Research Article

Intranasal Immunization with Chitosan/pCAGGS-*fla*A Nanoparticles Inhibits *Campylobacter jejuni* in a White Leghorn Model

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Campylobacter jejuni is the most common zoonotic bacterium associated with human diarrhea, and chickens are considered to be one of the most important sources for human infection, with no effective prophylactic treatment available. We describe here a prophylactic strategy using chitosan-DNA intranasal immunization to induce specific immune responses. The chitosan used for intranasal administration is a natural mucus absorption enhancer, which results in transgenic DNA expression in chicken nasopharynx. Chickens immunized with chitosan-DNA nanoparticles, which carried a gene for the major structural protein FlaA, produced significantly increased levels of serum anti-*Campylobacter jejuni* IgG and intestinal mucosal antibody (IgA), compared to those treated with chitosan-DNA (pCAGGS). Chitosan-pCAGGS-*fla*A intranasal immunization induced reductions of bacterial expellation by 2-3 $\log_{10}$ and 2 $\log_{10}$ in large intestine and cecum of chickens, respectively, when administered with the isolated *C. jejuni* strain. This study demonstrated that intranasal delivery of chitosan-DNA vaccine successfully induced effective immune response and might be a promising vaccine candidate against *C. jejuni* infection.

1. Introduction

Gram-negative bacterium *Campylobacter* is responsible for an estimated 400 million human cases of enterocolitis worldwide each year, making it the leading cause of bacterial foodborne disease and a major causative agent of traveller's disease. In a limited number of cases, the enteric manifestations are followed by sequelae, such as reactive arthritis and the life-threatening neuropathy Guillain-Barré syndrome (GBS) [1]. Two thermophilic species, *C. jejuni* and *C. coli,* are responsible for the vast majority of human campylobacteriosis (∼90% and ∼10%, resp.) [1]. Animals such as chickens, cattle, pigs, sheep, and dogs may act as asymptomatic reservoirs [2], by shedding *C. jejuni* in their stools, which results in the contamination of animal food products and surface water during slaughter and carcass dressing [3]. Chickens, often heavily colonized with *C. jejuni* without signs of pathology, are considered to be one of the most important sources for human infection [4]. It is generally assumed that *C. jejuni*-contaminated poultry constitutes a major risk to human health.

A wide array of interventions has been developed to reducing the carriage of *C. jejuni* in livestock and poultry [5], including attempts to eliminate *C. jejuni* from farms by increasing biosecurity, separating contaminated flocks, and improving hygiene during slaughter. Although these measures undoubtedly help to control shedding of *C. jejuni* in infected animals, and may reduce the number of positive flocks and the contamination level of their products, vaccination of poultry against *C. jejuni* will probably be the most effective measure, and remains a major goal [6, 7]. Considering the physiology of *Campylobacter* and their

intestinal ecological niche in poultry, conventional vaccines have a poor performance, including inactivated vaccines [8, 9].

Vaccines generally have a very good safety profile. The rapid advances in genomics, proteomics, and molecular design of vaccines provide hope that an effective vaccine for *Campylobacter spp.* can be developed in the near future [1, 10, 11]. In 1990, it was first reported that intramuscular injection of plasmid DNA in a simple saline solution could transfect muscle cells *in vivo* [12]. Genetic immunization is a novel vaccine strategy that conceptually combines some of the most desirable attributes of standard vaccine approaches. The immune responses comprise both cell-mediated and humoral (antibody) components. Most recent studies associated with the efficacy of DNA vaccine against *C. jejuni* focus on the potential vaccine antigens, plasmid vector, and DNA delivery (mucosal adjuvants). The gene *fla*A plays an important role in the pathogenicity of *C. jejuni* [13]. As a result of its biocompatibility, biodegradability, low cost, and ability to open intercellular tight junctions, chitosan can be used as a delivering vehicle for the mucosal vaccine and may have an adjuvant effect [14].

Here, we reported intranasal immunization of White Leghorn chickens using chitosan-DNA nanoparticles that carried the recombinant plasmid pCAGGS-*fla*A for the *fla*A gene encoding the major structural protein FlaA of *C. jejuni*, and demonstrated its efficacy in inducing specific immune responses and protection against homologous strain challenge.

2. Materials and Methods

2.1. Animals. White Leghorn chickens aged 1 day were obtained from the Comparative Medicine Center of Yangzhou University (Yangzhou University, Jiangsu, China). Animals were housed, handled, and immunized following approval by the institutional animal experimental committee.

2.2. Plasmids, Bacteria, and Antibodies. pCAGGS was a kind gift from Dr. Miyazaki (Faculty of Medicine, University of Tokyo, Japan). *C. jejuni* strain ALM-80 was isolated from chicken by the Jiangsu Key Laboratory of Zoonosis, Yangzhou University (Jiangsu, China). COS-7 cells were ordered from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), and grown in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; GIBCO). Chitosan, FITC-labeled rabbit antichicken IgG, horseradish peroxidase- (HRP-) labeled goat antichicken IgA, HRP-labeled rabbit antichicken IgG and IgM were obtained from Sigma (St Louis, MO, USA). All other chemicals and reagents were of analytical grade, and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.3. Construction of pCAGGS-flaA. A pair of primers were designed to amplify gene *fla*A of *C. jejuni* that contained

*Xba*I and *EcoR*I sites, forward: 5′-CATCTAGAAGCATT-TAACAAGTTCATGG-3′, reverse: 5′-TAGAATTCGTG-TTTATCCTAAAACCCAT-3′. Amplification was conducted in a volume of 50 μL, containing 5 μL 10× buffer, 3 pM dNTP, 20 pM primers, 2 μL (10 pg) DNA, and 1 U *Taq* polymerase (Takara Diagnostics, Dalian, China). The reactions were performed with a thermal cycler (Bio-Rad Ltd, Hercules, CA, USA) using the following temperature-cycling parameters: initial denaturation at 95°C for 5 min, followed by 32 cycles of 1 min at 94°C, 1 min at 58°C, 1.5 min at 72°C, with a final extension step of 5 min at 72°C. Amplification generated 1935-bp DNA fragments. Eight microliters of PCR products were separated on a 1% (w/v) agarose gel following electrophoresis at 60 V for 1.5 h. Gels were stained with ethidium bromide (0.3 mg/l) and visualized under UV illumination. A 100-bp DNA ladder (Promega, Madison, WI, USA) was used as a molecular size standard.

Then, the PCR product was cloned into pGEM-T easy vector (Sangon Biotech, Shanghai, China) to give rise to pT-*fla*A. The *EcoR*I-*Xba*I-restricted insert of pT-*fla*A was cloned into eukaryotic expression vector pCAGGS, and then the recombinant vector pCAGGS-*fla*A was constructed and inserted in *Escherichia coli* (DH5α) by electrotransformation.

For immunization purposes, homogeneous stocks of DNA were purified from 2.5 L cultures using the Plasmid Giga Preparation kit (Qiagen, Hilden, Germany). The DNA preparations were quantified by optical density (OD) measurements at 260 nm and analyzed by 1% (w/v) agarose gel electrophoresis.

2.4. Preparation of Chitosan-DNA Nanoparticles. The chitosan/pCAGGS-*fla*A DNA nanoparticles were synthesized by Mao et al. [15]. In brief, a chitosan (Sigma, St Louis, MO, USA) solution (200 μg/mL in 5 mM sodium acetate buffer, pH 5.5), 400 kD, 85% deacetylated, and a pCAGGS-*fla*A solution (100 μg/mL in 5 mM sodium sulfate solution) were separately preheated to 50°C–52°C in parallel. Then, the pCAGGS-*fla*A solution was added into an equal volume of the chitosan solution. Uniform particles were obtained by coacervation between chitosan and DNA, and were left to stand for at least 1 h at room temperature.

2.5. Characterization of Chitosan-DNA Complexes. Freshly prepared chitosan-DNA complexes were characterized by morphological examination by transmission electron microscopy (TEM). The samples were placed on a cotton grid, allowed to sit for a few minutes and air-dried. Then, they were negatively stained with uranyl acetate for visualization under a transmission electron microscopy (TEM, accelerating voltage/120 kV, Philips Tecnai 12, Holland).

Naked DNA (1 μg in 20 μL of 25 mM Na_2SO_4 solution) or chitosan-DNA suspension (20 μL, equivalent to 1 μg of DNA) was incubated with 0.5 U DNase I (final concentration of 28 U/mL) for 15 min at 37°C. EDTA was added to stop the reaction to a final concentration of 50 mM. Then, chitosan-DNA was subjected to chitosanase and lysozyme (final concentrations of 0.15 and 15 U/mL) digestion at

37°C for 4 h. Integrity of DNA was analyzed by agarose electrophoresis.

2.6. Gene Expression In Vitro.

Two micrograms of plasmid (Chitosan-pCAGGS-*fla*A) was transfected into 70% confluent COS-7 cells covering a 6 cm^2 dish using a LipofectAMINE 2000 kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. After 2 days incubation at 37°C in 5% CO_2, the cells were washed with phosphate-buffered saline (PBS) and fixed with 3% (v/v) paraformaldehyde in PBS for 20 min at room temperature. The cells were then washed three times and permeabilized with PBS containing 0.05% (v/v) Tween 20 for 30 min. After a further wash with PBS, the cells were incubated with a 1 : 50 dilution of mouse antiFlaA antiserum (from our laboratory) for 1 h followed by rinsing with PBS and incubation with fluorescein isothiocyanate- (FITC-) conjugated goat antimouse immunoglobulin G antibody (Sigma-Aldrich, St. Louis, MO, USA) for 45 min. The cells were washed again with PBS and visualized by a fluorescence microscope (Leica Microsystems GmbH,Wetzlar, Germany).

2.7. Association Efficiency of Chitosan-DNA Nanoparticles.

To assay the association efficiency (AE) of pCAGGS-*fla*A, the nanoparticles suspension was first centrifuged (Heraeus Fresco 21; Thermo Scientific, Germany) at 30,000 g, at 4°C for 30 min. The total amount of the original pCAGGS-*fla*A and free (or unconjugated) pCAGGS-*fla*A were then analyzed using a microspectrophotometer (Biophotometer; Eppendorf, Germany). The association efficiency was calculated as follows:

$$AE(\%) = \left\{ \frac{\text{total amount of pCAGGS-}flaA}{\text{total amount of pCAGGS-}flaA} - \frac{\text{total amount of free pCAGGS-}flaA}{\text{total amount of pCAGGS-}flaA} \right\} \times 100(\%) \tag{1}$$

2.8. Vaccination of Chickens and Challenge Experiment.

One-day-old White Leghorn chickens, randomly divided into three groups (30 per group), were immunized intranasally with chitosan/pCAGGS-*fla*A nanoparticles, chitosan/pCAGGS, and saline control, respectively. These chickens were immunized three times on days 1, 15, and 29 at a dose of 150 μg in 200 μL (100 μL each nostril). On day 42, all chickens were challenged orally with 5×10^7 ALM-80 strain. Three chickens were sacrificed every 3 days from day 45 to day 63 to harvest the small intestine, large intestine, and cecum to calculate the number of *C. jejuni* ALM-80. The method of isolation and enumeration of *Campylobacter* can be found in our preliminary study [4].

2.9. Serum and Mucosal Antibody Titers to Campylobacter Measured by Enzyme-Linked Immunosorbent Assay (ELISA).

Blood was collected after 4 and 6 weeks of primary immunization and sera were stored at −70°C until they were tested by ELISA for antibodies. The intestinal mucosal secretion was collected at 2 and 4 weeks after booster immunization. The abdominal cavity was opened aseptically, and the entire small intestine, including the duodenum, jejunum, and ileum, was collected. Pancreas, connective tissue, and fat were removed into PBS, and the intestine was cut longitudinally and then cut into 1-cm-long sections. The intestinal antibodies were extracted using a PBS solution containing Tween 20 (0.05%, v/v), soybean trypsin inhibitor (0.1 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA), EDTA (0.05 mg/mL), and phenylmethylsulfonyl fluoride (PMSF) (0.35 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). Intestinal lavage solutions were mixed with extraction solution and shaken for 2 h at 4°C. After centrifugation at 20,000 × g for 30 min at 4°C the supernatant was collected. Bovine serum albumin was added to a final concentration of 0.1% (w/v) and samples were preserved at −20°C. The titer of antibodies against *Campylobacter* in intestinal secretions was measured using an indirect enzyme-linked immunosorbent assay (ELISA) developed by laboratory procedure. Briefly, the ELISA involved the following steps: (1) 96-well microtitre plates were coated with the isolated strain ALM-80 (5 × 10^5 cfu/well) for 6 h at 42°C, and blocked with BSA solution (10%, w/v) for 1 h at 37°C, then washed with a 0.05% (v/v) solution of Tween 20 in PBS. (2) Samples were serially diluted and added. The plates were incubated for 2 h at 37°C and washed with PBS-Tween. (3) Goat antimouse IgG, IgM, or IgA was added. The plates were incubated for 1.5 h at 37°C and washed with PBS-Tween. (4) *o*-phenylenediamine dichloride (OPD; Sigma, USA) was added to the plates at room temperature. (5) H_2SO_4 (2.5 M) was added to stop the reaction 30 min after addition of the substrate. (6) The absorbance was recorded at 490 nm.

2.10. Ratio of CD4$^+$/CD8$^+$ T Cells in Cecal Tonsil and Spleen.

To analyze the changes in T-cell composition upon immunization, spleen and cecal tonsil of each animal (four per group on investigation days 42 and 56) were crushed in ice-cold PBS. The homogenates were centrifuged at 300 rpm for 2 min at below 4°C before the insoluble matter settled. The isolated cells were incubated with FITC- (fluoresceinisothiocyanate-) labeled CD4 and PE (phycoerythrin-) labeled CD8α monoclonal antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) for 30 min at 37°C in the dark. After the cells were washed, aliquots of 20,000 cells per sample were analyzed using a FACSAria flow cytometry system (Becton Dickinson, CA, USA).

2.11. Statistical Analysis.

The data were presented as mean ± standard deviation. The Student *t*-test for comparison of two independent samples was used to evaluate differences between the groups (immunized group versus control). Viable counts of bacteria were converted into logarithmic form. For the purpose of statistical analysis, a $\log_{10}$ viable count of <1.47 (limit for direct plate detection), obtained from a sample that became positive only after enrichment, was rated as a $\log_{10}$ value of 1.0. A sample which yielded no *C. jejuni* growth after enrichment was rated as a $\log_{10}$ value

of 0. Data were evaluated by multifactorial variance analysis. *P*-values less than .05 were considered to be significant, and *P*-values less than .01 were considered to be highly significant. The flow cytometry data were evaluated using BD Bioscience's FACS Diva software (Becton Dickinson, CA, USA).

3. Results

3.1. Identification of pCAGGS-flaA. *fla*A was amplified from *C. jejuni* ALM-80 by PCR, and sequence analysis showed that the sequence of amplified fragment was consistent with that of *fla*A published in GenBank. Recombinant plasmids pT-*fla*A and pCAGGS-*fla*A were confirmed by PCR and restriction enzyme digestion. It was demonstrated that recombinant plasmids contained the *fla*A gene.

3.2. Synthesis and Characterization of Chitosan-DNA. Chitosan-DNA complexes were synthesized when DNA (pCAGGS-*fla*A) solution (100 μg/mL in 5 mM Na_2SO_4) was added to 0.02% (w/v) chitosan (pH 5.5) at 55°C during high-speed vortexing. Uniform particles were obtained by coacervation between chitosan and DNA. TEM observation confirmed that freshly synthesized chitosan-DNA complexes were 80–100 nm in size and nearly spherical (Figure 1), with an association efficiency of 91.9%.

The ability of the chitosan/pCAGGS-*fla*A nanoparticles to protect pCAGGS-*fla*A from nuclease degradation was evaluated by enzymatic digestion and agarose gel electrophoresis. As shown in Figure 2, 1 μg naked DNA was completely degraded (lane 5) under 28 U/mL DNAse I digestion. However, even at such a high DNAse I concentration, chitosan-DNA complex remained intact (lane 2). As a control, 2 μg chitosan showed entire degradation after chitosanase and lysozyme digestion releasing most of the DNA inside the complex (lane 3). These results suggest that chitosan could protect DNA from markedly high concentration of DNAse I digestion. At the physiological condition such as mucosal membrane surfaces with large quantities but lower concentration of nucleases, this protection of DNA by chitosan would be meaningful for the maintenance of integration and function of DNA vaccine.

3.3. Specific Serum and Intestinal Antibody Responses. The ability of constructed chitosan-DNA vaccine complex to express *fla*A in eukaryotic cells (COS-7 cell line) transfected with pCAGGS-*fla*A, and the negative control pCAGGS was elucidated. At 48 h post transfection, only pCAGGS-*fla*A-transfected cells expressed protein FlaA, which was detected by immunofluorescent assay (Figure 3).

In order to evaluate the potential utility of chitosan-DNA immunization in inducing specific responses, 1-day-old commercial White Leghorn chickens were vaccinated orally with chitosan-plasmid nanoparticles at 2-week intervals. Negative control groups included blank and CS-pCAGGS controls. Low titers of specific antibodies were observed in all immunized groups at day 28. However, the titers of specific
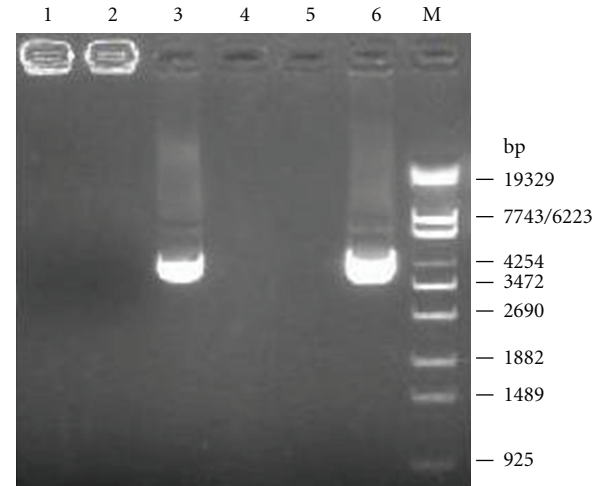

Figure 1: TEM image of chitosan-DNA. Freshly prepared chitosan-DNA solution was negatively stained with uranyl acetate. Scale bars represent 200 nm. The freshly synthesized chitosan-DNA complexes were approximately 80–100 nm in size and nearly spherical under TEM.
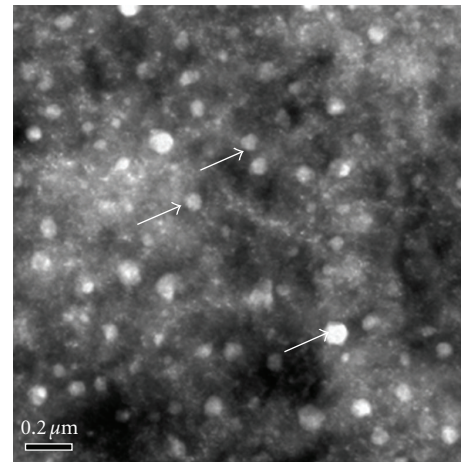

Figure 2: Protection of DNA from DNAse I digestion by chitosan. Naked DNA and chitosan-DNA complex containing 1 μg DNA were both incubated with DNAse I for 15 min at 37°C. Then the reaction was stopped by adding with 0.5 M EDTA. chitosan-DNA was then subjected to chitosanase and lysozyme digestion. Finally, all samples were run on 0.8% (w/v) agarose gel and stained with ethidium bromide. Lane 1: chitosan-DNA; 2: chitosan-DNA + DNAse I (28 U/mL); 3: chitosan-DNA + chitosanase (0.15 U/mL) + lysozyme (15 U/mL); 4: 3 + DNAse I (28 U/mL); 5: DNA+ DNAse I (28 U/mL); 6: DNA (pCAGGS-*fla*A, 4.1 kb).

antibodies in the CS-pCAGGS-*fla*A group were elevated significantly at day 42 ($P < .01$) (Table 1).

3.4. Ratio of $CD4^+/CD8^+$ T Cells in Spleen and Cecal Tonsil. The number of $CD4^+/CD8^+$ cells in cecal tonsil and spleen was detected by flow cytometry. Experimental data showed that the ratio of $CD4^+/CD8^+$ T cells in the spleen of the CS-pCAGGS-*fla*A group was significantly increased at day

TABLE 1: Immunogenicity assay of *C. jejuni* chitosan-DNA vaccine in chickens.

Group	Titers of antibodies in serum		Titers of antibodies in intestinal mucosa	
	Second immunization	Third immunization	Second immunization	Third immunization
CS-pCAGGS-*fla*A	96.0 ± 55.4[a]	426.7 ± 147.8[a]	65.3 ± 62.0[b]	170.7 ± 73.9[a]
CS-pCAGGS	26.7 ± 9.2	23.3 ± 18.5	34.7 ± 28.1	13.3 ± 4.6
PBS	26.7 ± 9.2	23.3 ± 18.5	34.7 ± 28.1	29.3 ± 30.3

[a]High significantly different ($P < .01$) among vaccine groups as calculated by Student's *t*-test.
[b]Significantly different ($P < .05$) among vaccine groups as calculated by Student's *t*-test.
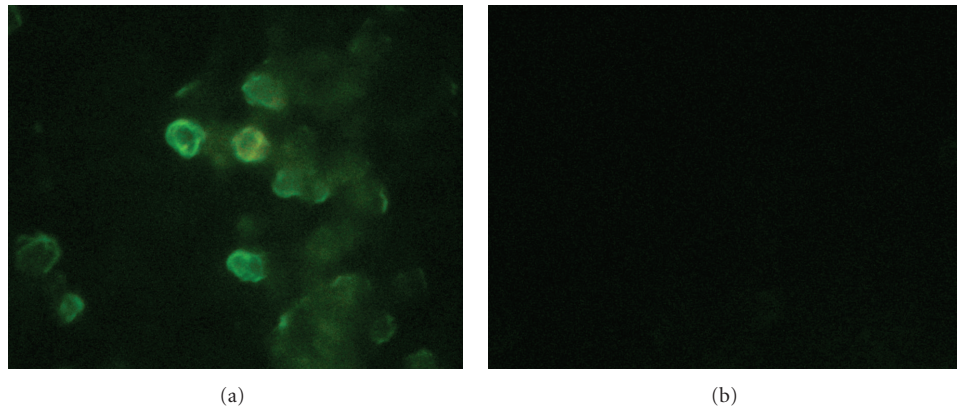"CS" is the short form for chitosan.

(a)

(b)

FIGURE 3: *In vitro* gene expression of chitosan-encapsulated pCAGGS-*fla*A in COS-7 cell line by immunofluorescent assay. Ability of constructed chitosan-DNA vaccine complex to express *fla*A in eukaryotic cells (COS-7 cell line) transfected with pCAGGS-*fla*A (a), and the negative control pCAGGS (b) was evaluated. After 48 h transfection, only pCAGGS-*fla*A-transfected cells expressed protein flaA, which was detected by fluorescent microscopy.

28 and day 42, and the number of CD4$^+$ cells was greater than the number of CD8$^+$ cells. In the cecal tonsils, the ratio of CD4$^+$/CD8$^+$ cells significantly increased after the third immunization compared to that in the control group. The results indicated that the ratio of CD4$^+$/CD8$^+$ T cells in the cecal tonsil and spleen of the CS-pCAGGS-*fla*A groups was increased after the third immunization, and the number of CD4$^+$ cells was greater than the number of CD8$^+$ cells (Table 2).

3.5. Shedding of C. jejuni in Immunized Chickens after Challenge. After oral challenge with 5×10^7 bacteria of strain ALM-80, the percentage of chickens with *C. jejuni*-positive cloaca showed a downward trend in the vaccine group, and bacteria were not detected at day 63. However, the negative control groups retained a high *C. jejuni*-positive ratio (Table 3).

3.6. Protection Effects of Immunized Chickens after Challenge with C. jejuni. After challenge, in vaccine group, the number of strain ALM-80 bacteria in the small intestine, large intestine, and cecum reached a peak from day 51 to day 57, and then decreased. The number of bacteria was significantly lower than control, which showed an upward trend of bacterial number in small intestine, large intestine and cecum. In the CS-pCAGGS-*fla*A group, ALM-80 was cleared from the small intestine on day 60 (Table 4). Compared with

the negative control groups, the number of *C. jejuni* was reduced by 2-3 $\log_{10}$ in the large intestine and by 2 $\log_{10}$ in the cecum when chickens were challenged with the homologous strain (Table 4). Infection tests induced expression of that chitosan-DNA nanoparticle vaccine that delivered *fla*A of *C. jejuni* could reduce the number of *C. jejuni* in small intestine, large intestine, and cecum effectively.

4. Discussion

Vaccination has played an important role in the protection of chickens against *C. jejuni* infections. However, the conventional killed vaccines against *C. jejuni* do not completely prevent infection and bacterial shedding [1]. Thus, an improved vaccine and/or vaccination schedule is needed to induce immunity in chickens. In the current study, we have developed chitosan/pCAGGS-*fla*A nanoparticles to inhibit *Campylobacter jejuni* in a White Leghorn model, which is a common layer chicken breed in China.

DNA vaccine can provide effective immunostimulation in terms of humoral and cell-mediated responses, therefore overcoming the influence of maternal antibody. Meanwhile, DNA vaccine has broad prospects in the prevention and treatment of bacterial, viral, parasitic, and other infections [12]. The strong immunogenicity and cross-protection of antigen genes are the prerequisites for constructing DNA vaccines, which is a good strategy to improve their immunological effects. For *C. jejuni*, adhesion to, invasion, and

TABLE 2: Ratio of CD4$^+$/CD8$^+$ T cells in spleen and cecal tonsil of immunized chickens.

Group	Ratio^a of CD4$^+$/CD8$^+$ T cells in spleen		Ratio^a of CD4$^+$/CD8$^+$ T cells in cecal tonsil	
	Second immunization	Third immunization	Second immunization	Third immunization
CS-pCAGGS-*fla*A	1.34 ± 0.53^b	1.54 ± 0.23^b	0.98 ± 0.17	1.94 ± 0.21^b
CS-pCAGGS	0.63 ± 0.22	0.94 ± 0.22	0.92 ± 0.26	1.29 ± 0.18
PBS	0.62 ± 0.05	1.00 ± 0.08	1.17 ± 0.30	1.27 ± 0.30

^{a}Values are means ± standard deviations (four samples per group) for ratio of CD4$^+$/CD8$^+$ T cells treated with CS-pCAGGS-*fla*A and CS-pCAGGS or PBS.
^{b}Significantly different ($P < .05$) among vaccine groups as calculated by Student's *t*-test.

TABLE 3: Average positive rate of swab specimens of White Leghorn chickens challenged with strain ALM-80.

Group	Average positive rate of swab specimens after oral challenge		
	7 days	14 days	21 days
CS-pCAGGS-*fla*A	47.62% (10/21)	30.77% (4/13)^a	0.00% (0/9)^a
CS-pCAGGS	47.62% (10/21)	53.33% (8/15)	44.44% (4/9)
PBS	42.86% (9/21)	53.33% (8/15)	55.56% (5/9)

^{a}Significantly different ($P < .05$) among vaccine groups as calculated by Student's *t*-test.

colonization of host intestinal cells require the participation of flagellin, which is essential for *C. jejuni* to adhere to host cells. Therefore, it is a key step in the process of host infection [16]. In our study, based on the analysis of virulence genes mainly carried by isolated strains, we selected flagellin gene *fla*A, which is closely involved in the infection process, as a protective antigen gene to construct a DNA vaccine. The flagella gene plasmid DNA constructed by Wyszyńska et al. can reduce the number of *Campylobacter* by 2 log$_2$ [17]. Widders et al. found that titers of IgG and IgM in serum and IgG in intestinal mucosa were high after primary immunization with intraperitoneal injection and secondary immunization with intraperitoneal injection or oral administration of purified flagellin protein, and results indicated that immunization could reduce the level of intestinal infection with *C. jejuni* by 1-2 log$_2$ [18].

DNA vaccine expression vectors with different promoters have significant impacts on expression of immunogenic genes [19]. The plasmid vector pCAGGS contains chicken β-actin promoter and cytomegalovirus enhancer of eukaryotic expression plasmid, therefore, the level of pCAGGS will be significantly high after transient transfection of most cell lines [20, 21]. Many studies have suggested that the expression efficiency of pCAGGS is high, therefore, it is presently being applied widely [22]. On the other hand, using chitosan, a natural bioadhesive material, as a gene carrier turned out to be an ideal way to stimulate the immune system. The aminoglucose and DNA can form complexes (polyplexes) via their electrostatic interaction. Chitosan can condense DNA into relatively compact nanoparticles and these can overcome physiological barriers when drugs are absorbed by the mucosa and arrive at the submucosal lymphoid tissue by endocytotic channels. The advantages of chitosan nanoparticles as gene vectors are as follows. (i) They can protect DNA from degradation *in vivo* [15]. (ii) Positively charged nanoparticles together with negatively charged mucosal cells and sialic acid can generate stronger electrostatic interactions and prolong the reaction time between vaccines and the mucosa, and increase vaccine uptake [23]. The preparation conditions of chitosan-DNA nanoparticles are mild, without using organic solvents, which solves the problem of DNA destruction by solvent residues. Chitosan is regarded as a potential nonviral gene carrier. Mao et al. were the first to combine DNA vaccine and chitosan to create a chitosan-DNA nanovaccine, which successfully generated immunological protection in a murine model of peanut allergy, and proved to be an effective oral gene delivery system [15]. In our study, high encapsulation efficiency was obtained (>90%), as demonstrated by electrophoresis and electron microscopy. Protection tests showed that chitosan-DNA nanoparticle vaccine that contained *fla*A of *C. jejuni* reduced effectively colonization of *C. jejuni* in the small intestine, large intestine, and cecum.

At present, investigation of the protective immune responses against *C. jejuni* is at the initial stage [24]. *Campylobacter* vaccine can induce significant immune protection against experimental infection of mice, ferrets, rabbits, or primates, and might relieve or eliminate clinical symptoms in these animals [25]. Nevertheless, vaccines have different effects on mammals and chickens, which needs to be considered when research is conducted using chicken models of colonization of the intestinal tract.

In addition, since *fla*A of *C. jejuni* is highly variable [26], it is interesting to assess the protective efficacy of the chitosan/pCAGGS-*fla*A nanoparticles against challenge with other *C. jejuni* strains in future studies. What's more, they need to replicate more efficiently in broilers by optimizing the immunization strategy.

5. Conclusions

This study demonstrated that intranasal delivery of chitosan-DNA vaccine successfully induced effective

TABLE 4: The number of *C. jejuni* in small intestine, large intestine, and cecum after oral challenge.

Organ	Group	CFU (CFU/g)						
		3 days	6 days	9 days	12 days	15 days	18 days	21 days
Small intestine	1	9.2×10^3	3.4×10^3	6.0×10^2	8.2×10^3	1.9×10^3	0	0
	2	3.5×10^2	7.6×10^2	1.2×10^2	1.4×10^4	5.9×10^4	6.3×10^3	7.6×10^4
	3	2.0×10^3	6.3×10^2	7.3×10^2	1.7×10^3	1.2×10^5	2.9×10^4	3.8×10^4
Large intestine	1	4.6×10^5	1.1×10^6	1.5×10^7	4.8×10^6	2.9×10^6	3.5×10^6	6.5×10^5
	2	5.4×10^7	3.2×10^7	2.2×10^7	1.1×10^7	1.8×10^8	4.2×10^7	1.5×10^8
	3	4.7×10^7	2.1×10^7	2.2×10^7	9.3×10^7	3.5×10^8	3.2×10^7	1.8×10^7
Cecum	1	2.2×10^6	4.5×10^6	2.8×10^7	2.9×10^7	3.7×10^7	3.0×10^7	1.2×10^7
	2	2.7×10^8	1.8×10^8	7.4×10^8	2.4×10^8	1.9×10^8	2.6×10^9	2.3×10^9
	3	4.3×10^8	2.2×10^8	4.0×10^8	4.0×10^8	2.6×10^8	4.7×10^9	2.7×10^9

Group 1: CS-pCAGGS-*fla*A group.
Group 2: CS-pCAGGS group.
Group 3: PBS group.

immune response and might be a promising vaccine candidate against *C. jejuni* infection.

Acknowledgments

References

[1] M. R. de Zoete, J. P. M. van Putten, and J. A. Wagenaar, "Vaccination of chickens against *Campylobacter*," *Vaccine*, vol. 25, no. 30, pp. 5548–5557, 2007.

[2] D. G. Newell, "Animal models of *Campylobacter jejuni* colonization and disease and the lessons to be learned from similar *Helicobacter pylori* models," *Journal of Applied Microbiology*, vol. 90, no. 30, pp. 57S–67S, 2001.

[3] D. J. Verwoerd, "Ostrich diseases," *Revue Scientifique et Technique*, vol. 19, no. 2, pp. 638–661, 2000.

[4] J. L. Huang, H. Y. Xu, G. Y. Bao et al., "Epidemiological surveillance of *Campylobacter jejuni* in chicken, dairy cattle and diarrhoea patients," *Epidemiology and Infection*, vol. 137, no. 8, pp. 1111–1120, 2009.

[5] J. A. Wagenaar, D. J. Mevius, and A. H. Havelaar, "*Campylobacter* in primary animal production and control strategies to reduce the burden of human campylobacteriosis," *Revue Scientifique et Technique*, vol. 25, no. 2, pp. 581–594, 2006.

[6] M. D. Lee and D. G. Newell, "*Campylobacter* in poultry: filling an ecological niche," *Avian Diseases*, vol. 50, no. 1, pp. 1–9, 2006.

[7] C. Zhao, B. Ge, J. De Villena et al., "Prevalence of *Campylobacter spp.*, *Escherichia coli*, and *Salmonella* serovars in Retail Chicken, Turkey, Pork, and Beef from the Greater Washington, D.C., Area," *Applied and Environmental Microbiology*, vol. 67, no. 12, pp. 5431–5436, 2001.

[8] D. D. Ringoir and V. Korolik, "Colonisation phenotype and colonisation potential differences in *Campylobacter jejuni* strains in chickens before and after passage *in vivo*," *Veterinary Microbiology*, vol. 92, no. 3, pp. 225–235, 2003.

[9] R. I. Walker, "Considerations for development of whole cell bacterial vaccines to prevent diarrheal diseases in children in developing countries," *Vaccine*, vol. 23, no. 26, pp. 3369–3385, 2005.

[10] S. Huang, O. Sahin, and Q. Zhang, "Infection-induced antibodies against the major outer membrane protein of *Campylobacter jejuni* mainly recognize conformational epitopes," *FEMS Microbiology Letters*, vol. 272, no. 2, pp. 137–143, 2007.

[11] N. E. Scott and S. J. Cordwell, "*Campylobacter* proteomics: guidelines, challenges and future perspectives," *Expert Review of Proteomics*, vol. 6, no. 1, pp. 61–74, 2009.

[12] J. A. Wolff, R. W. Malone, P. Williams et al., "Direct gene transfer into mouse muscle *in vivo*," *Science*, vol. 247, no. 4949, part 1, pp. 1465–1468, 1990.

[13] T. M. Wassenaar, N. M. C. Bleumink-Pluym, D. G. Newell, P. J. M. Nuijten, and B. A. M. Van der Zeijst, "Differential flagellin expression in a *fla*A⁻ / *fla*B⁺ mutant of *Campylobacter jejuni*," *Infection and Immunity*, vol. 62, no. 9, pp. 3901–3906, 1994.

[14] E. A. McNeela, D. O'Connor, I. Jabbal-Gill et al., "A mucosal vaccine against diphtheria: formulation of cross reacting material (CRM197) of diphtheria toxin with chitosan enhances local and systemic antibody and Th2 responses following nasal delivery," *Vaccine*, vol. 19, no. 9-10, pp. 1188–1198, 2000.

[15] H.-Q. Mao, K. Roy, V. L. Troung-Le et al., "Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency," *Journal of Controlled Release*, vol. 70, no. 3, pp. 399–421, 2001.

[16] J. Müller, F. Schulze, W. Müller, and I. Hänel, "PCR detection of virulence-associated genes in *Campylobacter jejuni* strains with differential ability to invade Caco-2 cells and to colonize the chick gut," *Veterinary Microbiology*, vol. 113, no. 1-2, pp. 123–129, 2006.

[17] A. Wyszyńska, A. Raczko, M. Lis, and E. K. Jagusztyn-Krynicka, "Oral immunization of chickens with avirulent *Salmonella* vaccine strain carrying *C. jejuni* 72Dz/92 *cja*A gene elicits specific humoral immune response associated with protection against challenge with wild-type *Campylobacter*," *Vaccine*, vol. 22, no. 11-12, pp. 1379–1389, 2004.

[18] P. R. Widders, L. M. Thomas, K. A. Long, M. A. Tokhi, M. Panaccio, and E. Apos, "The specificity of antibody in chickens immunised to reduce intestinal colonisation with *Campylobacter jejuni*," *Veterinary Microbiology*, vol. 64, no. 1, pp. 39–50, 1998.

[19] D. L. Suarez and S. Schultz-Cherry, "The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model," *Avian Diseases*, vol. 44, no. 4, pp. 861–868, 2000.

[20] Z. Bu, L. Ye, R. W. Compans, and C. Yang, "Enhanced cellular immune response against SIV Gag induced by immunization with DNA vaccines expressing assembly and release-defective SIV Gag proteins," *Virology*, vol. 309, no. 2, pp. 272–281, 2003.

[21] Z. Bu, L. Ye, M. J. Skeen, H. K. Ziegler, R. W. Compans, and C. Yang, "Enhancement of immune responses to an HIV env DNA vaccine by a C-terminal segment of listeriolysin O," *AIDS Research and Human Retroviruses*, vol. 19, no. 5, pp. 409–420, 2003.

[22] H. Niwa, K. Yamamura, and J. Miyazaki, "Efficient selection for high-expression transfectants with a novel eukaryotic vector," *Gene*, vol. 108, no. 2, pp. 193–200, 1991.

[23] R. J. Soane, M. Frier, A. C. Perkins, N. S. Jones, S. S. Davis, and L. Illum, "Evaluation of the clearance characteristics of bioadhesive systems in humans," *International Journal of Pharmaceutics*, vol. 178, no. 1, pp. 55–65, 1999.

[24] O. Sahin, N. Luo, S. Huang, and Q. Zhang, "Effect of *Campylobacter*-specific maternal antibodies on *Campylobacter jejuni* colonization in young chickens," *Applied and Environmental Microbiology*, vol. 69, no. 9, pp. 5372–5379, 2003.

[25] S. Baqar, A. L. Bourgeois, L. A. Applebee et al., "Murine intranasal challenge model for the study of *Campylobacter* pathogenesis and immunity," *Infection and Immunity*, vol. 64, no. 12, pp. 4933–4939, 1996.

[26] R. J. Owen, A. Fayos, J. Hernandez, and A. Lastovica, "PCR-based restriction fragment length polymorphism analysis of DNA sequence diversity of flagellin genes of *Campylobacter jejuni* and allied species," *Molecular and Cellular Probes*, vol. 7, no. 6, pp. 471–480, 1993.