Development and Evaluation of CmeC Subunit Vaccine against Campylobacter jejuni

Ximin Zeng, Fuzhou Xu and Jun Lin*

Department of Animal Science, The University of Tennessee, Knoxville, TN 37996, USA

Abstract

Campylobacter jejuni is the leading bacterial cause of human enteritis in many industrialized countries. There is no commercial vaccine against C. jejuni available to date. CmeC is an essential outer membrane component of CmeABC multidrug efflux pump that plays a critical role in antibiotic resistance and in vivo colonization of C. jejuni. CmeC is prevalent in C. jejuni strains and is dramatically induced and immunogenic in vivo. In this study, we analyzed CmeC sequence homology, examined in vitro immune protection of CmeC peptide antibodies, and produced full-length recombinant CmeC (rCmeC) for evaluating immunogenicity and protective efficacy of the CmeC subunit vaccine against C. jejuni using chicken model system. Amino acid sequences of CmeC from 24 diverse C. jejuni strains were determined and subjected to alignment, which revealed that CmeC is highly conserved in C. jejuni with a identity ranging from 97.3% to 100%. CmeC peptide antibodies inhibited the function of CmeABC efflux pump and enhanced susceptibility of C. jejuni to bile salts, the natural antimicrobial present in the intestine. Two full-length rCmeC proteins with N- or C-terminal His tag were produced in E. coli; the N-terminal His-tagged rCmeC with high purity and yield was obtained by single step affinity purification. The purified rCmeC was used in two vaccination trials using a chicken model of C. jejuni infection. Stimulation of CmeC-specific serum IgG responses via oral vaccination required immunization with higher doses of rCmeC (200μg) together with 70μg of mucosal adjuvant mLT (modified E. coli heat-labile enterotoxin). Subcutaneous vaccination of chickens with rCmeC remarkably stimulated both serum IgG and IgA responses. However, CmeC-specific intestinal secretory IgA response was not significantly stimulated regardless of vaccination regimen and the rCmeC vaccination did not confer protection against C. jejuni infection. Together, these findings provide further compelling evidence that CmeC is a promising subunit vaccine candidate against C. jejuni infection. However, the CmeC vaccination regimen should be optimized to enhance CmeC-specific mucosal immune response in the intestine for protection against C. jejuni.

Keywords: Campylobacter jejuni; Subunit vaccine; Multidrug efflux pump

Introduction

Campylobacter jejuni is the leading bacterial cause of human enteritis in the United States and other industrialized countries [1]. This pathogenic organism causes watery diarrhea and/or hemorrhagic colitis in humans and is also associated with Guillain-Barré syndrome, an acute flaccid paralysis that may lead to respiratory muscle compromise and death [2,3]. Poultry are the major reservoir of Campylobacter and thus the main source for human campylobacteriosis [1,4]. At the same time that prevalence of infection is increasing, Campylobacter has become increasingly resistant to antibiotics, including fluoroquinolones and macrolides, the major drugs of choice for treating human campylobacteriosis [5]. Despite the growing need for new antibiotics due to increasing drug resistance in Campylobacter and other bacteria, many pharmaceutical companies have been placing less emphasis on antibiotic discovery [6]. Therefore, alternative intervention strategies, such as vaccination, are needed to prevent and control Campylobacter infections. To dates, vaccines against Campylobacter infection are still not available, primarily due to the antigenic complexity of this organism and a lack of understanding of the mechanisms of pathogenesis. Information concerning protective antigens as vaccine candidates in C. jejuni is limited and vaccinations against C. jejuni using animal models including chickens have had only partial success [7-9].

It has been well established that prior infection with C. jejuni can induce protective immunity against Campylobacter infections in humans and animals, strongly supporting the feasibility of development of immunization-based approaches to control Campylobacter infections [7]. Outer membrane proteins (OMPs) of C. jejuni are considered the major mediators of pathogen-host interactions and are promising candidates for the design of protective vaccines. Recently, we characterized a unique OMP CmeC, an essential component of multidrug efflux pump CmeABC that plays a critical role in antibiotic resistance and pathogenesis of C. jejuni [10-13]. The CmeC is a promising subunit vaccine candidate against C. jejuni because of following compelling evidences. First, CmeC is essential for C. jejuni colonization in animal intestine by mediating bile resistance [10,11,13]. Compared to the wild type strain that colonized the chickens as early as day 2 post-inoculation with a density as high as 10^7 CFU/g feces, the isogenic CmeC mutant failed to colonize any of the inoculated chickens throughout the study [12]; the minimum infective dose for CmeABC mutant is at least 2.6×10^4 folds higher than that of the wild-type strain [12]. Second, PCR and immunoblotting analyses showed that CmeC is widely existed and constitutively expressed among different C. jejuni strains, suggesting that CmeC is highly conserved in terms of sequence and antigenicity [11]. Third, expression of CmeC is dramatically induced by bile salts present in the intestine, further highlighting the critical role of CmeC.

*Corresponding author: Dr. Jun Lin, Department of Animal Science, The University of Tennessee, 2640 Morgan Circle Drive, Knoxville, TN 37996-4574, USA, Tel: (865)974-5598; Fax: (865)974-7297; E-mail: jlin6@utk.edu

Received November 30, 2010; Accepted December 30, 2010; Published December 31, 2010


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in *Campylobacter* pathogenesis [13]. This notion also is supported by a recent microarray study by Stintzi et al. [14], in which expression of *cmeABC* operon was found to be highly up-regulated in *in vivo*. In addition, using multiple chicken sera for immunoblot analysis, we also demonstrated that CmeC is expressed during *Campylobacter* infection of chickens and elicited a specific antibody response in the host [12], supporting the feasibility of targeting CmeC for immune protection against *C. jejuni* colonization. Finally, we also demonstrated that inhibition of CmeABC by efflux pump inhibitors increased susceptibility of *Campylobacter* to various antimicrobials, prevented emergence of macrolide resistant *C. jejuni*, and reduced in *in vivo* colonization of *C. jejuni* using a chicken model system [15,16]. Based on these observations, we hypothesize that CmeC antibodies could inhibit functions of CmeABC pump and that CmeC is a promising subunit vaccine candidate to prevent and control *C. jejuni* colonization in the intestine.

In this study, we determined sequence homology of CmeC in diverse *Campylobacter* strains as well as in *in vitro* immune protection of CmeC peptide antibodies, which further support the feasibility of targeting CmeC for immune intervention against *C. jejuni* infection. We also constructed plasmids for producing full-length rCmeC and optimized conditions for purification of large quantities of rCmeC with high purity. The purified rCmeC was then used in two large chicken vaccination trials to evaluate the immunogenicity and protective efficacy of the CmeC subunit vaccine with different vaccination regimens.

**Materials and Methods**

**Bacterial strains, plasmids and culture conditions**

The major bacterial strains and plasmids used in this study are listed in Table 1. Fourteen *C. jejuni* isolates (JL7, 10, 12, 36, 78, 81, 83, 85, 90, 91, 93, 94, 95 and 118) from various sources (human, chicken, turkey, bovine, and environment) were used for PCR amplification and sequence analysis of the complete *cmeC* gene. In vitro colonization of *C. jejuni* using a chicken model system [15,16] and in vivo colonization of *C. jejuni* using a chicken model system [15,16]. Based on these observations, we hypothesize that CmeC antibodies could inhibit functions of CmeABC pump and that CmeC is a promising subunit vaccine candidate to prevent and control *C. jejuni* colonization in the intestine.

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**PCR amplification and sequence analysis of the CmeC gene**

Genomic DNA was extracted from each isolate by using Wizard*®* Genomic DNA Purification Kit (Promega, Madison, WI). Primers CmeCF (5’-CAGCAAAACTTCGTTTTCGTC-3’) and CmeCR (5’-YCAGGTATTGGTTTATATG-3’) were used to amplify the entire *cmeC* gene. Amplified PCR products were purified by QiAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced in Molecular Biology Resource Facility at the University of Tennessee (Knoxville, TN). The *cmeC* sequences were converted to amino acid sequences and subjected to alignment together with other publicly available *C. jejuni* sequences (Accession #: ABC59229, AAT38952, CAL34515, YP_001000076, ZP_01100537, ZP_01069571, ZP_01067179, YP_178433, ZP_01071951) using DNAsStar package (version 6.0). To predict the secondary structure of CmeC, the amino acid sequence of CmeC was analyzed by using the program TransMembrane protein Re-Presentation in 2 Dimensions tool (TMRPres2D) [17]. Homology Modeling was performed using an automated model on SWISS Model Workspace (http://swissmodel.expasy.org/workspace/), using NodT (1wp1.pdb) as a template.

**Effect of CmeC peptide antibodies on susceptibility of *C. jejuni* to bile salts**

We have prepared a recombinant strain of *E. coli* JM109 expressing a portion of CmeC (aa 41 to 248 of total 492 aa in length) and high-titer of specific rabbit polyclonal antiserum directed against this CmeC peptide was also generated [11]. This CmeC peptide antiserum was used to examine the effect of CmeC peptide antibodies on susceptibility of *C. jejuni* 81-176 to cholic acid, a representative bile salt. Briefly, a log-phase culture of *C. jejuni* 81-176-

### Table 1: Key bacterial strains and plasmids used in this project.

<table>
<thead>
<tr>
<th>Plasmids or Strains</th>
<th>Description</th>
<th>Source or Reference</th>
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<td>pQE-30</td>
<td>Vector for N-terminal His-tagged protein construction</td>
<td>Qiagen</td>
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<tr>
<td>pQE-70</td>
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<td>pQE-30 ligated with cmeC segment encoding mature CmeC</td>
<td>This study</td>
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<tr>
<td>pCmeC-CSHS</td>
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Production and purification of full-length rCmeC

To construct a recombinant strain producing full-length N-terminal His-tagged rCmeC, a 1,444-bp fragment encoding mature 473-aa CmeC (aa 20 to 492) with the removal of the 19 aa signal peptide was PCR amplified from \( C.\) jejuni NCTC 11168 using primers CCF (5’-AAAGGATCCGTTGCTTTTGGTCAATATTTATTATTGTT-3’) and CCR (5’-AACCCGGGCTATTCTCTAAAAGATATCTAAATT-3’). The restriction sites (\( \text{Bgl II} \) and \( \text{Sma I} \), underlined in the primer sequences) were underlined. The amplified DNA was cloned into the pQE-70 vector (Qiagen, Hilden, Germany). The amplified DNA was digested with \( \text{Bgl II} \) and \( \text{Sma I} \), and was cloned into the vector pQE-30 (Qiagen). The amplified CmeC fragment was digested with \( \text{Bam HI} \) and \( \text{Sma I} \), and was cloned into the vector pQE-30, which previously had been digested with \( \text{Bam HI} \) and \( \text{Sma I} \), creating a C-terminal His-tagged rCmeC.

CmeC vaccination and \( C.\) jejuni challenge: Trial 1

The experimental design is detailed in Table 2. Briefly, newly hatched broiler chickens (\( n = 120 \)) were allocated randomly into 6 groups (20 per group). Each group of chickens were maintained in a sanitized wire-floored cage and provided with unlimited access to water and commercial chicken starter feed without antibiotic additives. At 1-week-old age, chickens were immunized orally with 200 μl of CmeC vaccine in PBS via oral gavage. CmeC doses used were 50 and 200 μg, either alone or coadministered with 10 μg of mLT (general gift from Dr. John Clements in Tulane University Medical Center). Chickens receiving PBS only or mLT only were used as control groups. Booster doses were administered 2 wk after primary immunization. Blood samples were collected from the wing vein of each chicken at 1, 3, 5, 7 wk of the experiment to monitor circulating CmeC specific IgA and IgG antibodies; intestinal lavage samples were collected and prepared at 3, and 5 wk from euthanized chickens (5 bird/group) as described previously [20] to monitor mucosal IgA antibody response. Prior to challenge, chickens were screened again with conventional culture methods to ensure that they were \( C.\) jejuni free. At week 5, 100 μl culture of \( C.\) jejuni NCTC 11168 was inoculated orally into broilers (10 CFU/bird). After challenge, cloacal swabs were collected every 2 d for 10 d. Swabs were diluted serially, and plated on MH agar containing selective supplement (SR117E; Oxoid, Basingstoke, Hampshire, England) for enumeration of \( C.\) jejuni cells. The randomly selected colonies were tested by PCR [12] to ensure that output \( C.\) jejuni was the same as the inoculum and there were no contamination of chickens by other sources.

CmeC vaccination and \( C.\) jejuni challenge: Trial 2

The second vaccination trial (Table 3) had four major modifications compared to Trial 1 (Table 2). First, white leghorn chickens, which grow slower than broilers, were used in this trial. Second, to compare effects of vaccination route on immune response and protective efficacy of rCmeC, subcutaneous administration was included in this trial. Third, the dose of mucosal adjuvant mLT was increased to 10 to70 μg/chicken. Finally, the dose of CmeC NCTC 11168 used for challenge was reduced to 103 CFU/bird.

Enzyme-linked immunoabsorbant assay (ELISA)

CmeC-specific antibodies in intestinal lavage samples and sera were measured by indirect ELISA, which was performed using a previously published protocol [21] with the following modifications.
Development and Evaluation of CmeC Subunit Vaccine against *Campylobacter jejuni*. J Vaccines Vaccin 1: 112.
doi:10.4172/2157-7560.1000112

Microtiter plates (Nunc-Immuno Plate, Thermo Fisher scientific, Fairlawn, NJ) were coated with 100 μl of rCmeC (30 ng/well) in coating buffer (ammonium acetate plus ammonium carbonate [pH 8.2]) overnight at room temperature. For vaccination Trial 1, to examine the levels of circulating CmeC specific IgA and IgG antibodies and mucosal IgA antibodies, chicken serum and intestinal lavage samples were routinely diluted 1:100 and 1:4, respectively. For vaccination Trial 2, chicken intestinal lavage samples were routinely diluted 1:4 and 1:100 respectively. For vaccination Trial 2, chicken intestinal lavage samples were routinely diluted 1:4

Results

CmeC is highly conserved in *C. jejuni*

The full-length CmeC genes from 14 *C. jejuni* strains were PCR amplified and sequenced in this study. The new sequences were translated into deduced protein sequence and aligned with the CmeC sequences from 10 *C. jejuni* strains deposited in the NCBI public database (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). As shown in Figure 1, amino acid sequences of CmeC displayed a high level of homology (97.3% to 100% aa identity) among *C. jejuni* strains.

![Phylogenetic relationship of CmeC from diverse C. jejuni strains](image)

Figure 1: Phylogenetic relationship of CmeC from diverse *C. jejuni* strains. The amino acid based dendrogram was constructed by neighbor-joining methods in MEGA 4.0. The strains with ‘JL’ prefix were used for cmeC gene amplification and sequencing in this study and the complete sequences of these strains have been deposited in GenBank. The CmeC sequences of other strains are the public database NCBI (http://www.ncbi.nlm.nih.gov/).

![Growth responses of C. jejuni 81-176 to anti-CmeC serum and control serum](image)

Figure 2: Growth responses of *C. jejuni* 81-176 to anti-CmeC serum and control serum (pre immune serum). The log-phase culture of 81-176 was diluted to approximately 2×10^6 cfu/ml in MH broth containing sublethal concentrations of cholic acid (2 mg/ml). Anti-CmeC and control sera were added to cells with 1:10 dilution and cells were incubated for 6 hours at 42°C under microaerophilic conditions. Samples were diluted in MH broth and plated on MH agar plates to determine bacterial viability. Each bar represents the mean value obtained from triplicate assays.

![SDS-PAGE analysis of rCmeC production and purification from E. coli](image)

Figure 3: SDS-PAGE analysis of rCmeC production and purification from *E. coli*. (A) Expression and purification of N-terminal His-tagged rCmeC. M, standard molecular mass markers (Bio-Rad); 0h and 3h, noninduced and 3 h-induced whole-cell lysate, respectively; E, eluted rCmeC fraction using Ni-NTA affinity chromatography (Qiagen). The putative GroEL, an *E. coli* molecule chaperone, was consistently co-purified with rCmeC using the standard protocol. (B) Expression and purification of C-terminal His-tagged rCmeC. FL: flow through; Ag, Ni-NTA agarose after elution; W1-W5, washing fractions; E1-E3, elution fractions. (C) Efficient removal of GroEL contaminant by ATP-Mg²⁺ treatment. Eluted fractions (E3 to E10) during Ni-NTA purification with (right panel) or without (left panel) addition of 5 mM of ATP-Mg²⁺ were subjected to SDS-PAGE analysis.
CmeC peptide antibodies enhanced susceptibility of C. jejuni to bile salt

Supplementation of anti-CmeC serum in MH broth containing a sublethal concentration of cholic acid resulted in moderate but significant growth reduction (Δlog$_{10}$ unit, $P < 0.05$, Figure 2) when compared to growth in the presence of control serum (pre-immune serum, negative for CmeC). This finding suggests that anti-CmeC antibodies specifically bind to surface-exposed CmeC, inhibit the function of CmeABC efflux pump, and increases the susceptibility of C. jejuni to bile salt.

Production of high purity His-tagged rCmeC

The anti-CmeC serum used in above in vitro immune protection assay was directed against a portion of CmeC (aa 41 to 248) [11] and may not contain all protective epitopes of CmeC. To better study immune protection conferred by CmeC vaccination in this study, two E. coli constructs were constructed for producing full-length rCmeC proteins. Both N-terminal (Figure 3A) and C-terminal His-tagged rCmeC (Figure 3B) were successfully produced in E. coli and purified by Ni-NTA agarose affinity column. However, the yield of C-terminal His-tagged rCmeC was significantly lower than that of N-terminal His-tagged rCmeC. Approximately 1.5 mg of N terminal His-tagged rCmeC was consistently purified from 100 ml of induced culture using 1 ml of Ni-NTA resin. Thus, to produce large amount of rCmeC for vaccination studies, we then focused on optimization of purification for N-terminal His-tagged rCmeC. As shown in Figure 3A, a protein band with approximate MW of 60 kDa was consistently co-purified with rCmeC when following the standard protocol. Based on the molecular weight of this co-purified protein, it is likely this contaminated protein is E. coli molecule chaperone GroEL [19]. Since it has been reported that including Mg$^{2+}$-ATP in washing buffer could efficiently remove the GroEL contamination [23], the purification procedure was modified to improve the purity of the extracted rCmeC. As shown in Figure 3C, addition of 5 mM of Mg$^{2+}$-ATP in lysis buffer completely removed contaminated bands, resulting in eluents containing rCmeC with high purity. Large quantities of high-purity rCmeC was obtained using the modified procedure for the vaccination and ELISA in this study.

Immunological responses and protective efficacy following CmeC vaccination

In Trial 1, oral vaccination of chickens with or without mLT adjuvant did not significantly ($P > 0.05$) enhance IgG and IgA levels in serum and in intestinal lavage at different days postimmunization (Figure 4). Despite the variations observed among individual chickens, chickens in group 6 (200 µg rCmeC + mLT) at 28 d postimmunization...
showed consistently and slightly higher serum IgG level ($P = 0.57$) than PBS control group. Interestingly, all groups displayed relatively higher IgG and IgA levels immediately prior to primary CmcC immunization (Figure 4), likely due to the effect of maternal antibodies [21,24]. Consistent with the patterns of these antibody responses, challenge of chickens with NCTC 11168 at 28 days after primary CmcC vaccination did not show a significant difference of colonization among groups ($P > 0.05$) (Data not shown). All chickens in each group were quickly colonized by C. jejuni NCTC 11168 two days after challenge with an average shedding level of $\sim 10^8 \text{CFU/g}$ feces, likely due to the high dose of inoculums used for challenge ($10^8 \text{CFU/bird}$).

In Trial 2, oral immunization of chickens with a higher dose of CmcC (200μg) and mucosal adjuvant (70μg) significantly ($P < 0.05$) elevated the IgG titer than those in the control groups (receiving mLT or CmcC only) and the group administered a low dose of CmcC (50μg) plus mLT at 28 and 42 d postimmunization (Figure 5A). As shown in the embedded figure in Figure 5A, the OD$_{405nm}$ values for higher dose of CmcC (200μg) together with 70μg of mLT were 0.89 ± 0.16 and 0.88 ± 0.10 at 28 d and 42 d postimmunization, respectively, which are significantly higher ($P < 0.05$) than the mean OD$_{405nm}$ values from other groups. However, serum IgA titer were similar among the groups that received oral vaccination (Figure 5B). Subcutaneous vaccination of chickens with CmcC together with Freund’s incomplete adjuvant dramatically elevated serum IgG and IgA titers ($P < 0.05$) at 14, 28, and 42 d postimmunization compared with the control group vaccinated with Freund’s incomplete adjuvant only. However, CmcC vaccination did not trigger significantly higher level of intestinal secretory IgA against rCmcC, regardless of vaccination route and dosage (Figure 6). Consistent with the lack of significantly induced local IgA response to CmcC, CmcC vaccination in Trial 2 did not significantly reduce C. jejuni colonization in the intestine (Figure 7).

**Discussion**

Previous studies have reported that CmcC is prevalent in C. jejuni, is induced and immunogenic in vivo, and is essential for C. jejuni colonization [10-13]. These findings suggest that CmcC is a promising subunit vaccine candidate against C. jejuni colonization in the intestine. The *in vitro* studies in this project provided further compelling evidence that CmcC is an attractive candidate for C. jejuni vaccine development. Alignment of complete CmcC sequences from diverse strains demonstrated that CmcC is highly conserved in C. jejuni (Figure 1). This finding is consistent with a recent report in which a small portion of cmeC was PCR amplified from different *Campylobacter* strains for sequencing [25]. It is likely that CmcC displayed variation between different *Campylobacter* species, such as C. jejuni and C. coli, because alignment of partial CmcC sequence showed only 83% aa identity between C. jejuni and C. coli [25]. However, sequence analysis of full length CmcC in this study clearly indicated that the genetic variation within C. jejuni, which is responsible for ~ 90% of human campylobacteriosis [1], is extremely limited. This evidence together with *in vitro* inhibitory effect of CmcC peptide antiserum on the function of CmeABC efflux pump (Figure 2) provides a strong rationale to develop CmcC-based subunit vaccine.

To overcome the potential limitations of using partial CmcC peptide for vaccination studies, such as lack of enough protective epitopes, the full-length rCmcC proteins were produced and purified in this study. Expression and purification of recombinant membrane proteins in *E. coli* system are always coupled with problems such as low yield, insolubility, unfolding or misfolding, co-purification of contaminations, proteolytic degradation, and less biological activity. In the past decade, extensive efforts have been placed on the modification of promoter, design of fusion with tags, refolding after purification, chaperone co expression/knockout, and protease gene knockout [26-28]. In this study, the N-terminal His tagged rCmcC is located in cytoplasm due to removal of the 19-aa signal peptide. Thus, cytoplasmic rCmcC proteins which have hydrophobic transmembrane domains are likely to attract/arrest the chaperones [28]. During our purification of rCmcC, we consistently observed the co-purification of a protein with molecular weight of ~ 60 kDa together with the target rCmcC band (Figure 3A and 3C), suggesting that the GroEL (60 kDa)-GroES (10 kDa) chaperone system in *E. coli* [19,26] may bind newly synthesized rCmcC. Since the binding of ATP can trigger the turnover of substrates in GroEL-GroES systems by reducing the affinity of GroEL-GroES with substrates [23], 5 mM of Mg$^{2+}$-ATP was added into the lysate to facilitate the dissociation of rCmcC from GroEL-GroES. This single and simple modification increased the purity of extracted rCmcC without affecting yield (Figure 3C). It is also important to mention that the detergent Empigen BB was used to facilitate solubilization of rCmcC during purification in this study because Empigen BB is a mild zwitterionic detergent and is known for its ability to preserve the antigenicity and functional activity of isolated proteins [29]. Together, the rCmcC protein with high purity has been obtained in this study for various research efforts, such as vaccination described in this study and crystallization in the future studies.
We have established a chicken model of *C. jejuni* infection and used this model to study the critical role of CmeC in colonization of *C. jejuni* [12]. We chose to use chickens for vaccination and *C. jejuni* challenge studies primarily for the following reasons. First, chickens are a natural host for *C. jejuni*. The high susceptibility of chicken to *Campylobacter* colonization, the ease of handling, and the low cost of chickens have provided an ideal model system to study the colonization and immunogenicity of *C. jejuni* in the host [8, 12, 15, 16, 30]. Second, newly hatched chickens are always Campylobacter-free, providing a clean and economical host for evaluating *Campylobacter* colonization [21]. Third, to date, there are no appropriate disease models for *C. jejuni* infection; a recently developed non-human primate model system may partly mimic symptoms caused by *C. jejuni* infection in humans [31]. Although *C. jejuni* does not cause clinical disease in chickens (an infection model, not a disease model), chickens are a sufficient and powerful colonization model to evaluate adaptation and survival of *Campylobacter* in response to harsh *in vivo* conditions (e.g. bile salts relevant to this study). Fourth, the heat labile enterotoxin (LT) has been used as a mucosal adjuvant with chicken oral vaccines [32, 33], which provides a rationale for us to use mLTI together with the CmeC oral vaccine in chicken model system described in this study. Finally, poultry is considered a major source for *C. jejuni* in humans [7]. Reduction of *Campylobacter* in poultry by vaccination of chickens will reduce the risk of exposure by humans who consume poultry products. Therefore, findings from chicken studies are also directly relevant to food safety and public health.

Oral vaccination of chickens, other animals as well as humans with subunit vaccine in conjunction with adjuvant LT or mLTI have been demonstrated to induce protective immunity [32-35]. Unexpectedly, oral vaccination of CmeC with mLTI in two trials performed in this study did not trigger significant local immune response to CmeC and thus failed to confer protection of chickens against *C. jejuni* colonization. However, the findings from this study provided useful information for future development and evaluation of CmeC subunit vaccine using the chicken as a model. First, white leghorn appears a better animal host than broiler for vaccination evaluation, primarily due to its slow growth rate, which makes animal handling easier and also allows us to initiate late primary vaccination (e.g. 3 wk of age) when *Campylobacter* specific maternal antibodies decrease to a low level. Second, although dramatic systemic immune response to CmeC was induced using the subcutaneous vaccination route in this study, the elevated antibody titters in serum did not result in increased levels of intestinal antibodies, which suggests that vaccination via mucosal route, such as oral vaccine, should be used to induce a strong gut mucosal immune response to control *Campylobacter* colonization. Third, development of an oral subunit vaccine faces a common difficulty: weak immunogenicity due to the choice of adjuvant and antigen degradation in the GI tract. Previous studies have shown that *Campylobacter*-specific secretory and serum IgA antibodies correlate with protection against *Campylobacter* infection [36]. In this study, sufficient levels of CmeC-specific antibodies, particularly intestinal IgA, were not reached for protection against *C. jejuni* infection. Given that the mLTI used in this study has been shown to be effective with oral vaccines in different animals including chickens at low dose (e.g. 5μg single dose for pig) [35], it is unlikely that the mLTI fails to stimulate immune response if it reaches chicken gut together with rCmeC. We speculate that orally administered rCmeC and/or mLTI may be absorbed to the upper gastrointestinal tract and significantly degraded in the intestine before it can prime the host immune system. To solve this problem, appropriate delivery systems may be explored to enhance mucosal immune response. For example, encapsulation of rCmeC using the chitosan microsphere, an effective adjuvant/carrier system [37, 38], may be a promising approach to deliver rCmeC to the target site and trigger a strong local intestinal mucosal immune response. In addition, identification of protective epitopes of CmeC followed by construction of live *Salmonella*- vectored vaccine is also a promising approach for persistent deliver specific protective epitopes to induce local mucosal response [39]. Elucidation of the structure of CmeC by taking advantage of the high purity rCmeC obtained from this study for crystallization will facilitate construction of a *Salmonella* live vaccine expressing specific surface region(s) of CmeC. These hypotheses will be tested in future studies.

Successful development of CmeC-based vaccine may lead to a novel bifunctional vaccine that not only prevents *Campylobacter* colonization but also combats antibiotic resistance in *C. jejuni*. It has been demonstrated that inhibition of multidrug efflux pumps by efflux pump inhibitors (EPIs) is an effective approach to improve the clinical performance of antibiotics [40-42]. We also observed that inhibition of CmeABC pump by an efflux pump inhibitor increased susceptibilities of *C. jejuni* to various antibiotics and significantly reduced the frequency of emergence of macrolide resistant *C. jejuni* [15, 16]. However, several key issues (e.g. toxicity, *in vivo* stability, production cost) should be well addressed before EPIs can be used clinically and accepted by medical community. Given the significant role of CmeC in multidrug resistance, we hypothesize that CmeC antibodies may function similarly as EPI by targeting an essential component of CmeABC pump and immunization of chickens with CmeC vaccine may enhance the activity of clinical antibiotics against *C. jejuni*. Compared to EPIs, the CmeC vaccine may be a more realistic approach to potentiate the activity of clinical antibiotics against *Campylobacter*. Thus, CmeC may also represent a novel vaccine that can enhance the efficacy of clinical antibiotics and even reduce the frequency of *in vivo* emergence of antibiotic resistant *C. jejuni*. This hypothesis needs to be determined after optimization of CmeC vaccine regimen in the future.

Acknowledgments

We are grateful to Dr. John D. Clements (Tulane University Medical Center) for kindly providing the mucosal adjuvant mLTI-R192G as a gift for the vaccination study in this paper. We thank Ky Van Hoang, Andree A. Hunkapiller, and Azrell Martinez for technical support. This study was supported by grant 1R21AI072551-01 from NIH.

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