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Evaluation of *in ovo* vaccination of DNA vaccines for *Campylobacter* control in broiler chickens

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ABSTRACT

Campylobacter is the leading bacterial cause of human enteritis in developed countries. Chicken is a major natural host of *Campylobacter*. Thus, on-farm control of *Campylobacter* load in poultry would reduce the risk of human exposure to this pathogen. Vaccination is an attractive intervention measure to mitigate *Campylobacter* in poultry. Our previous studies have demonstrated that *Campylobacter* outer membrane proteins CmeC (a component of multidrug efflux pump) and CfrA (ferric enterobactin receptor) are feasible and promising candidates for vaccine development. In this study, by targeting these two attractive vaccine candidates, we explored and evaluated a new vaccination strategy, which combines the *in ovo* vaccination route and novel DNA vaccine formulation, for *Campylobacter* control in broilers. We observed that direct cloning of *cfrA* or *cmeC* gene into the eukaryotic expression vector pCAGGS did not lead to sufficient level of production of the target proteins in the eukaryotic HEK-293 cell line. However, introduction of the Kozak consensus sequence (ACCATGG) in the cloned bacterial genes greatly enhanced production of inserted gene in eukaryotic cells, creating desired DNA vaccines. Subsequently, the validated DNA vaccines were prepared and used for two independent *in ovo* vaccination trials to evaluate their immune response and protective efficacy. However, single *in ovo* injection of specific DNA vaccine at 18th day of embryonation, regardless using neutral lipid-protected vector or not, failed to trigger significant IgG and IgA immune responses and did not confer protection against *C. jejuni* colonization in the intestine of chickens. In conclusion, this study demonstrates that the Kozak sequence is critically important for construction of the DNA vaccine expressing prokaryotic gene. The optimal regimen for *in ovo* vaccination of DNA vaccine for *Campylobacter* control in poultry needs to be determined in future studies.

1. 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 [1]. This pathogenic organism causes watery diarrhea, fever, and abdominal cramping in patients and is unusually associated with Guillain-Barre Syndrome, an acute flaccid muscular paralysis that may result in respiratory muscle compromise and death [2,3]. Poultry is considered one of the major reservoir of *Campylobacter* and therefore the main source of human campylobacteriosis [4,5]. On-farm control of *Campylobacter* in poultry would therefore reduce the risk of human campylobacteriosis and have a significant impact on food safety and public health. However, to date, there is still no effective and practical vaccine available to control *Campylobacter* infections in poultry, primarily due to lack of effective and practical vaccination strategies [6,7].

To achieve the goal of developing effective, safe, inexpensive, and convenient vaccination strategies that could be practically used to in poultry for mitigation of *C. jejuni*, 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, we have identified and characterized two surface-exposed proteins, CmeC and CfrA, that play an essential role in *C. jejuni* colonization in the chicken intestine [8–13]. CfrA is a surface-exposed 'gatekeeper' that is essential for *C. jejuni* colonization by mediating ferric enterobactin high affinity iron acquisition [11]. CmeC is an essential outer membrane protein component of CmeABC multidrug efflux that plays a critical role in multidrug resistance and *C. jejuni* colonization [8,9,12,13]. The following findings from our previous studies provided compelling evidence that both CmeC and CfrA have significant advantages compared to other

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immunogenic/protective antigens identified in *C. jejuni*: (1) both CfrA and CmeC are immunogenic in poultry and elicit a specific antibody response during *C. jejuni* infection in poultry [9–11]; (2) CfrA and CmeC specific antibodies greatly inhibited the function of corresponding target and significantly reduced growth of *C. jejuni* [11,12]; (3) both CfrA and CmeC are prevalent and highly conserved in diverse *C. jejuni* strains [9,11,12]; (4) CfrA and CmeC are highly induced and produced in the intestinal tract [9–12]; 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 [13]. Clearly, these comprehensive molecular, immunogenic, functional studies have shown that CmeC and CfrA are promising candidates for developing an effective vaccine against *C. jejuni* in poultry.

Vaccination at embryonation day 18 has proven to be a safe, effective, and convenient method for vaccination of chickens against viral, bacterial and protozoal diseases [14]. In addition, DNA vaccine, the eukaryotic expression plasmid bearing the gene of desired target antigen, has been successfully developed to prevent and control infectious diseases and other disease in human and animals [15,16]. DNA vaccination has various advantages, including the ability to induce both cellular and humoral immune response, lack of risk for infection, long-term persistence of immunogen, easiness of manufacture, and stability for shipping and storage [16,17]. More importantly, when combined with *in ovo* vaccination, DNA vaccination could offer various advantages for poultry [18]. Therefore, by targeting the two attractive vaccine candidates CfrA and CmeC, in this study, we explored and evaluated the immunogenicity and protective efficacy of a new vaccination strategy, which includes the *in ovo* vaccination route and DNA vaccine formulation, for *Campylobacter* control in broilers.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The major bacterial strains and plasmids used in this study, and their sources, are listed in Table 1. The standard *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 *Campylobacter* Growth and Preston *Campylobacter* Supplements (Oxoid, Bashingstoke, Hampshire, England). The *E. coli* strains were grown on Luria-Bertani (LB, BD Difco) plates containing 50 μg/mL of ampicillin (Amp) or in LB broth containing 50 μg/mL of Amp with shaking (250 rpm) at 37 °C overnight.

2.2. PCR

Primers used in this study and the expected sizes of the products are listed in Table 2. 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 slightly varied according to estimated product sizes (1 min per kb for extension at 72 °C). PCR products were further purified with the QIAquick Purification Kit (Qiagen) for cloning procedures or sequencing analysis.

2.3. Construction of DNA vaccines.

The pCAGGS vector (Table 1) 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) [19]. The full-length *cfrA* and *cmeC* fragments from *C. jejuni* NCTC 11168 were first 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 products were 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. 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 using vector-specific primer together with inserted gene-specific primer for identification of the recombinant plasmids with correct orientation of specific inserted gene. The identified desired recombinant plasmids pCAGGS_CfrA and pCAGGS_CmeC (DNA vaccines) were finally subjected to sequence analysis to confirm the orientation and integrity of the inserted fragment.

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	[29]
pCAGGS_CmeC	Full-length *cmeC* cloned in pCAGGS vector, Amp^r	This stud
pCAGGS_CfrA	Full-length *cfrA* cloned in pCAGGS vector, Amp^r	This study
pCmeC-K	Full-length *cmeC* with Kozak sequence, Amp^r	This study
pCfrA-K	Full-length *cfrA* with Kozak sequence, Amp^r	This study
ptCmeC-K	Truncated *cmeC* with Kozak sequence, Amp^r	This study
ptCfrA-K	Truncated *cfrA* with Kozak sequence, Amp^r	This study
pR-M02	Control vector (with EGFP) for pReceiver-M02	Genecopoeia
Strains		
JL241	*Campylobacter jejuni* NCTC 11,168	[11]
JL275	*E. coli* JM109 containing pCfrA-NHIS for production of rCfrA	[11]
JL243	*E. coli* JM109 containing pCmeC-NHIS for production of rCmeC	[12]
JL1102	*E. coli* Top10 containing pCAGGS_CmeC	This study
JL1103	*E. coli* Top10 containing pCAGGS_CfrA	This study
JL1187	*E. coli* Top10 containing ptCmeC-K	This study
JL1186	*E. coli* Top10 containing ptCfrA-K	This study
JL1185	*E. coli* Top10 containing pCmeC-K	This study
JL1118	*E. coli* Top10 containing pCfrA-K	This study

Table 2
The primers used for construction of 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_F pCAGGS_CmeC_R	CCGCTCGAGATGAATAAAATAATTTCAATTAGTGCTATAGC CCGCTCGAGCTATTCTCTAAAAGACATATCTAAATTTTTTGA	1479 bp	Full-length *cmeC*
pCAGGS_CmeC_F2 PCAGGS_CmeC_R PCAGGS_CmeC_TM2_F PCAGGS_CmeC_TM3_R	CCGCTCGAG***ACCATGG***ATAAAATAATTTCAATTAGTGCTATAGC CCGCTCGAGCTATTCTCTAAAAGACATATCTAAATTTTTTGA CCGCTCGAG***ACCATGG***CTTATGAAAATGAAAATGCTCTT CCGCTCGAGTTACTTGGCTAAATTTACATTTTGGTAAA	1500 bp 562 bp	Full-length *cmeC* with Kozak sequence Truncated *cmeC* Kozak sequence
pCAGGS_CfrA_F pCAGGS_CfrA_R	CCGCTCGAGATGAAAAAAATATGTCTATCAGTTTGC CCGCTCGAGTTAAAAGTTACCATTGATAGAAATATACATTC	2091 bp	Full-length *cfrA*
pCAGGS_CfrA_F2 pCAGGS_CfrA_R pCAGGS_CfrA-B1-R	CCGCTCGAG***ACCATGG***AAAAAATATGTCTATCAGTTTGC CCGCTCGAGTTAAAAGTTACCATTGATAGAAATATACATTC CCGCTCGAGTTACCATTTATCACTTACTTTTTTGGTAATG	2112 bp or 513 pb	Full-length or truncated *cfrA* Kozak sequence

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

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). The procedure for cloning the PCR fragments into pCAGGS vector are the same as described above.

2.4. Validation of DNA vaccines

Transfection was performed to validate the production of CfrA or CmeC in eukaryotic cells by specific DNA vaccine. Briefly, 4 μg of recombinant plasmid 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. The cells transfected with PBS, the pR-M02 plasmid expressing eGFP, or the original pCAGGS vector served as control. After 5–6 h 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 h 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 below. Transfections were performed in duplicate.

2.5. Production of high-purity recombinant CfrA (rCfrA) and rCmeC

The *E. coli* constructs for producing N-terminal Histidine-tagged rCmeC (JL243) and rCfrA (JL275) were from our recent studies [11,12]. The full-length Histidine-tagged rCmeC and rCfrA were purified from *E. coli* culture using Ni^{2+}-NTA affinity chromatography as described previously with modifications [11,12]. 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 prior to use. The concentration of the recombinant proteins was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce).

2.6. SDS-PAGE and immunoblotting

Five μL of the above whole cell lysate suspension was loaded in each lane and separated by SDS-PAGE with a 12% (w/v) polyacrylamide gel at 80 V for 25 min followed by 160 V for 40 min by electrophoresis. Following SDS-PAGE, proteins in gels were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) in transfer buffer at 90 V for 1 h. The membrane was then incubated in blocking buffer (5% Nestle skim milk powder in PBS) for 1 h 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-CmeC 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.

2.7. Production of DNA vaccine

The control plasmid pCAGGS and the two modified DNA vaccine vectors (pCmeC-K and pCfrA-K) that can produce full-length target proteins in eukaryotic cells were extracted from the *E. coli* JL894, JL1185, and JL1118 (Table 1), respectively, using the QIAGEN Plasmid Maxi Kit (Qiagen, Hilden, Germany). The concentration and purity of plasmid DNA was determined by NanoDrop®.

2.8. In ovo DNA vaccination experiments

In Trial #1 (Table 3), 70 embryonated eggs laid by Cobb 500 broiler breeder hens 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 μL of sterile water, pCAGGS plasmid, 50 μg pCmeC-K in 100 uL sterile water, or 50 μg pCfrA-K in 100 μL sterile water into the amniotic fluid as described previously [20,21]. The 23 gauge needle with 1 in. in length was used for injection and transparent scotch tape was used to seal the injection site. The

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

Group	Agent for *in ovo* injection	Number of hatched chicks	Sample collection	*C. jejuni* challenge on day 14
1	100 μL ddH_2O	16	**Blood**: 14d, 21d, 28d;	Yes
2	50 μg pCAGGS	15	**Cloacal swabs:** 14d, 16d, 19d, 21d, 26d	Yes
3	50 μg pCmeC-K	17		Yes
4	50 μg pCfrA-K	12		Yes

treated eggs were put back into the incubator together with the eggs in control group for an additional three days of incubation until chicks were hatched. After hatch, all chicks were kept in clean wire-floored cages and provided with water and antibiotic-free feed *ad libitum*. At day 14 post-hatch, all the chickens were challenged orally with *C. jejuni* NCTC 11168 with a dose of 10^4 CFU per chicken. Cloacal swabs from each bird were collected every 2–5 days from day 14 to day 28 post-hatch and suspended in 100 μL of MH broth. The samples were then spread on MH plates with a dilution of 1:1, 1:100 and $1:10^4$ and incubated at 42 °C under microaerophilic condition for 48 h 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 Trial #2 (Table 4), there were several significant modifications 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 [22]. Second, rCmeC and rCfrA proteins (50 μg per egg) were included as controls. Finally, the challenge date was delayed to 21 days of age. Briefly, at day 18 of embryonation, eggs were randomly divided into 6 treatment groups (15–17 eggs per group) for *in ovo* injection (Table 4). Chicken management, *C. jejuni* challenge, blood and cloacal sampling are 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, 0.05 g/mL of EDTA, and complete mini protease inhibitor [Roche, prod. No: 04693159001]) by a ratio of 1:10 (1 g of intestinal content:10 mL of lavage extraction buffer); the intestinal lavages were used for determining specific mucosal immune response using ELISA as detailed below.

2.9. Enzyme-linked immunosorbent assay (ELISA)

An anzyme-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 μL 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 h. Plates were washed with 200 μL washing solution (0.5% Tween 20 in PBS) using a plate washer and blotted dry. Plates were then blocked with 100 μL 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. 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 incubation at 37 °C for one hour, plates were washed and ABTS peroxidase substrate (KPL) was added to each well. 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).

3. Results

3.1. Construction of DNA vaccines

The pCAGGS_CfrA and pCAGGS_CmeC recombinant plasmids that carry the original cloned genes (Table 1) were successfully constructed as shown by no any frameshift or mutations detected in the cloned complete full-length of *cfrA* and *cmeC* gene. However, production of CfrA or CmeC by the DNA vaccines in transfected HEK-293 cells were not detected by immunoblotting assay even if large quantities of transfected HEK-293 cells were loaded for SDS-PAGE (data not shown). We have ruled out the issue with respect to transfection. As shown in Fig. 1A, the control plasmid pR-M02 that encodes eGFP could transfect HEK-293 cells as visualized through fluorescent microscopy, demonstrating the success of the standard transfection procedure. Given that immunoblotting using specific antibodies failed to detect CfrA and CmeC from the cells transfected with pCAGGS_CfrA and pCAGGS_CmeC, respectively, the production levels of cloned *cfrA* and *cmeC* are too low to be detected.

We speculated such low production level of the cloned bacterial gene is likely due to the lack of Kozak consensus sequence that plays a major role in the initiation of the translation process in eukaryotic cells. To test this, subsequently we constructed new DNA vaccines by introducing Kozak consensus sequence (ACCATGG) immediately upstream of specific cloned gene. Two

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

Group	Agent for *in ovo* injection	Number of hatched chicks	Sample collection	*C. jejuni* challenge on day 21
1	100 μL ddH_2O + 100 μL neutral lipid	14	**Blood**: 14d, 21d, 30d	Yes
2	50 μg pCAGGS + 100 μL neutral lipid	11	**Intestinal lavage:** 30d	Yes
3	50 μg pCmeC-K + 100 μL neutral lipid	13	**Cloacal swabs:** 21d, 23d, 25d, 28d, 30d	Yes
4	50 μg pCfrA-K + 100 μL neutral lipid	14		Yes
5	100 μg rCmeC + 100 μL neutral lipid	14		Yes
6	100 μg rCfrA + 100 μL neutral lipid	9		Yes

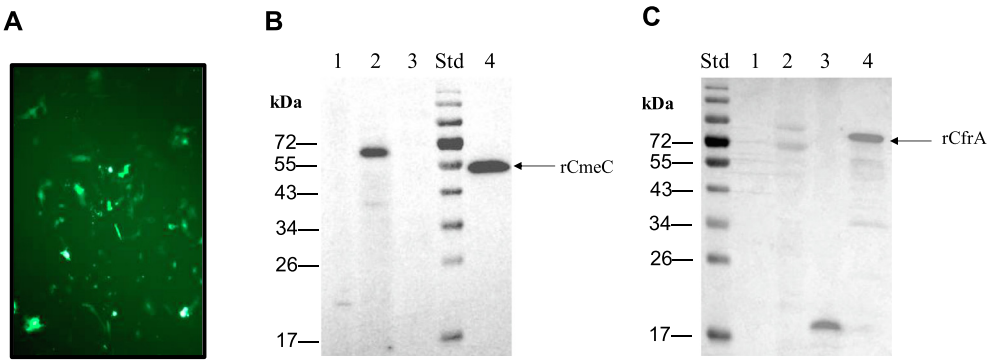

Fig. 1. Validation of DNA vaccine for production of CmeC or CfrA in transfected eukaryotic cells. (A) Fluorescent microscopy of the HEK-293 cells transfected with an eGFP expressing plasmid, (B) Immunoblot analysis of CmeC expression by the DNA vaccines with Kozak sequence modification in HEK-293 cells. The cells were transfected with ptCmeC-K (lane 1), pCmeC-K (lane 2), or control pCAGGS (lane 3). The purified rCmeC was used as positive control (lane 4), and (C) Immunoblot analysis of CfrA expression by the DNA vaccines with Kozak sequence modification in HEK-293 cells. The cells were transfected with control pCAGGS (lane 1), pCfrA-K (lane 2), and ptCfrA-K (lane 3). The purified rCfrA was used as positive control (lane 4).

CmeC DNA vaccines with modified Kozak sequence pCmeC-K and ptCmeC-K, were successfully constructed and were expected to produce a full-length and truncated CmeC, respectively (Table 1). Similarly, two modified 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). The reason for construction of recombinant plasmids expressing truncated version of CmeC or CfrA is that production of full-length CfrA or CmeC may be toxic for the transfected host cells.

With molecular manipulation by introducing Kozak consensus sequence, immunoblotting assay showed that both truncated and full-length CmeC (Fig. 1B) and CfrA (Fig. 1C) were produced in HEK-293 cells. Regarding CmeC, the production level of full-length CmeC (Lane 2, Fig. 1B) appears to be much higher than the truncated one with molecular mass approximately 20 kDa (Lane 1, Fig. 1B); it is also possible the truncated CmeC only has weak epitopes for the CmeC antiserum used for immunoblotting assay. Compared to the control His-tagged rCmeC (~53 kDa in Lane 4, Fig. 1B), the CmeC produced in HEK-293 cells showed larger molecular mass by displaying a significant band shift (Lane 2, Fig. 1B); this phenomenon is not surprising because the CmeC is very likely subjected to posttranslational modifications, such as glycosylation and phosphorylation. With respect to CfrA, both full-length CfrA (Lane 2, Fig. 1C) and truncated product (Lane 3, Fig. 1C) were produced in HEK-293 cells. Compared to the control rCfrA (76 kDa in lane 4, Fig. 1C), the full-length CfrA produced in HEK-293 cells showed two bands; the band with molecular mass more than 76 kDa is likely the intact CfrA with postlational modification while the one with molecular mass less than 76 kDa is likely the partly degraded product. Together, these findings clearly demonstrated that introduction of the Kozak sequence plays a critical role in developing the DNA vaccines for effective production of bacterial gene products in eukaryotic cells.

3.2. In ovo DNA vaccination trials in broilers

To avoid missing functionally important epitopes, subsequently we only chose the two validated DNA vaccines pCmeC-K and pCfrA-K, which express full length of CmeC and CfrA, respectively, for *in ovo* vaccination trials in this study.

In Trial #1, the pCmeC-K and pCfrA-K DNA vaccines were directly used for *in ovo* injection at day 18 of embryonation. As shown in Fig. 2, the pCmeC-K (denoted as 'pCmeC' hereinafter) and pCfrA-K (denoted as 'pCfrA' hereinafter) vaccines failed to enhance serum IgG titre in chickens compared to the two negative controls (injected with water or control pCAGGS plasmid) at 21 and 28 days of age. Great individual variations were observed for IgG response among groups (Fig. 2). Upon *C. jejuni* challenge, all chickens were colonized by *C. jejuni* NCTC 11168 two days post-challenge and the colonization level peaked at 7 days post-challenge, with an average shedding level of approximately 10^7 CFU/g feces (data now shown). The shedding levels of *C. jejuni* are not significantly ($P > 0.05$) different between control groups and the vaccination groups (data not shown).

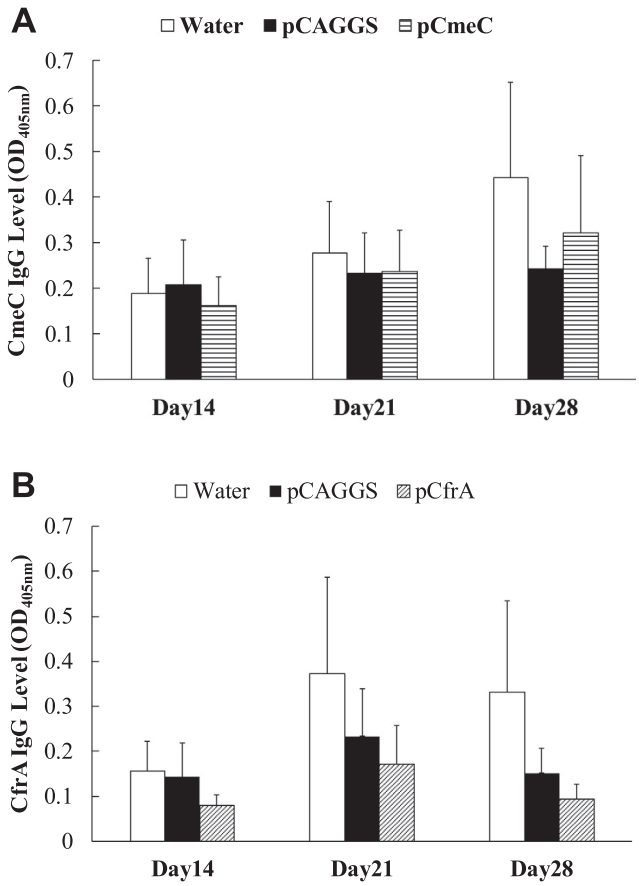

Fig. 2. Systemic IgG response following *in ovo* vaccination (Trial #1). Indirect ELISA analysis of systemic IgG level to CmeC (A) or CfrA (B). Serum samples were collected on day 14 (immediately before *C. jejuni* challenge), day 21 and day 28 for ELISA analysis (1:100 dilution). Error bars represent standard deviation.

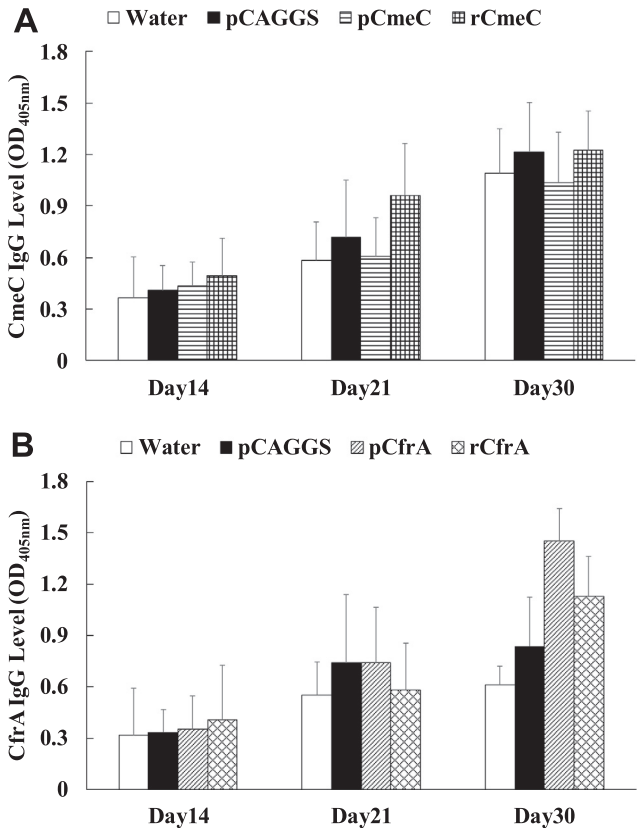

Fig. 3. Systemic IgG response following *in ovo* vaccination (Trial #2). Indirect ELISA analysis of systemic IgG level to CmeC (A) or CfrA (B). Serum samples were collected on day 14, day 21(immediately before challenge) and day 30 for ELISA analysis (1:100 dilution). Error bars represent standard deviation.

In Trial #2, the *in ovo* vaccination strategy was modified primarily by including protection of DNA vaccines with neutral lipid and adding control group with single *in ovo* injection of specific subunit vaccine (rCmeC or rCfrA protein). However, the two DNA vaccines still failed to trigger systemic IgG response (Fig. 3). Similarly, *in ovo* vaccination of broilers using purified rCfrA or rCmeC did not induce significant systemic IgG immune response either, likely due to interference of maternal antibodies (Fig. 3). In terms of IgA immune response, both systemic IgA levels (Fig. 4A and B) and intestinal mucosal IgA levels (Fig. 4C and D) did not significantly differ between control and treatment groups. As expected, approximately 9 days after challenging chickens with *C. jejuni* NCTC 11169 (Day 30), specific IgG and IgA immune responses were induced (Figs. 3 and 4). In particular, the birds vaccinated with pCfrA DNA vaccine group displayed significantly ($P < 0.05$) higher level of anti-CfrA IgG in serum than control groups (Fig. 3B). Consistent with the patterns of weak systemic and mucosal immune responses observed on Day 14 and Day 21 (Figs. 3 and 4), challenge of chickens with NCTC 11168 at age of 21 days did not show a significant difference in *C. jejuni* colonization levels at different time points among the four groups (data not shown). Generally, in all groups, chickens were successfully colonized by *C. jejuni* two days post-challenge with average shedding level at $5x10^5$ CFU/g feces. Shedding levels peaked at four days post-challenge with an average of 10^8 CFU/g feces in all groups.

4. 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 [23], a variety of DNA vaccines have been tested and licensed, including those designed to prevent and control cancer, allergies, and infectious diseases [15–17]. The principle of DNA

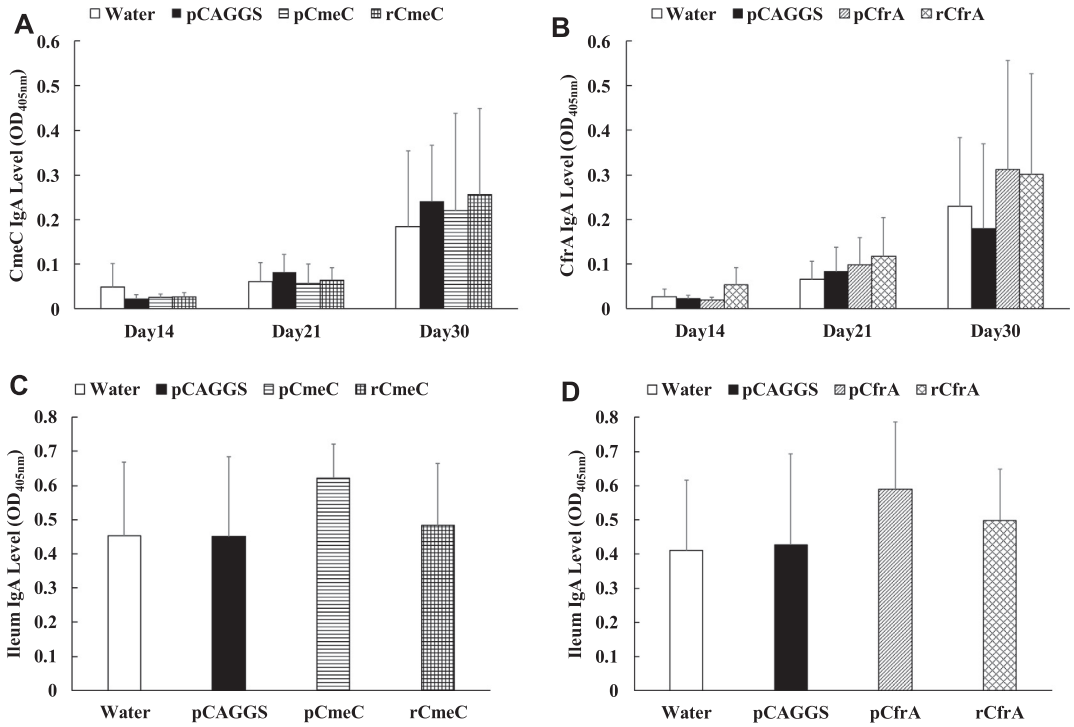

Fig. 4. Systemic and intestinal mucosal IgA responses following *in ovo* vaccination (Trial #2). Indirect ELISA was performed to examine systemic IgA level to CmeC (A) or CfrA (B), as well as intestinal mucosal IgA level to CmeC (C) or CfrA (D). To analyze intestinal mucosal IgA, the intestinal lavages collected at day 30 were used for ELISA (1:50 dilution). Error bars represent standard deviation.

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. In chicken, various DNA vaccines also have been developed, most of which were against viruses [24,25]. A number of DNA vaccines against coccidiosis in poultry also have been reported [26].

In this study, we chose the eukaryotic expression vector pCAGGS [19] for construction of DNA vaccine expressing the CfrA or CmeC of *C. jejuni* because this vector has a high expression efficiency and has been widely chosen for the construction of DNA vaccine used in animals including chicken [27–30]. However, despite no any difficulties encountered during recombinant plasmid construction, initial DNA vaccines (pCAGGS_CmeC and pCAGGS_CfrA, Table 1) failed to produce detectable target protein by western blot analysis. We speculated that such surprisingly low expression is likely due to the lack of the Kozak sequence of the cloned bacterial genes. The Kozak sequence is the sequence adjacent to the translational start site (AUG) on eukaryote mRNA molecules, which can be recognized by the eukaryote ribosome for efficient protein translation. 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 [31], where upper case letters denote a high level of conservation while lower case letters denote a relatively low level conservation. In this study, we introduced the core Kozak consensus sequence (ACCATGG) to the upstream of the cloned *cmeC* or *cfrA* genes, leading to the construction of desired DNA vaccines that displayed enhanced production of CfrA or CmeC in eukaryotic cells, as confirmed by immunoblotting analysis (Fig. 1).

Of various studies focused on construction of DNA vaccine expressing bacterial genes, to date, only limited publications reported the similar Kozak sequence modification strategy used in this study. In one study, *Brucella abortus* lumazine synthase gene including the Kozak consensus sequence was cloned in pcDNA3 plasmid and expression of the cloned gene was confirmed by transient transfection of COS-7 cells [32]. In another study by Cassataro et al. [33], a pCIOmp31 DNA vaccine vector containing the Kozak sequence was constructed and demonstrated to express the Omp31 gene from *Brucella.* Similarly, the DNA vaccines 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 [34]. Notably, none of these DNA vaccine studies [32–34] examined the production level of the vector carrying original bacterial gene (without Kozak sequence), impeding evaluation of the impact of Kozak sequence on the magnitude of production of specific bacterial gene product. In this study, with availability of both the plasmids directly carrying original *C. jejuni* genes and the plasmids carrying the genes with Kozak modification, our immunoblotting analysis (Fig. 1) provided compelling evidence that the Kozak sequence is critically important for constructing desired DNA vaccine expressing cloned prokaryotic gene in eukaryotic host.

In ovo delivery has been proposed as an attractive vaccination route for chickens, particularly when automatic *in ovo* injection system has been available and adopted in commercial poultry production. Notably, *in ovo* vaccination various advantages particularly when combined with DNA vaccine in poultry [18]. 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 [35]. The immune system of chickens has been proposed to be well developed by day 18 of inoculation, providing rationale for stimulating immune response via *in ovo* vaccination [14]. In addition, *in ovo* vaccination is a fully automatic method to vaccinate massive numbers of eggs (20,000–30,000 per hour), and has been applied to various vaccines for viral, bacterial and protozoal diseases in broilers, without compromising embryo viability [14,35]. This method also reduces eggs handling, improves hatchery manageability, and reduces cost and labor [35]. To date, many poultry vaccines have been approved by the USDA for *in ovo* administration [35].

However, in this study, we were frustrated to observe that the tested DNA vaccines failed to induce strong immune response, consequently not conferring protection in broiler chickens against *C. jejuni* infection. There are several factors determining the success of *in ovo* DNA vaccination. The dosage of specific DNA vaccine is recognized as a critical factor [36]. It has been reported that 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 [22]. Therefore, the dosage of 50 μg of DNA chosen in this vaccination study was reasonable and expected to work normally for *in ovo* vaccination. In addition, the injected DNA vector may be degraded by DNase in the amniotic fluid [22]. To address this issue, in Trial #2, we emulsified the DNA vaccine with the neutral lipid for protection of injected DNA vaccine as recommended in a previous study [22]. To ensure successful *in ovo* vaccination, we also placed specific attentions on other factors including the embryonic stage of development and the protocol for injection. We performed *in ovo* injection at day 18 of embryo development because the immune system of chicken is expected to be well developed at this stage for *in ovo* injection [14,37]. In addition, the specific type of needle used in this study has been widely and successfully used for *in ovo* injection [37–40]. Taken together, in this study, we are confident the DNA vaccines were injected successfully into the amniotic fluid and taken up by the bird embryos in both *in ovo* vaccination trials. Based on the findings from a recent DNA vaccine evaluation for *C. jejuni* control in poultry [41], the avian strain and/or immune status may cause the lack of induction of protective immunity in this study. In addition, it is possible that a single *in ovo* vaccination regimen is not sufficient to o induce strong protective immune response using the developed DNA vaccines in this study. A boost vaccine regimen following the single *in ovo* vaccination needs to be examined in the future. Finally, in terms of the DNA vaccine that expressing bacterial gene, to date, no information exists concerning the impact of specific posttranslational modification of bacterial gene product on the efficacy of bacterial DNA vaccine. At least, such posttranslational modification may trigger different immune response, leading to misinterpretation of ELISA results when using unmodified recombinant protein as coating antigen as reported in this and other studies.

Ethics approval

All chicken studies were approved by The University of Tennessee Institutional Animal Care and Use Committee (IACUC No. 2099-0412).

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Declaration of Competing Interest

The authors declare that here are no any potential conflicts of interest regarding publication of this paper.

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