atrophy of lymphoid organs (spleen, gut-associated lymphoid tissue and lumbar lymph nodes) and other histologic lesions. Another study has shown that high levels of dietary selenomethionine resulted in splenocyte proliferation, reduced B cell numbers, IL-4, and IL-12 secretion in C57BL/6N female mice (Vega et al., 2007). Excess

dietary sodium selenite has also been demonstrated to cause apoptosis of bursa of Fabricius, lesions of thymus and decreased percentages of the peripheral blood T-cell subsets in chickens (Peng et al., 2011; Peng et al., 2009). Overall, lack of vitamins and high doses of Se in chicken feed led to the poor growth and immunity of birds in our vaccination trial. Therefore, the chicken vaccination experiments need to be repeated in the future with specific emphasis on the quality control of chicken feed.

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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, amp ^r	(Hitoshi et al., 1991)
pCAGGS_CmeC	Full-length <i>cmeC</i> in pCAGGS vector, amp ^r	Jones (2013)
pCAGGS_CfrA	Full-length <i>cfrA</i> in pCAGGS vector, amp ^r	Jones (2013)
pCmeC-K	Full-length <i>cmeC</i> with Kozak sequence, amp ^r	This study
pCfrA-K	Full-length <i>cfrA</i> with Kozak sequence, amp ^r	This study
ptCmeC-K	Truncated <i>cmeC</i> with Kozak sequence, amp ^r	This study
ptCfrA-K	Truncated <i>cfrA</i> with Kozak sequence, amp ^r	This study
Strains		
JL894	<i>E. coli</i> Top10 containing pCAGGS	Dr. Miyazaki (University of Tokyo, Japan)
JL1102	<i>E. coli</i> Top10 containing pCAGGS_CmeC	Jones. (2013)
JL1103	<i>E. coli</i> Top10 containing pCAGGS_CfrA	Jones. (2013)
JL1187	<i>E. coli</i> Top10 containing ptCmeC-K	This study
JL1186	<i>E. coli</i> Top10 containing ptCfrA-K	This study
JL1185	<i>E. coli</i> Top10 containing pCmeC-K	This study
JL1118	<i>E. coli</i> Top10 containing pCfrA-K	This study
JL243	<i>E. coli</i> JM109 containing pCmeC-NHIS	(Zeng et al., 2010)
JL275	<i>E. coli</i> JM109 containing pCfrA-NHIS	(Zeng et al., 2009)

Table 2: Primers used in construction of the DNA vaccines.

Primer	DNA Sequence (5'-3')^a	Product Size	Target gene
pCAGGS_F pCAGGS_R	GAGCCTCTGCTAACCATGTTC TTTTGGCAGAGGGAAAAAGA	N/A	The sequence upstream and downstream of multiple cloning site
pCAGGS_CmeC_F2 PCAGGS_CmeC_R	CCGCTCGAG <i>ACCATGG</i> GATAAAAATAAT TTCAATTAGTGCTATAGC CCGCTCGAGCTATTCTCTAAAAGACA TATCTAAATTTTTTGA	1500 bp	Full-length <i>cmeC</i>
PCAGGS_CmeC_TM2_F PCAGGS_CmeC_TM3_R	CCGCTCGAG <i>ACCATGG</i> CCTTATGAAAA TGAAAATGCTCTT CCGCTCGAGTTACTTGGCTAAATTTA CATTTTGGTAAA	562 bp	Truncated <i>cmeC</i>
pCAGGS_CfrA_F2 pCAGGS_CfrA_R pCAGGS_CfrA-B1-R	CCGCTCGAG <i>ACCATGG</i> AAAAAATAT GTCTATCAGTTTGC CCGCTCGAGTTAAAAGTTACCATTGA TAGAAATATACATTC CCGCTCGAGTTACCATTTATCACTTA CTTTTTTGGTAATG	2112 bp or 513 pb	Full-length or truncated <i>cfrA</i>

^a The restriction enzyme site was underlined. The Kozak sequence (ACCATGG) was highlighted with bold and italic letters.

Table 3. Evaluation of *in ovo* DNA vaccination (Trial 1).

Group	Number of hatched chicks^a	Immunization at 18d of embryonation	Sample collection	<i>C. jejuni</i> challenge on day 14
1	16	100 µL ddH ₂ O	Blood: 14d, 21d, 28d; Cloacal swabs: 14d, 17d, 21d, 24d, 28d	Yes
2	15	50 µg pCAGGS		Yes
3	17	50 µg pCmeC-K		Yes
4	12	50 µg pCfrA-K		Yes

^a To ensure the number of hatched chicks in each group, 17-18 eggs in each group were subjected to *in ovo* vaccination.

Table 4. Evaluation of *in ovo* DNA vaccination (Trial 2).

Group	Number of hatched chicks^a	Immunization at 18d of embryonation	Sample collection	<i>C. jejuni</i> challenge on day 21
1	14	100 µL ddH ₂ O + 100 µL neutral lipid	Blood: 14d, 21d, 30d; Intestinal lavage: 30d; Cloacal swabs: 14d, 17d, 21d, 24d, 30d	Yes
2	11	50 µg pCAGGS + 100 µL neutral lipid		Yes
3	13	50 µg pCmeC-K + 100 µL neutral lipid		Yes
4	14	50 µg pCfrA-K + 100 µL neutral lipid		Yes
5	14	100 µg rCmeC + 100 µL neutral lipid		Yes
6	9	100 µg rCfrA + 100 µL neutral lipid		Yes

^a To ensure the number of hatched chicks in each group, 15-17 eggs were subjected to *in ovo* vaccination.

Table 5. Evaluation of intranasal immunization of chitosan-encapsulated subunit vaccines in chickens (Trial 3).

Group	Number of Chickens	Day 7 Primary Immunization	Day 21 Booster Immunization	Sample collections	<i>C. jejuni</i> challenge on day 35
1	20	PBS		Blood: 21d, 35d, 44d; Intestinal lavage: 21d, 44d; Cloacal swabs: 35d, 37d, 40d, 42d, 44d	Yes
2	20	CM			Yes
3	20	CM-rCmeC(100 µg)			Yes
4	20	CM-pCmeC-K (50 µg)			Yes
5	20	CM-rCfrA(100 µg)			Yes
6	20	CM-pCfrA-K (200 µg)			Yes

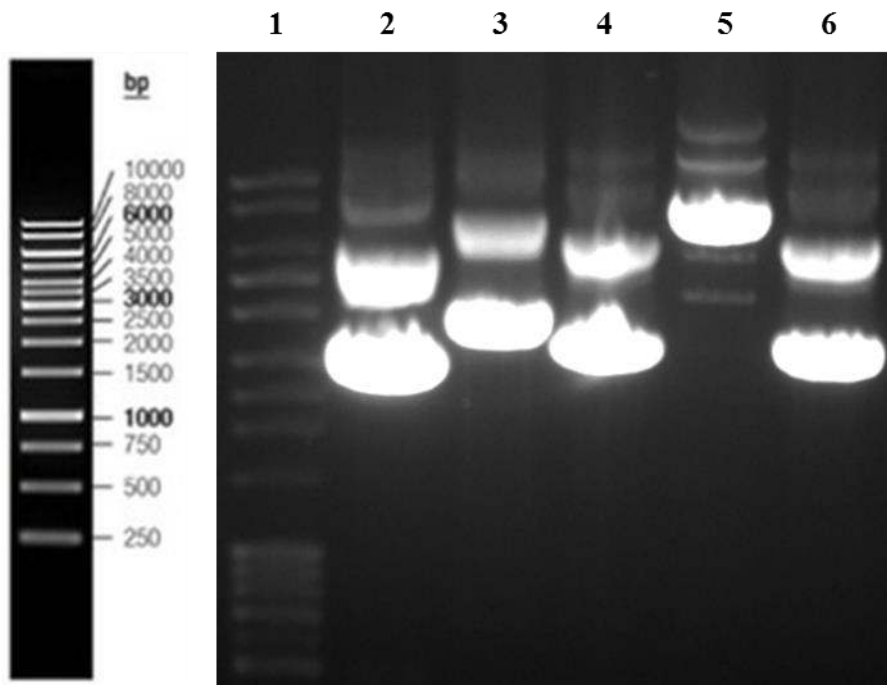


Figure 1. Construction of modified DNA vaccine vectors.

The plasmids were analyzed by 0.8% agarose gel electrophoresis. Lane 1: standard 1 kb ladder; Lane 2: pCAGGS; Lane 3: pCmeC-K; Lane 4: ptCmeC; Lane 5: pCfrA-K; Lane 6: ptCfrA-K

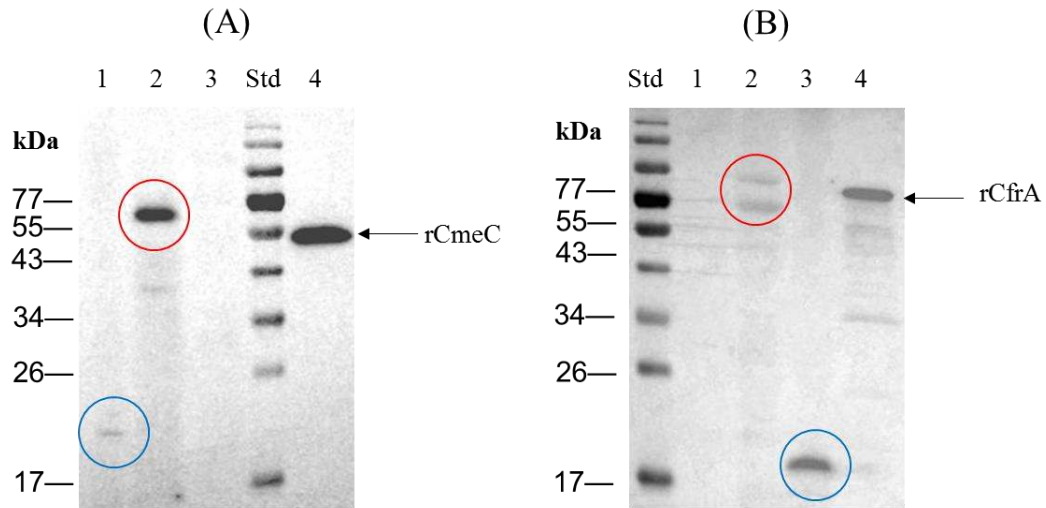


Figure 2. Expression of target gene by the modified DNA vaccine vectors in transfected eukaryotic cells.

A). Immunoblot analysis of CmeC expression by the modified DNA vaccine vectors in HEK-293 cells. Lane 1: the HEK-293 cells transfected with ptCmeC-K; Lane 2: the HEK-293 cells transfected with pCmeC-K; Lane 3: the HEK-293 cells transfected with pCAGGS (negative control); Lane 4: purified recombinant CmeC (positive control). B). Immunoblot analysis of CfrA expression by modified DNA vaccine vectors in HEK-293 cells. Lane 1: the HEK-293 cells transfected with pCAGGS (negative control); Lane 2: the HEK-293 cells transfected with pCfrA-K; Lane 3: the HEK-293 cells transfected with ptCfrA-K; Lane 4: purified recombinant CfrA (positive control). The inserted gene products are highlighted by circles.

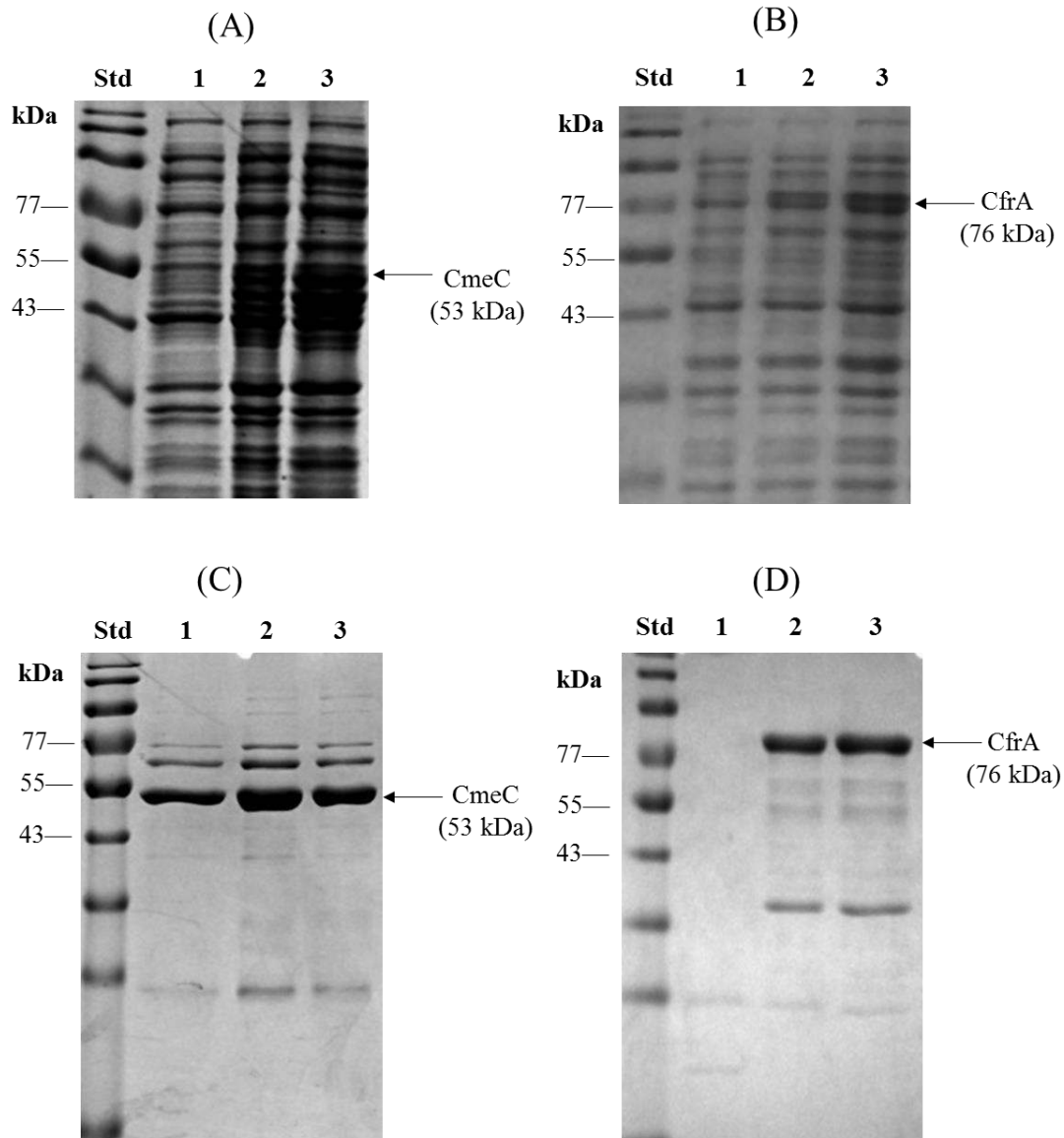


Figure 3. SDS-PAGE analysis of purified recombinant proteins.

A). IPTG induction of CmeC. The whole cell lysate of *E. coli* JL243 after 1 hr (lane 1), 2 hr (lane 2), and 3 hr (lane 3) of IPTG induction. B). IPTG induction of CfrA. The whole cell lysate of *E. coli* JL275 after 1 hr (lane 1), 2 hr (lane 2), and 3 hr (lane 3) of IPTG induction. C). Purification of His-tagged rCmeC using Ni-NTA affinity chromatography. Lane 1: Elution 1; Lane 2: Elution 2; Lane 3: Elution 3. D). Purification of His-tagged rCfrA using Ni-NTA affinity chromatography. Lane 1: Elution 1; Lane 2: Elution 2; Lane 3: Elution 3.

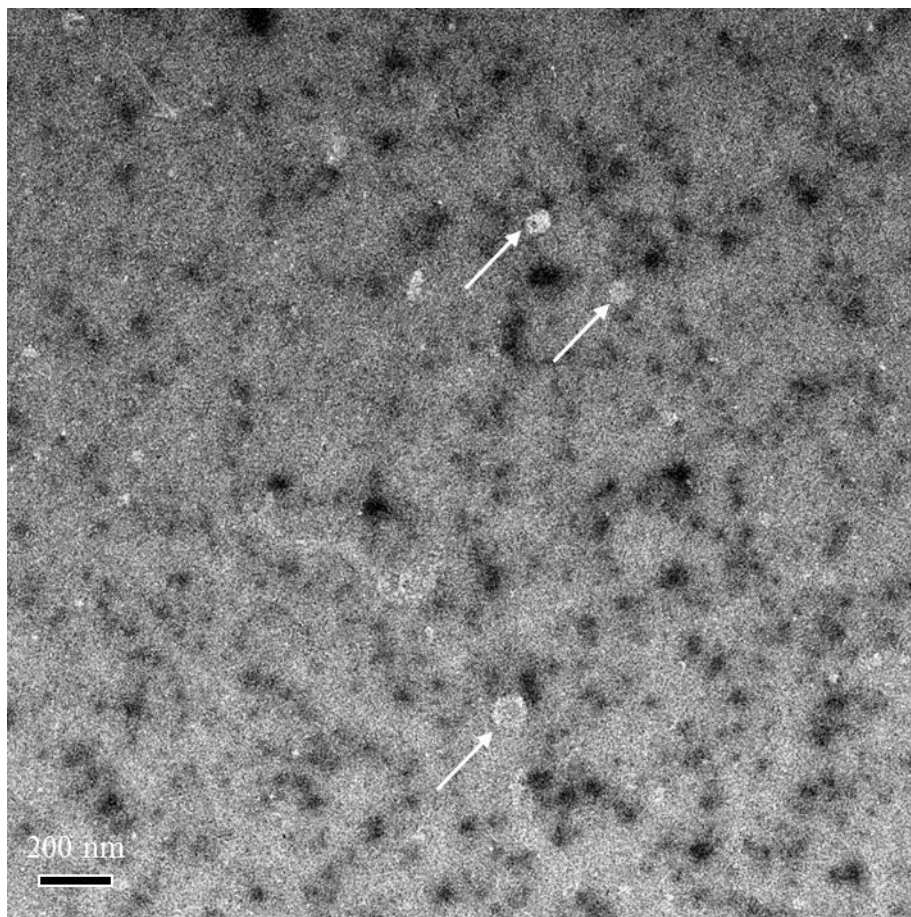


Figure 4. Transmission electron microscopy image of CM-pCmeC.

Scale bar represents 200 nm. Freshly prepared CM-pCmeC nanoparticles were approximately 50~100 nm in size and nearly spherical under TEM.

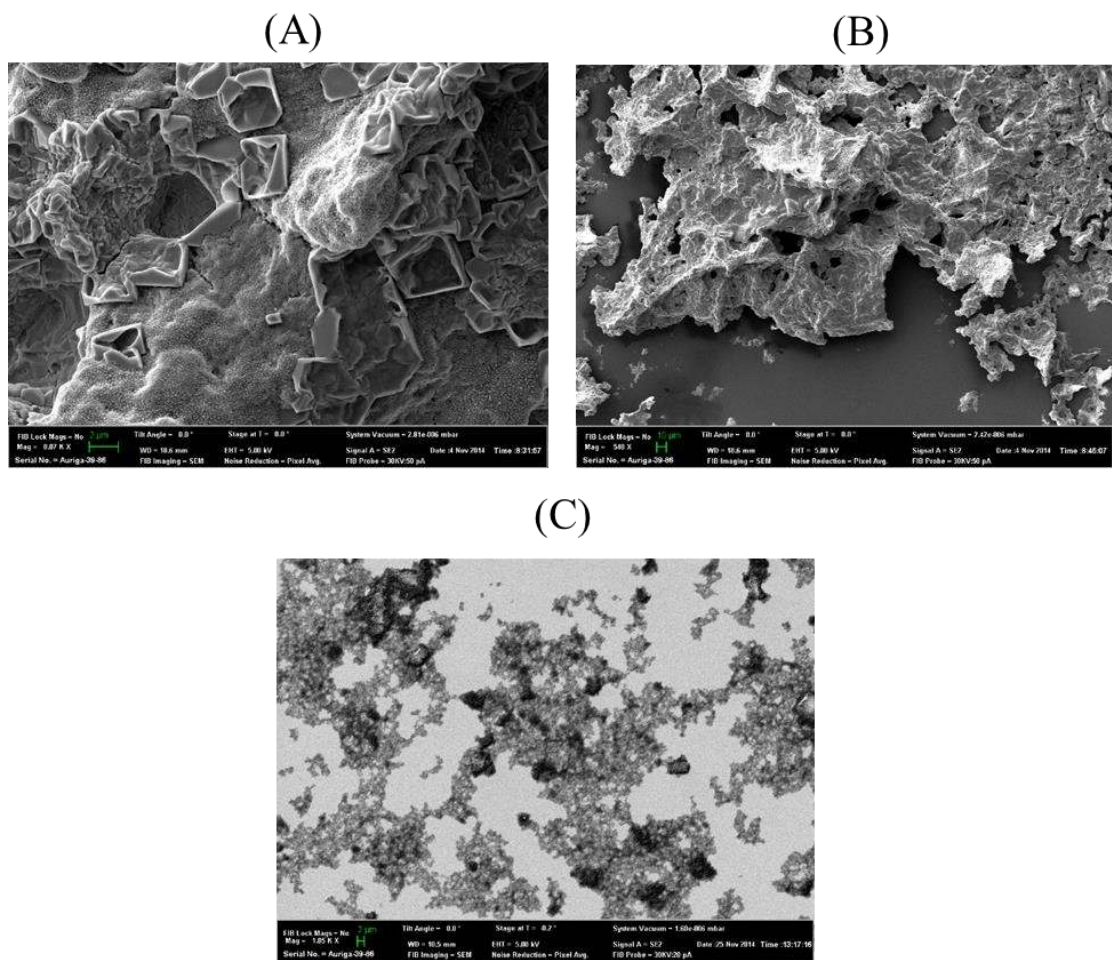


Figure 5. Scanning electron microscopy of Chitosan/Pluronic-F127-proteins with tripolyphosphate (TPP).

A). Chitosan/Pluronic-F127-PBS control. B). 3:1 Chitosan/Pluronic-F127-CmeC. C). 3:1 Chitosan/Pluronic-F127-CmeC diluted ten times in ddH₂O.

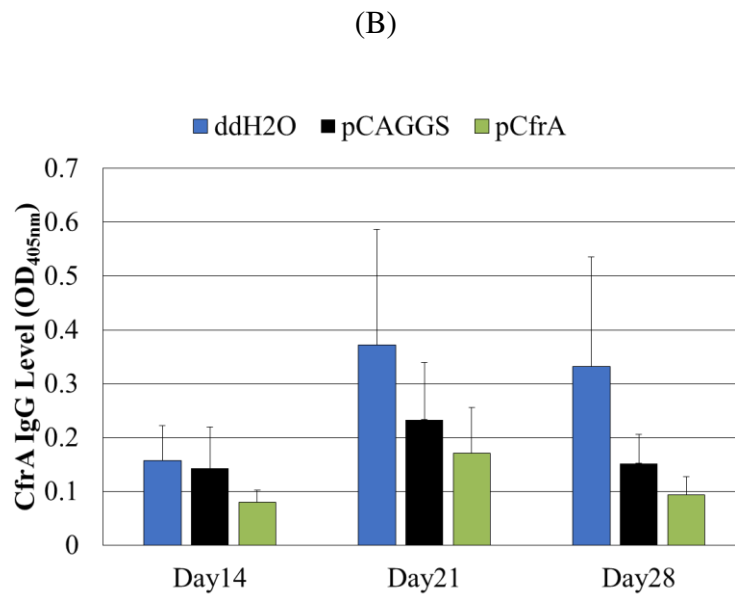
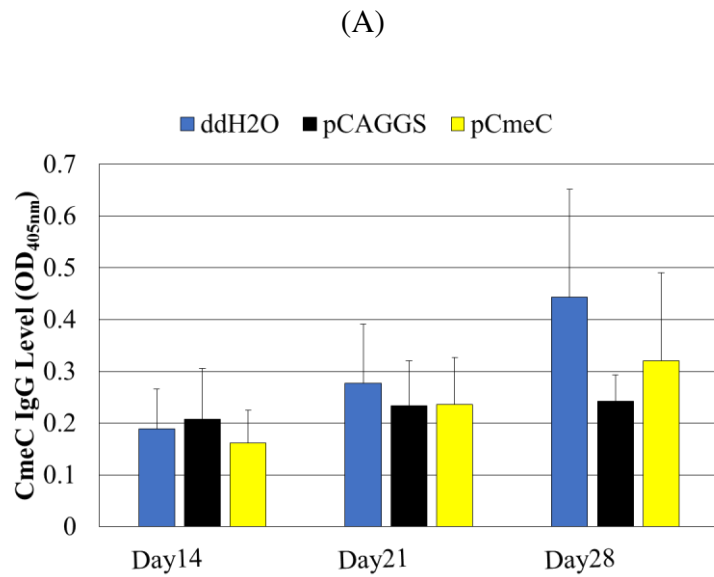


Figure 6. Systemic IgG response following *in ovo* vaccination (Trial 1).

Indirect ELISA analysis of systemic IgG level to CmeC (A) or CfrA (B). Serum samples were collected at day 14 (prior to challenge), day 21 and day 28. Error bars represent standard deviation.

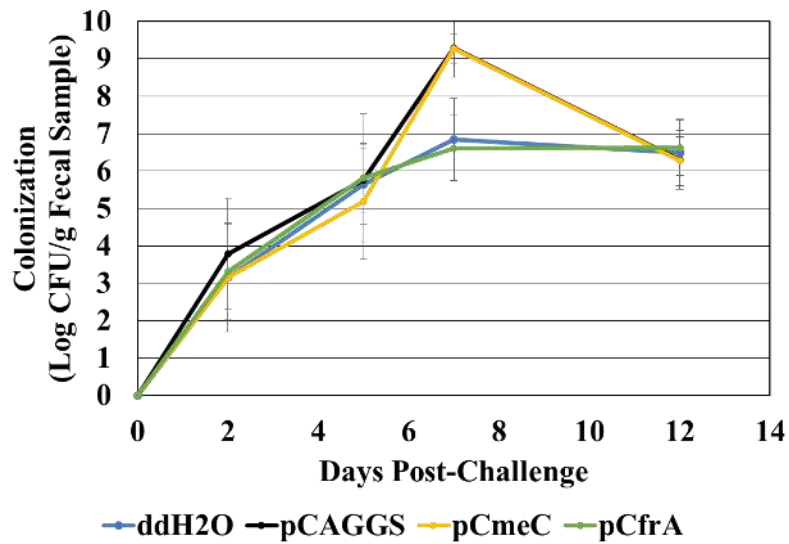
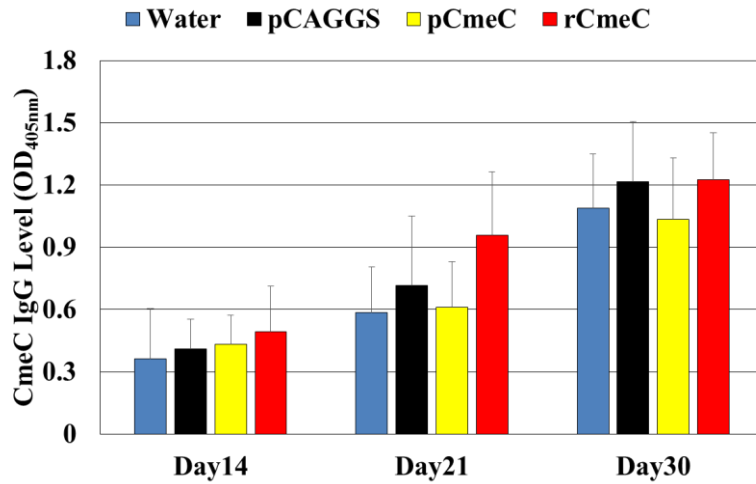


Figure 7. Shedding levels of *C. jejuni* in different vaccination groups (Trial 1).

Colonization levels of *C. jejuni* in different vaccination groups at day 0, day 2, day 5, day 7 and day 12 after chickens were challenged with *C. jejuni* NCTC 11168. Error bars represent standard deviation.

(A)



(B)

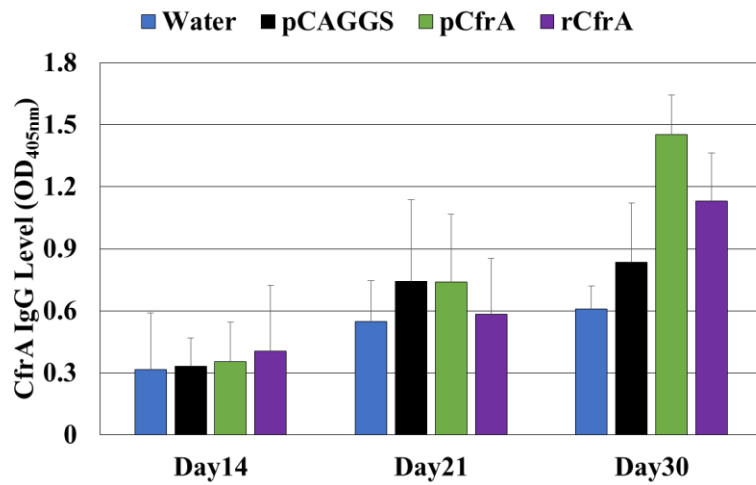
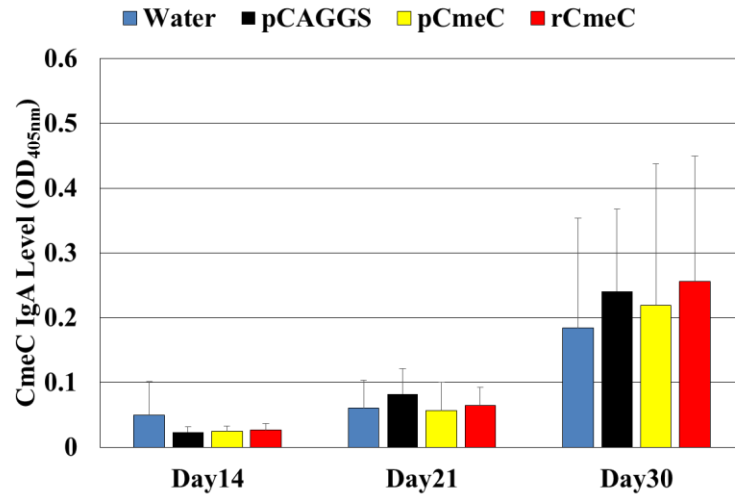


Figure 8. Systemic IgG response following *in ovo* vaccination (Trial 2).

Indirect ELISA analysis of systemic IgG level to CmeC (A) or CfrA (B). Serum samples were collected at day 14, day 21 (prior to challenge), and day 30. Error bars represent standard deviation.

(A)



(B)

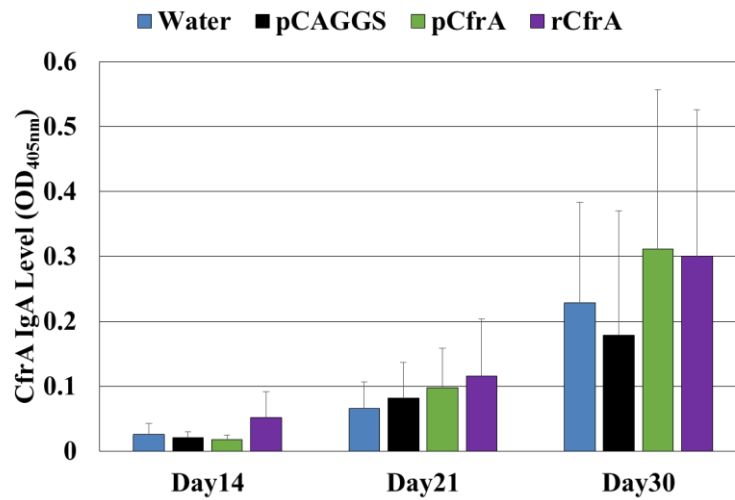
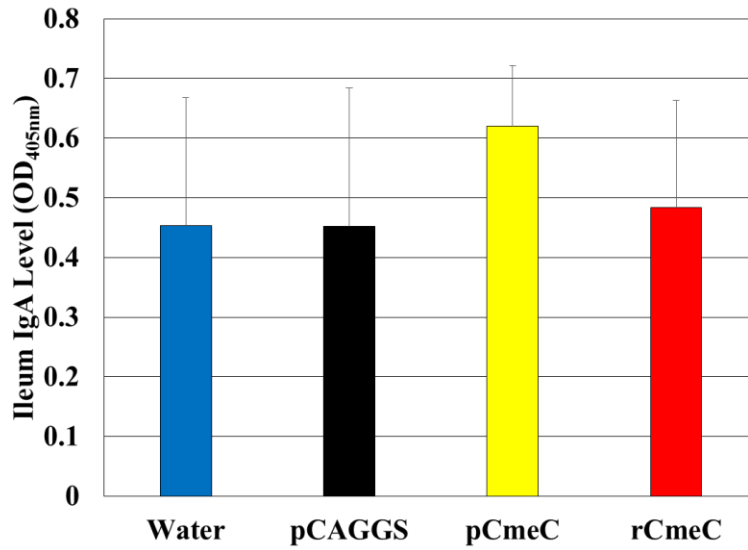


Figure 9. Systemic IgA response following *in ovo* vaccination (Trial 2).

Indirect ELISA analysis of systemic IgA level to CmeC (A) or CfrA (B). Serum samples were collected at day 14, day 21 (prior to challenge), and day 30. Error bars represent standard deviation.

(A)



(B)

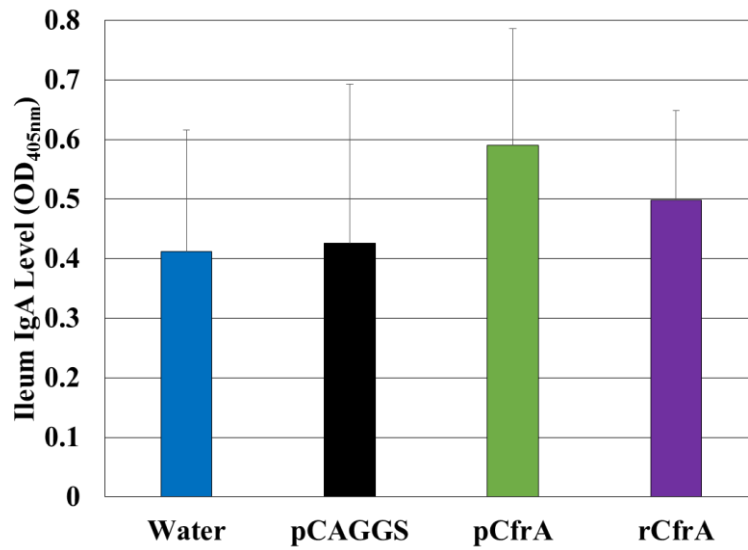


Figure 10. Intestinal mucosal IgA response following *in ovo* vaccination (Trial 2).

Indirect ELISA analysis of intestinal mucosal IgA level to CmeC (A) or CfrA (B). Intestinal lavages were collected at day 30. Error bars represent standard deviation.

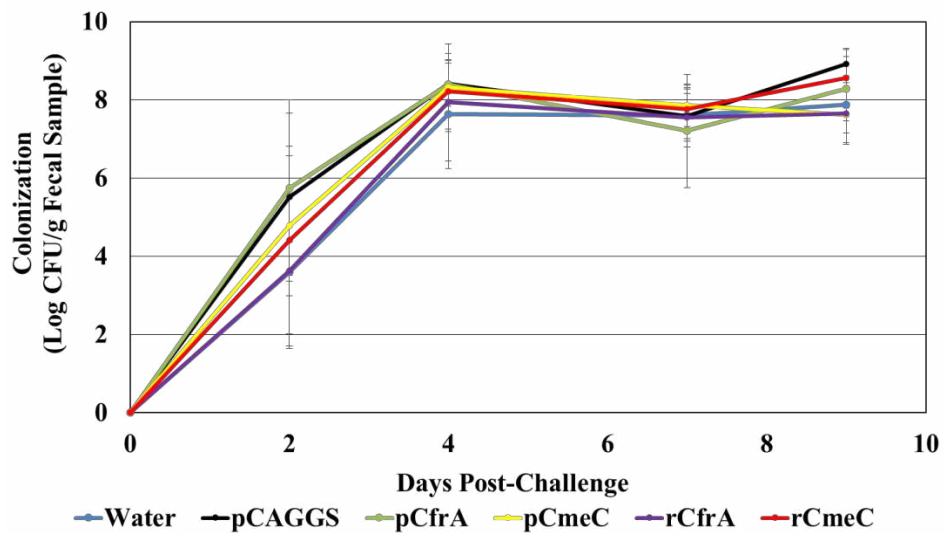


Figure 11. Shedding levels of *C. jejuni* in different vaccination groups (Trial 2).

Colonization levels of *C. jejuni* in different vaccination groups at day 0, day 2, day 4, day 7 and day 9 after chickens were challenged with *C. jejuni* NCTC 11168. Error bars represent standard deviation.

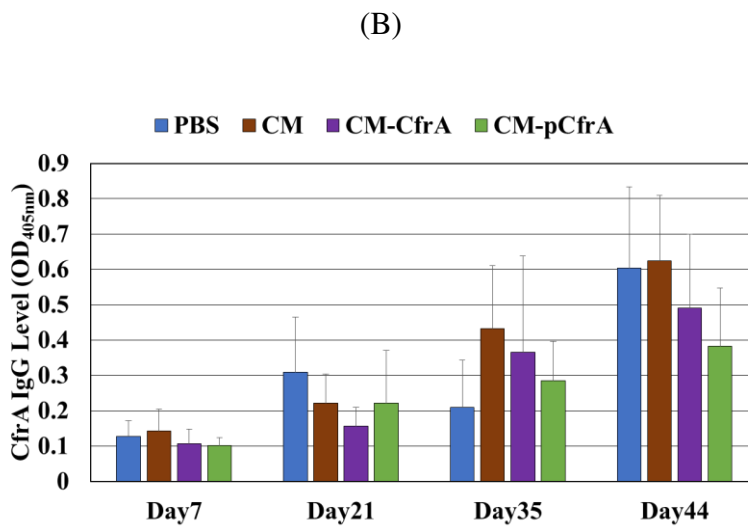
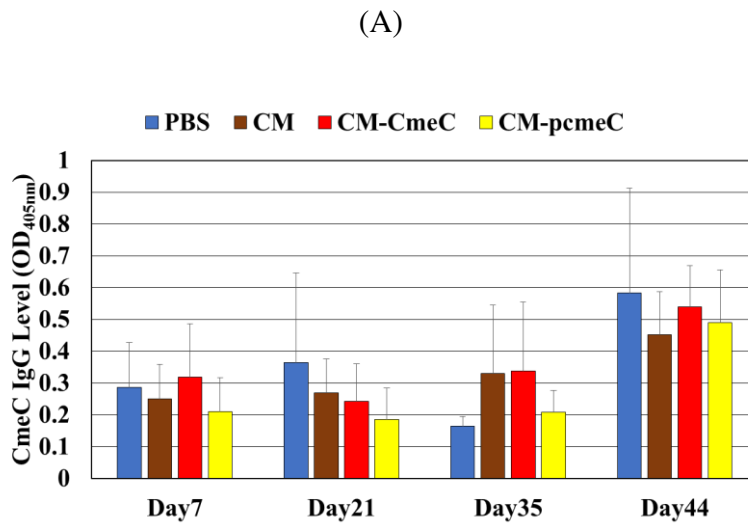
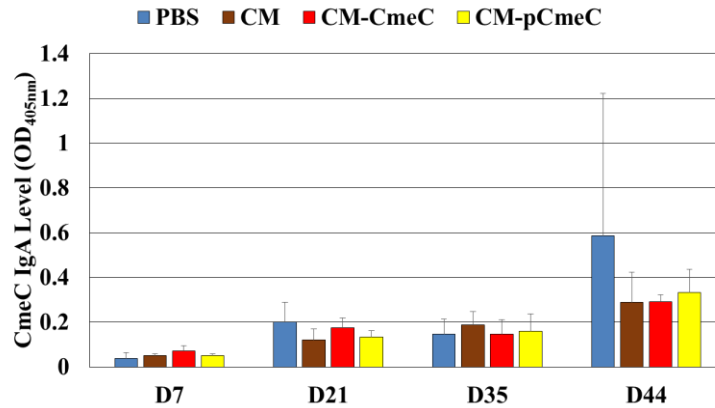


Figure 12. Systemic IgG response following intranasal vaccination with chitosan-encapsulated subunit vaccines (Trial 3).

Indirect ELISA analysis of systemic IgG level to CmeC (A) or CfrA (B). Serum samples were collected at day7, day 21 (prior to challenge), day 35 and day 44. Error bars represent standard deviation.

(A)



(B)

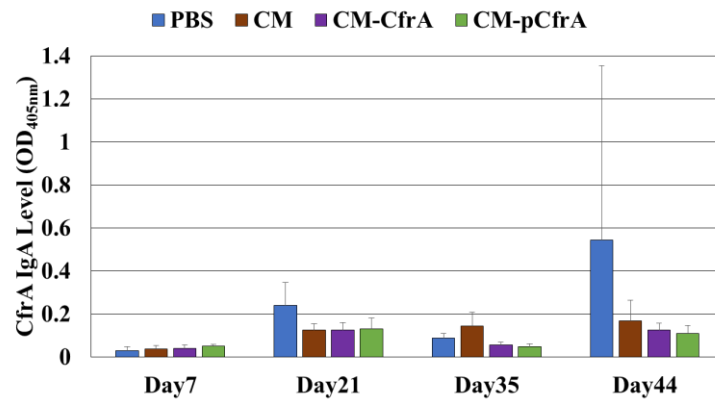


Figure 13. Systemic IgA response following intranasal vaccination with chitosan-encapsulated subunit vaccines (Trial 3).

Indirect ELISA analysis of systemic IgA level to CmeC (A) or CfrA (B). Serum samples were collected at day7, day 21(prior to challenge), day 35 and day 44. Error bars represent standard deviation.

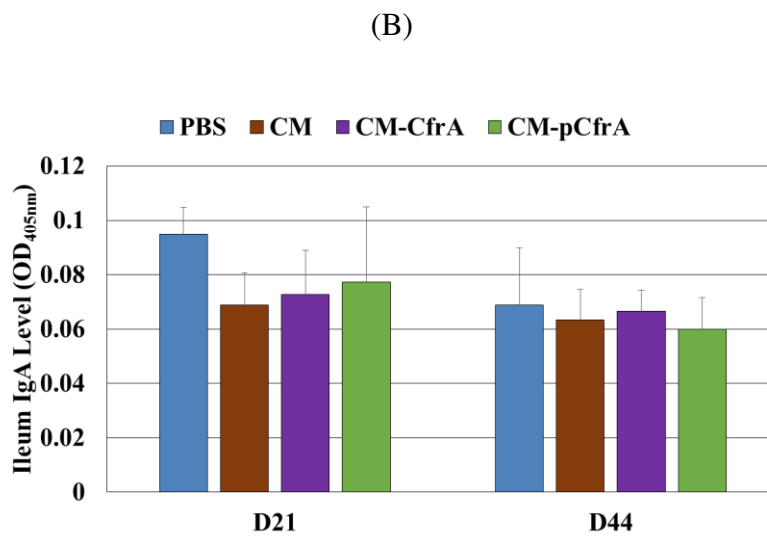
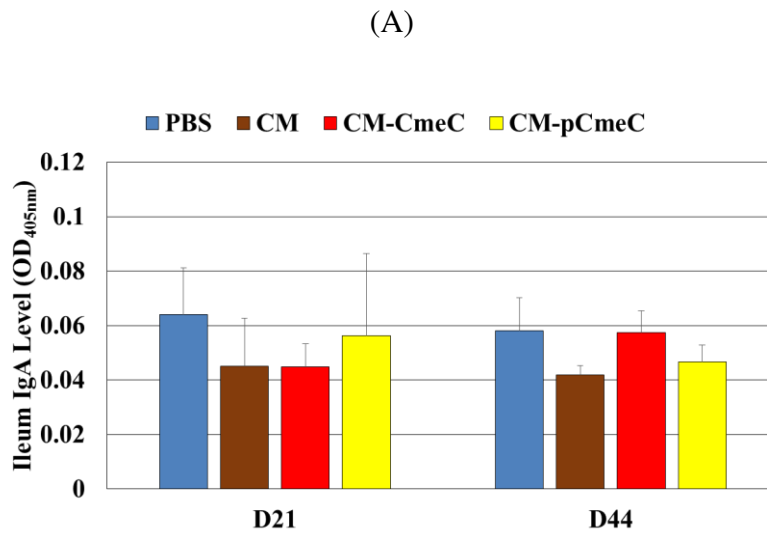


Figure 14. Intestinal mucosal IgA response following intranasal vaccination with chitosan-encapsulated subunit vaccines (Trial 3).

Indirect ELISA analysis of intestinal mucosal IgA level to CmeC (A) or CfrA (B). Intestinal lavages were collected at day 21(prior to challenge) and day 44. Error bars represent standard deviation.

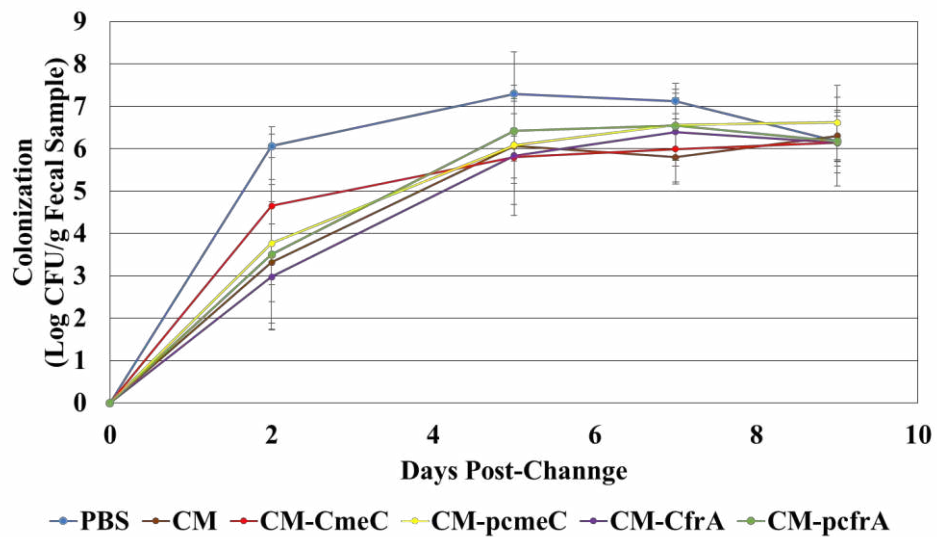


Figure 15. Shedding levels of *C. jejuni* in different vaccination groups (Trial 3).

Colonization levels of *C. jejuni* in different vaccination groups at day 0, day 2, day 5, day 7 and day 9 after chickens were challenged with *C. jejuni* NCTC 11168. Error bars represent standard deviation.

VITA

Xiang Liu was born on August 9, 1986 and grew up in Wuhan, China. In 2005, he was enrolled in Huazhong Agricultural University, where he received a bachelor's degree in Veterinary Medicine in 2010. In the same year, he began employment as a veterinarian in Wuhan Zoo in Wuhan, China. In 2013, he enrolled in the Master's Program in the Department of Animal Science at the University of Tennessee in Knoxville, TN. In the winter of that year, he married Liang Yin.