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**Epitope Mapping of *Campylobacter jejuni* Flagellar Capping Protein (FliD) by
Chicken (*Gallus gallus domesticus*) Sera**

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ABSTRACT

Campylobacter jejuni, a Gram-negative rod, is a zoonotic pathogen associated with human acute bacterial gastroenteritis worldwide. The flagellum, composed of more than 35 proteins, is responsible for colonization of *C. jejuni* in the host gastrointestinal tract as well as inducing protective antibodies against the homologous serotype. In our previous study, we demonstrated that the flagellar capping protein (FliD) is an immunodominant protein that reacted strongly to sera from field chickens. In this communication, we mapped linear immunoreactive epitopes on FliD using a set of 158 synthetic peptides of 15-mer overlapping with 11 amino acid residues on peptide microarrays with sera from field chickens. The results from peptide microarrays showed (1) no cross-reactivity of the immobilized peptides with the secondary anti-chicken antibody in the control incubation, and (2) heterogeneous patterns of sera reacting to the immobilized peptides. The peptides that strongly reacted to more than three chicken sera were selected for further validation by the peptide ELISA. The results showed peptides 24, 91 and 92 had relatively high reactivity and less variation among 64 individual serum samples, indicating these peptides represented the shared immunodominant epitopes on the *C. jejuni* FliD protein. These peptides were also recognized by sera from chickens immunized with the purified recombinant FliD protein. The findings of the specific shared linear immunodominant epitopes on FliD in this study provide a rationale for further evaluation to determine their utility as epitope vaccines covering multiple serotypes for chicken immunization, and subsequently, for providing safer poultry products for human consumption.

KEYWORDS

Campylobacter jejuni, Flagellar capping protein, FliD, Epitope mapping, Zoonosis,

Foodborne pathogen

INTRODUCTION

Campylobacter jejuni, a Gram-negative, curved-rod, microaerophilic and flagellated bacterium [1], is the most common etiological agent of human bacterial gastroenteritis worldwide with 400 – 500 million culture-confirmed cases annually [2-6]. The clinical spectra of *Campylobacter* infection range from asymptomatic carriage to the severe diseases in the forms of Guillain-Barré syndrome and reactive arthritis [7-9]. Animals including poultry and wild birds are important reservoirs of this bacterium. Consumption of contaminated poultry products is regarded as a major source for human infection [10-12]. Although *C. jejuni* has been intensively studied [13, 14 for review], the pathogenesis of this bacterium in poultry as well as in humans is not yet fully understood. Many *C. jejuni* genes and their gene products have been recognized as important factors for bacterial colonization and invasion of intestinal tracts [15-17]. The flagellum is one of these factors.

The flagellum of microorganisms has a complex structure, composed of three regions-the basal body, the hook and the extracellular filaments [13]. The major extracellular component is synthesized as flagellin monomers, which require capping by flagellar capping protein (FliD) at the distal tip of the mature flagellum [18-23]. The *fliD* gene of *C. jejuni* contains 1,929 nucleotides, potentially encoding 642 amino acids with a calculated molecular mass of 69.6 kDa [24]. The immunoblot analysis has demonstrated that the chicken sera from broilers and layers reacted strongly to the FliD protein, suggesting that the anti-FliD antibodies are prevalent in the poultry flocks [24, 42]. Therefore, this protein provides us with potential applications: (1) FliD may be used

as a vaccine for chickens to reduce colonization of *C. jejuni* in the gastrointestinal tract, and (2) this protein may be used for serology to rapidly identify *C. jejuni* in samples.

Anti-*Campylobacter* antibodies have long been regarded as an effective means for reducing *Campylobacter* colonization in chickens [e.g. 25-27]. Two recent studies further explored the specificity of immune responses to the *Campylobacter* FlaA and cj0669 proteins [28, 29]. However, little information about the immune responses of chickens to other flagellar proteins, including FliD, is available.

In this communication, we used chicken sera from the field in conjunction with the peptide microarray to screen potential epitopes in the FliD protein, validated the epitopes with peptide ELISA, and compared immune responses of the FliD-immunized chickens to the epitope peptides.

MATERIALS AND METHODS

Bacterial strains and culture conditions: *Campylobacter jejuni* D1-39 was cultured in Mueller-Hinton agar plates at 42 °C for 48 hours in a microaerobic condition (5% O₂, 10% CO₂ and 85% N₂) [30]. *Campylobacter jejuni* D1-39 was originally isolated from broiler fecal samples in Georgia, U.S., and is a robust colonizer in the chicken gastrointestinal tract. Competent *E. coli* cells were cultured according to the manufacturer's instructions (Lucigen Corp., Middleton, WI).

Expression and purification of FliD: The recombinant FliD protein was expressed in and purified from *E. coli* cells as described previously [31, 32]. The purity of the recombinant FliD protein was determined by SDS-PAGE [33].

Epitope mapping of FliD: The epitope mapping experiments were carried out by JPT Peptide Technologies GmbH (Berlin, Germany). Briefly, a set of 158 peptides of 15-mer

overlapping with 11 amino acid residues were synthesized and printed in triplicate on microarray slides (Supplemental Table 1). The slide was incubated with chicken sera (1:200 dilution in a SuperBlock T20 blocking buffer [Thermo Scientific, Rockford, IL]) for 60 min at 30 °C, followed by incubation in 0.1 µg/ml of anti-chicken IgY conjugated with Cy5 for 60 min at 30 °C. Twenty individual chicken sera (see below) as primary antibodies were used for probing the peptide microarrays. To assess the potential non-specific binding between the peptides and the labeled secondary antibody, a sub-microarray with the immobilized peptides was incubated with the anti-chicken IgY labeled with Cy5 at a concentration of 0.1 µg/ml. The slide was extensively washed after antibody incubation with 50 mM Tris-buffered saline-0.1% Tween 20 (pH 7.2). The slide was scanned using an Axon Geneoix scanner. Laser settings and applied resolution were identical for all performed measurements. The images were analyzed and quantified using spot-recognition software GenePix (Molecular Devices, Sunnyvale, CA). For each spot, the mean signal intensity was extracted between 0 and 65,535 in arbitrary units. The value of each peptide per serum was the mean of three spots on the microarray. If the coefficient of variance (CV) was larger than 0.5, the mean of the two closest values was used.

Immunization of broiler chickens with FliD: One-day-old broilers were purchased from local hatcheries. Five broilers were given intraperitoneally (ip) two doses of 100 µg of the FliD protein emulsified in Freund's incomplete adjuvant (Sigma-Aldrich Co., St. Louis, MO) at one and three weeks of age. PBS mixed with the same adjuvant was also given ip to five broilers as a negative control. All broilers from the same litter were raised in isolators with access to commercial food and water *ad libitum*. *Campylobacter*

in chickens was monitored by direct plating of fecal contents [43] from one week old to six weeks old. All samples were negative to *Campylobacter*. The use of broilers in the experiments followed the AVMA Guidelines for the Euthanasia of Animals [34], and was approved by the Institutional Animal Care and Use Committee of the Richard B. Russell Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Athens, GA. The proposal no. was PMSRU-08-2014-B.

Chicken sera: Blood from chickens was withdrawn from the brachial wing vein [35]. Blood was clotted by incubation at 37 °C for one hour, followed by at 4 °C overnight. Sera were collected by low-speed centrifugation, aliquoted and stored at -80 °C. Sixty-four sera (including 20 for the above peptide microarrays) were collected from a commercial flock for the routine serology, and were obtained from the Cornell University Animal Health Diagnostic Center, Ithaca, NY. The ages of chickens at bleeding were 44 - 52 weeks old. Previously these sera were tested positively to FliD by immunoblot [42]. However, the status of *Campylobacter* in the flock was not determined.

For the immunized and PBS groups, broilers were bled at the ages of five and six weeks (i.e. two and three weeks after second vaccination, respectively).

ELISA: ELISA was performed essentially as described previously [36]. Peptides of 15-mer, which were synthesized by LifeTein, LLC (Hillsborough, NJ), were dissolved in 100 mM sodium bicarbonate-carbonate buffer (pH 9.6). Hydrophobic peptides were first dissolved in methanol and then in bicarbonate-carbonate buffer for coating. Wells were coated with 5 µM of peptide in 100 µl buffer at 4 °C overnight. The next day, wells were blocked with a SuperBlock T20 blocking solution (Thermo Scientific) according to the manufacturer's instructions. The wells were incubated with broiler sera (1:100 dilution in

a PBS-0.5% Tween-20 solution) at 37 °C for one hour, followed by incubation with horseradish peroxidase (HRP) conjugated goat anti-chicken IgG antibody (KPL, Gaithersburg, MD) at the same condition. The HRP was detected with a TMB microwell peroxidase substrate system (KPL), and the reaction was terminated by the addition of an acid. The optical density of the reaction was read at a wavelength of 450 nm in a Thermo Multiskan MCC microplate reader (Thermo Scientific). The plates were intensively washed with a PBS-0.5% Tween-20 solution after each antibody incubation. No peptide, no broiler sera and no HRP-conjugated antibody wells were included as negative controls. The OD readings of the tested samples were subtracted from the average of the OD readings of negative controls. In one experiment, the ratios of each peptide OD readings to the peptide 52 OD readings were calculated (Fig. 1).

Statistical analysis: For ranking the reactivity of 64 sera from 44 - 52 weeks old chickens to the peptides, one-way ANOVA, two-way ANOVA and non-parametric methods were used to analyze the ELISA data. The student *t* test was used to compare serum ELISA reactivity between immunized and non-immunized chicken groups. A *p* value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

It is well known that the initial step of antigen and antibody interaction involves the recognition of the epitopes on an antigen by antigen-binding sites in antibodies [37]. Mapping the epitopes of an antigen provides insight into the specificity of the host immune response and thus provides a rationale for effective vaccine development [37]. To define the linear epitopes on the FliD protein, a total of 158 15-amino-acid peptides with overlapping 11 amino acid residues were synthesized and printed on a slide as a

21-multiwell peptide microarray format. As depicted in Supplemental Fig. 1S, we did not observed cross-reactivity between the peptides and the secondary antibody (< 290 arbitrary fluorescent units [FU]). Supplemental Fig. 1S shows the heatmap diagram, which compared differential antibody binding to each immobilized peptide. In general, the reactions of the sera to the peptides showed the heterogeneous patterns without the shared epitopes among 20 chicken sera. In addition, 16 peptides reacted strongly to chicken sera (> 3300 FU in an average of 20 chicken sera) (Table 1). With the exception of peptides 24 and 70, fourteen peptides were hydrophilic as calculated by the Kyte and Doolittle method (Table 1) [38], suggesting that they may be located on surface regions of the FlhD protein. The results are in agreement with the common notion that antigenic sites are located on the protein surface [37]. In further analysis, six chicken sera showed reactions to the several sets of overlapping peptides (Supplemental Table 1S). For example, serum sample 50 reacted to consecutive peptides 36 - 38. The short consensus sequence from these overlapping peptides is VDKNTTY, which is conserved among *C. jejuni* groups. We originally expected to use the chicken sera from the field to identify the common shared overlapping peptides in the peptide microarray, but were unable to determine the shared epitopes in this assay. Nonreactivity of other sera to the immobilized peptides may be due to the affinity and/or concentrations of the antibodies in chicken serum samples. A study on the *Campylobacter* FlaA epitope mapping showed that some peptides (P8, P16 and P21) reacted with 89%, 74% and 78%, respectively, of sera tested [28]. Furthermore, a recent study demonstrated that some monoclonal antibodies with well-defined epitopes

still cannot recognize the epitopes in broad peptide microarrays [39], reflecting the complicated nature of the peptide-protein interaction.

Because we could not identify the shared epitopes across 20 chicken sera, we selected five peptides (as highlighted in Table 1) that reacted to more than three chicken sera and had higher average of fluorescence unit for further analysis by peptide ELISA. Peptide 52 was also included in the peptide ELISA as a negative control. A total of 64 individual chicken sera, including 20 sera used for peptide microarray, were used for ELISA. The ratios of means of peptide ELISA OD values (in triplicate from individual serum samples against peptides) to the peptide 52 are depicted in Fig. 1. Individual chicken serum reacted to each peptide at various degrees. Based on the means of OD values of 64 sera for each peptide, we noted peptides 24, 91 and 92 had relatively high reactivity to sera, and had less variation among chickens (Table 2), indicating these peptides be the shared immunodominant epitopes for the *C. jejuni* FliD protein among chickens. Further ranking the reactivity of these peptides to chicken sera, each peptide is statistically significantly different from the other: peptide 24 > peptide 91 > peptide 92 > peptide 70 > peptide 155. This ranking of the reactivity is in an agreement with the results obtained from the microarray (Table 1). Whether these anti-epitope antibodies in chickens were elicited by other bacterial proteins remains to be determined. We previously analyzed that the degree of conservation of the FliD proteins ranges from >95% (vs. *C. jejuni*) to 77% (vs. *C. coli*) to <40% (vs. other *Campylobacter* spp.) [24]. Additionally, the *C. jejuni* FliD amino acid sequence shares 26% and 27% identity with those of *Escherichia coli* and *Salmonella*, respectively. These three peptides are conserved among *C. jejuni* groups, but not with other species.

Thus the possibility of chicken anti-epitope antibodies induced by other proteins is unlikely, but cannot be ruled out completely.

We further evaluated whether these epitope peptides identified by field chicken sera were also recognized by antibodies from chickens immunized with the purified recombinant FliD protein. Chickens were immunized with the FliD protein prepared in Freund's incomplete adjuvant as described in the Materials and Methods section. The reason for using commercial broilers for the FliD immunization is that there are no specific *Campylobacter* free chickens available. Immunoblot analysis was used to monitor chicken immune response to the FliD protein. As expected, all sera from one-day old chickens reacted strongly to the FliD protein (unpublished data). At three weeks of age (i.e. two weeks after first immunization), the FliD protein was not detected by sera from the PBS group. On the other hand, eight out of ten sera from the immunization group reacted to FliD (unpublished data). Similar results were also observed at five weeks of age (i.e. two weeks after second immunization). All chickens were negative to *Campylobacter* by bacterial cultures. Our results show that (1) maternal antibodies against FliD existed in high concentrations, but declined as they grew up. This is in agreement with the recent study by others [25]. (2) The FliD protein was immunogenic. Because no *Campylobacter* was isolated under our housing condition, it is very unlikely that the antibodies against FliD were induced by this microorganism in the environments. (3) It seems that the maternal antibodies may not affect the FliD immunization in our study.

As depicted in Fig. 2, variations in the OD readings among individual chickens in both PBS and FliD immunized groups were observed. In further analysis, the chickens

immunized with the FliD protein had statistically higher serum reactivity than the PBS groups to peptides 70 ($p = 0.0034$) and 155 ($p = 0.0003$) and peptides 91 ($p = 0.0150$) and 92 ($p = 0.0355$) after two and three weeks, respectively, of second immunization. Although the serum reactivity of the FliD-immunized chickens to the peptide 24 was higher than that from the PBS groups after two and three weeks immunization, both groups showed no statistical differences, due to outliers. It is interesting to note that the OD values from the FliD immunized chickens were lower than those from field chickens (Table 2 vs. Fig. 2). Several factors may play roles in these differences, such as various forms of antigens, routes of antigen administration, and age of the chickens. These results indicate that these peptides can be recognized by sera from field chickens as well as FliD immunized chickens, so these peptides may be potential candidates for vaccines.

Currently, there is no effective *Campylobacter* vaccine available. The use of live attenuated *C. jejuni* vaccines for poultry is attractive, but colonization in chicken guts and shedding of this microorganism in the environment have potential risks in food safety. Previous studies have demonstrated that antibodies against *Campylobacter* have protective ability, but are limited to the homologous serotype [e.g. 40, 41]. Our ultimate goal is to develop effective cross-reactive protein subunit vaccines against *C. jejuni*. We previously observed that sera from layers with ages of 42 – 51 weeks reacted strongly to the FliD protein in the Western blot analysis (unpublished data). Therefore, this protein may be an excellent target for vaccine development. To achieve this goal, identification of immunodominant and cross-reactive epitopes is needed. This study identified three peptides (peptides 24, 91 and 92) in the FliD protein that reacted

strongly to 64 individual serum samples from field chickens and that were recognized by sera from chickens immunized with FliD. These three peptides are also conserved among *C. jejuni* groups, suggesting that they merit further evaluation as to their immunogenicity and potential for use in epitope vaccines.

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FIGURE LEGEND

Fig. 1. Reaction of 64 chicken serum samples to the selected 15-mer peptides by ELISA. Each point indicates the ratio of the peptide OD mean value (in triplicate from each serum sample) to the peptide 52. The x-axis indicates the individual chicken serum samples collected from the field for ELISA. The y-axis indicates the ratio. The peptides for ELISA are shown at the right side of the figure.

Fig. 2. Reaction of serum samples from chicken non-immunized and immunized with the purified recombinant *C. jejuni* FliD protein to the selected peptides by ELISA. The y-axis indicates OD readings at a wavelength of 450 nm. The x-axis indicates the serum samples from non-immunized and immunized chickens reacted to the indicated peptides. Serum samples were collected two weeks (**A**) and three weeks (**B**) after second vaccination. Each point indicates the average of the OD values in triplicate for each individual serum sample. The horizontal bars indicate the means of the OD values of five serum samples.

Table 1. Synthetic peptide sequences in single-letter codes reacted strongly to chicken serum samples by peptide microarray assay

Peptide No ^a .	Amino Acid Sequence	Position in FliD Protein ^b	Average of Arbitrary Fluorescence Unit ^c	Hydropathy of Peptide ^d
10	TKKIEENTTKQKDLT	37 - 51	3388.62	-27.6
24	NSGVALPSMNINVTQ	93 - 107	7114.73	2.1
30	KGLANDGGFVNAQLN	117 - 131	4674.55	-4.4
33	QLNGTADLTFFSNGK	129 - 143	4511.15	-5.9
37	YTVTVDKNTTYRDLA	145 - 159	5544.33	-10.3
38	VDKNTTYRDLADKIN	149 - 163	4990.97	-18.2
46	GTPYRLTLTSKETGE	181 - 195	4397.83	-15.1
48	TSKETGEDSAISFYA	189 - 203	4841.30	-9.0
70	DLGVGMTLTLNKTGE	277 - 291	6553.60	-0.2
74	NFDVQQDFEGVTKAM	293 - 307	4860.70	-8.3
91	DGTTEDANGNKVNTK	361 - 375	7023.37	-25.7
92	EDANGNKVNTKVMLS	365 - 379	6791.73	-11.3
104	SFFSNITKYEDINHT	413 - 427	5258.33	-10.8
151	MIDTRYDTMANQWLQ	601 - 615	7216.25	-11.7
153	MANQWLQYESILNKL	609 - 623	4991.40	-4.8
155	ESILNKLNQQNLNTVT	617 - 631	5775.50	-7.0

^a The highlighted peptides (nos. 24, 70, 91, 92 and 155) were synthesized and used in peptide ELISA.

^b The amino acid positions of the peptides in the FliD protein are numbered after the *Campylobacter jejuni* D1-39 FliD protein (GenBank accession no. KC618388)

^c The numbers indicate the signal intensity of the averages of 20 sera of reactions and are assigned as an arbitrary fluorescence unit.

^d Hydropathy of each peptide was calculated by the Kyte and Doolittle method (1982).

Table 2 The OD values of chicken serum samples reacted to the peptides by ELISA^a

Peptide No.	OD Ranges	Mean ^b	Standard Deviation ^b	Coefficient of Variation ^c
24	2.391 - 3.411	2.880	0.196	0.068229
70	1.312 - 2.648	1.947	0.281	0.144382
91	2.079 - 3.012	2.555	0.240	0.094020
92	1.544 - 2.989	2.256	0.298	0.132098
155	0.711 - 2.585	1.333	0.339	0.254328
52	0.137 - 0.972	0.480	0.156	0.324036

^a ELISA were performed in triplicate for each serum as previously described (Croix et al. 1993). A total of 64 serum samples were used.

^b The mean and standard deviation of each peptide from 64 OD values were calculated with the Microsoft Office Excel program.

^c Coefficient of variation = standard deviation/mean

Figure 1

Figure 2A

Figure 2B