



Epitope mapping of *Campylobacter jejuni* flagellar capping protein (FliD) by chicken (*Gallus gallus domesticus*) sera

Hung-Yueh Yeh^{a,*}, Arife Ezgi Telli^b, Jarra F. Jagne^c, Christopher L. Benson II^d, Kelli L. Hiatt^a, John E. Line^a

^a United States Department of Agriculture, Agricultural Research Service, U.S. National Poultry Research Center, Poultry Microbiological Safety and Processing Research Unit, 950 College Station Road, Athens, GA 30605-2720, USA

^b Laboratory of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Selcuk, 42003, Konya, Turkey

^c Animal Health Diagnostic Center, Cornell University, Ithaca, NY 14853, USA

^d University of Georgia, Athens, GA 30605, USA

ARTICLE INFO

Article history:

Received 28 May 2015

Received in revised form 14 October 2016

Accepted 17 October 2016

Keywords:

Campylobacter jejuni

Flagellar capping protein

FliD

Epitope mapping

Zoonosis

Foodborne pathogen

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 reacted to more than three chicken sera and had higher averages of fluorescence units 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.

Published by Elsevier Ltd.

1. 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 reviews], 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 1929 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

* Corresponding author.

E-mail address: hungyueh.yeh@ars.usda.gov (H.-Y. Yeh).

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.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Campylobacter jejuni D1-39 was cultured in Mueller-Hinton agar plates at 42 °C for 48 h in a microaerobic condition (5% O₂, 10% CO₂ and 85% N₂) [30]. *C. 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, USA).

2.2. 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].

2.3. 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 S1 in the online version at DOI: <http://dx.doi.org/10.1016/j.cimid.2016.10.003>). The slide was incubated with chicken sera (1:200 dilution in a SuperBlock T20 blocking buffer [Thermo Scientific, Rockford, IL, USA]) 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 GenePix 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, USA). 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.

2.4. 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, USA) 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.

2.5. 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, USA. The ages of these 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).

2.6. ELISA

ELISA was performed essentially as described previously [36]. Peptides of 15-mer, which were synthesized by LifeTein, LLC (Hillsborough, NJ, USA), 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, USA) 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 (OD) 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).

2.7. Statistical analysis

For ranking the reactivity of 64 sera from 44 to 52 weeks old chickens to the peptides, one-way ANOVA, two-way ANOVA and

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