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SHORT COMMUNICATION

Reactions of chicken sera to recombinant *Campylobacter jejuni* flagellar proteins

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Abstract *Campylobacter jejuni* is a Gram-negative spiral rod bacterium and is the leading but underreported bacterial food-borne pathogen that causes human campylobacteriosis worldwide. Raw or undercooked poultry products are regarded as a major source for human infection. *C. jejuni* flagella have been implicated in colonization and adhesion to the mucosal surface of chicken gastrointestinal tracts. Therefore, flagellar proteins would be the excellent targets for further investigation. In this report, we used the recombinant technology to generate a battery of *C. jejuni* flagellar proteins, which were purified by His tag affinity chromatography and determined antigenic profiles of these recombinant flagellar proteins using sera from chickens older than 6 weeks of age. The immunoblot results demonstrate that each chicken serum reacted to various numbers of recombinant flagellar proteins. Among these recombinant proteins, chicken sera reacted predominantly to the FlgE1, FlgK, FlhF, FliG and FliY proteins. These antibody screening results provide a rationale for further evaluation of these recombinant flagellar proteins as potential vaccines for chickens to improve food safety as well as investigation of host immune response to *C. jejuni*.

Keywords *Campylobacter jejuni* · Flagellar proteins · Food-borne pathogen · Zoonoses · ε-Proteobacteria · Campylobacteriosis

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Introduction

Food-borne diseases caused by various agents are one of the several major global public health issues. Among agents, *Campylobacter jejuni* is the leading but underreported food-borne pathogen that causes human acute bacterial gastroenteritis worldwide (Altekruse et al. 1999; Janssen et al. 2008; Kubota et al. 2011). The major source for human infection with this microorganism has been linked to consumption of *Campylobacter*-contaminated poultry products (Hermans et al. 2012b; Sasaki et al. 2013). Therefore, many strategies for reduction of this microorganism in broiler chickens have been extensively investigated (e.g., Wagenaar et al. 2006; de Zoete et al. 2007; Line et al. 2008; Lin 2009; Hermans et al. 2011, 2012a; Bahrndorff et al. 2013; Robyn et al. 2013). However, so far, there are no effective strategies available for controlling colonization of *C. jejuni* in chicken gastrointestinal tracts.

C. jejuni is a Gram-negative, microaerophilic, thermophilic, spiral rod bacterium with one or both polar flagella (Ursing et al. 1994; Ryan et al. 2004). Like other bacterial flagella, the *C. jejuni* flagella are under strict coordination at both transcriptional and translational expression that involves more than 35 genes and their gene products (Gilbreath et al. 2011; Lertsethtakarn et al. 2011). The structure of the flagella consists of (1) the basal body, (2) the hook and (3) extracellular filaments (Gilbreath et al. 2011; Lertsethtakarn et al. 2011). The flagella have been implicated in colonization and invasion during *C. jejuni* pathogenesis (Wassenaar et al. 1991; Grant et al. 1993; Yao et al. 1994; Guerry 2007) so it is reasonable to target these flagellar proteins for potential vaccine development. For example, the flagella of *Pseudomonas aeruginosa* were evaluated as a vaccine component in clinical trials for cystic fibrosis patients (Döring et al. 2007; Campodónico et al. 2010), and

it was demonstrated that the vaccine lowered the risk for infection with this microorganism and prolonged the survival of the patients (Döring et al. 2007).

It was demonstrated that anti-*Campylobacter* antibodies reduce colonization of *C. jejuni* in the broiler chicken gastrointestinal tracts (Sahin et al. 2003; Cawthraw and Newell 2010; Layton et al. 2011). Further, chicken antibody studies with mass spectrometry revealed that a battery of epitopes including two flagellar proteins (FlgE2 and flagellins) in peptide fragments from *C. jejuni* whole-cell lysates were recognized by maternal antibodies (Sahin et al. 2003; Shoaf-Sweeney et al. 2008). However, no other flagellar proteins were detected by the maternal antibodies. Because the annotation of the *C. jejuni* genome sequence is available, we were interested in exploring the antigenicity of these flagellar proteins to identify novel antigens for potential vaccine development for broiler chickens.

We cloned and expressed the *C. jejuni* flagellar proteins in an *E. coli* expression system (Yeh et al. 2013), but whether these flagellar proteins are antigenic for broiler chickens is yet to be determined. Therefore, in this communication, we report using an immunoblot method to analyze the reactivity of 12 recombinant flagellar proteins to chicken sera.

Materials and methods

Production of recombinant flagellar proteins

Flagellar proteins of *C. jejuni* were generated by recombinant DNA technology as described previously (Yeh and Klesius 2011; Yeh et al. 2013). Briefly, each flagellar gene was PCR-amplified and cloned in the pRham expression vector (Lucigen Corp., Middleton, WI, USA). At least, three colonies from each flagellar gene were randomly selected for screening. The recombinant proteins from positive clones were produced by induction of the plasmid-harboring *E. coli* cells with 0.2 % of L-rhamnose, and the proteins were purified by affinity chromatography using cobalt- or nickel-chelating resins (Thermo Scientific, Rockford, IL, USA). The purity of each protein was examined by SDS-PAGE analysis.

SDS-PAGE and immunoblot analysis

Proteins were solubilized in 2X Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) and separated on 10–20 % SDS–Tris–HCl polyacrylamide precasted gels (Bio-Rad Laboratories). After electrophoresis, proteins were stained with Bio-Safe Coomassie G-250 Stain™ (Bio-Rad Laboratories) according to the manufacturer's instructions.

For immunoblot analysis, proteins in the SDS-PAGE gels were electrotransferred onto Immun-Blot® PVDF membranes using the Trans Blot®-Turbo™ Transfer System (Bio-Rad Laboratories) according to the manufacturer's instructions. The membranes were blocked with a solution of 10 mM Tris-0.5 M NaCl-0.5 % Tween 20–5 % skim milk (TNT-milk solution) at room temperature for 2–3 h, followed by incubation with broiler chicken sera (1:500 dilution) at 4 °C overnight. To detect broiler serum IgY, the membranes were incubated with donkey anti-chicken IgY antibody conjugated with horseradish peroxidase (Abcam Plc, Cambridge, MA, USA) and TMB peroxidase substrate (KPL, Inc., Gaithersburg, MD, USA), and membranes were extensively washed between each antibody incubation. The six-His tag fused to the recombinant protein was detected with a Nickel HisDetector™ Western blot kit (KPL, Inc.) according to the manufacturer's instructions. Images were recorded and processed with an AlphaImager HP System (version 3.4.0) (ProteinSimple™, Santa Clara, CA, USA) and its associated software.

Collection of chicken sera

Blood from broiler chickens of 5–6 weeks of age were withdrawn from the brachial wing vein (Kelly and Alworth 2013). Blood was clotted by incubation at 37 °C for 1 h, followed at 4 °C overnight. Sera were collected by low-speed centrifugation, aliquoted and stored at −80 °C. All broilers from the same litter were raised on the floor pens with access to commercial food and water ad libitum. The *Campylobacter* status was not checked.

The experimental uses of broiler chickens were approved by the Institutional Animal Care and Use Committee, Richard B. Russell Agricultural Research Center, Agricultural Research Service, US Department of Agriculture, Athens, GA, USA.

Pre-adsorption of chicken sera with *E. coli* 10G cells

The procedures for adsorption were described previously (Zhang et al. 2011). Briefly, the *E. coli* 10G cells were grown in LB broth at 37 °C with vigorously shaking overnight. The cells were harvested and washed twice with PBS. About 1×10^8 cells were resuspended in chicken sera, and the mixtures were incubated at 4 °C overnight. The adsorbed sera were collected by centrifugation at 10,000×*g* for 20 min. The sera were aliquoted and stored at −80 °C.

Results and discussion

Before identification of immunoreactivities for these recombinant *C. jejuni* flagellar proteins, the purity of these proteins was examined by SDS-PAGE analysis and His tag

detection. One major Coomassie blue-stained band corresponding to the expected molecular mass of each recombinant proteins was observed (Fig. 1). Further, because the sequence of six-His tag was included during the expression vector construction, the recombinant proteins were separated by SDS-PAGE and electrotransferred to PVDF membranes. A Nickel His Detection kit was used for the His tag detection. Each recombinant protein showed a major dense band corresponding to the respective expected protein mass on the blot, indicating these recombinant proteins are fusion proteins. In our previous study, the peptide fragments of the FlgE1, FlgG, FlgH, FlgK, FliD, FliE and FliI proteins were obtained from LC–ESI–MS/MS and were identified as parts of flagellar proteins after database search (Yeh et al. 2013). Therefore, we concluded that our recombinant proteins expressed in the *E. coli* system were indeed flagellar proteins.

In our previous study, we found chicken sera in a 1:500 dilution were optimal for immunoblot analysis to detect the FliD protein (Yeh et al. 2014), so the same dilution factor was used in this study. Many minor protein bands with molecular masses of about 30 kDa and smaller than 20 kDa were also observed in the gels (Fig. 2a). The reason is not known, but we assumed these proteins were originated from host *E. coli* 10G cells. We previously attempted to remove minute amounts of these proteins, but this contamination persisted (Yeh et al. 2013). Alternatively, the broiler chicken sera were pre-adsorbed with the *E. coli* 10G cells, which were used as host cells for the recombinant protein production, as described in the "Materials and methods" section to reduce background reactions. As depicted in Fig. 2b, we observed that except for two protein bands (molecular mass about 30 and 15 kDa), no other minor proteins were detected by chicken sera pre-adsorbed with *E. coli* 10G host cells, suggesting that these minor proteins may originate from *E. coli* during the purification process. Because *E. coli* is an important member in microbiota in chicken gastrointestinal tract, it is not surprising that chicken sera may have antibodies against the *E.coli* proteins. In addition, we observed that the specific antibody reactivity to the recombinant *C. jejuni* flagellar proteins was enhanced after sera were pre-adsorbed with *E. coli* cells (Fig. 2 as an example). Sera, therefore, from broiler chickens were pre-adsorbed with the *E. coli* cells before use for immunoblot analysis as described in "Materials and methods" section.

We wanted to determine whether sera from broiler chickens older than 6 weeks of age contained antibodies that reacted to the recombinant *C. jejuni* flagellar proteins. Twelve recombinant proteins were separated by

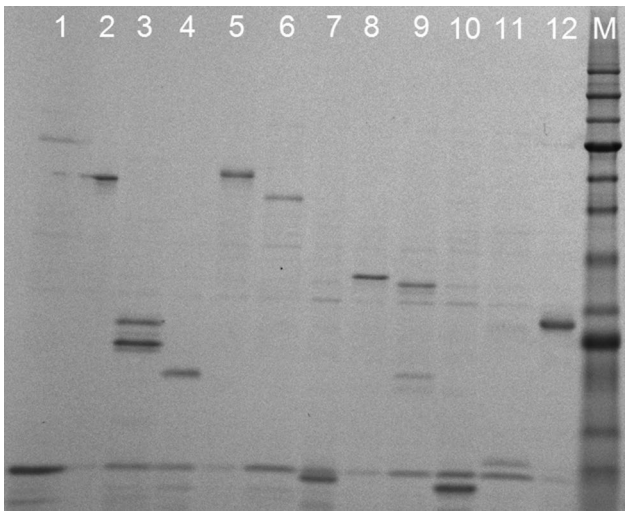

Fig. 1 SDS-PAGE analysis of the purified recombinant flagellar proteins. The samples were separated on 10–20 % SDS-PAGE gels and stained with Bio-Safe Coomassie G-250 Stain™. Lanes 1, Cjj0894; 2, FlgE1; 3, FlgG; 4, FlgH; 5, FlgK; 6, FlhF; 7, FliE; 8, FliG; 9, FliH; 10, FliN; 11, FliS; and 12, FliY. M, molecular mass markers in kilodalton (kDa) as follows: 230, 150, 100, 80, 60, 50, 40, 30, 25, 20, 15 and 10

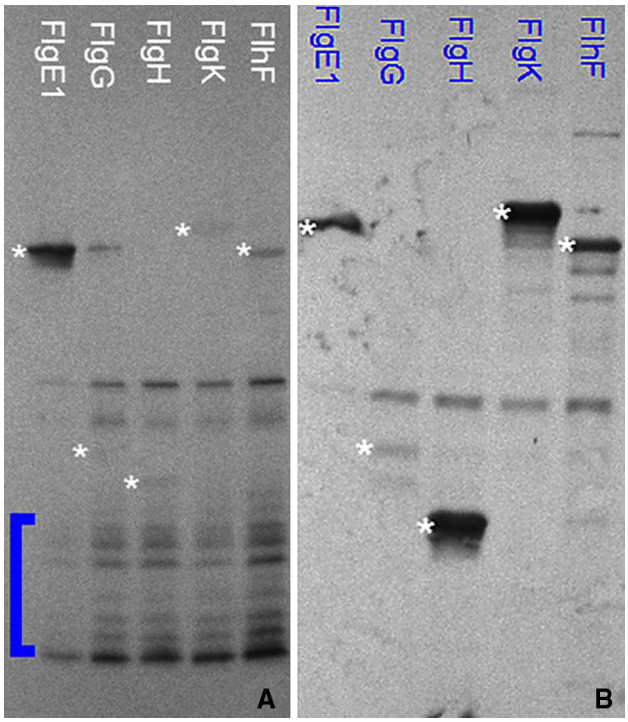

Fig. 2 Immunoblot analysis of the recombinant *Campylobacter jejuni* flagellar proteins to a chicken serum treated without **a** and with **b** preadsorption with *E. coli* 10G cells. The proteins were separated on 10–20 % SDS-PAGE and electrotransferred to the Immun-Blot PVDF membranes, followed by incubation in a 1:500 dilution of a chicken serum as described in "Materials and methods" section. The *bracket* at the *left column* in **a** indicates that these small minor proteins may originate from *E. coli* 10G cells. *Asterisk*, indicates the recombinant *Campylobacter jejuni* flagellar proteins

Table 1 Reactivities of chicken sera to recombinant *C. jejuni* flagellar proteins

Protein[a]	Molecular mass (kDa)[b]	Chicken serum no.[c]														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	Tested positive[d]
Cjj0894	82.0	−	−	+	−	+	−	−	−	−	−	−	+	−	−	3/14
FlgE1	58.2	+	+	+	+	+	+	+	+	+	−	−	−	−	+	10/14
FlgG	27.7	+	+	+	+	+	+	−	−	−	−	−	−	−	−	6/14
FlgH	25.2	+	+	+	−	+	−	+	−	+	+	−	−	−	−	7/14
FlgK	67.1	+	+	+	+	+	+	+	+	−	−	+	−	−	+	10/14
FlhF	55.4	+	+	+	+	+	−	−	+	+	−	−	+	+	−	9/14
FliE	10.8	+	+	−	−	−	−	−	−	−	−	−	−	−	−	2/14
FliG	38.4	+	+	+	+	+	+	+	−	+	−	+	−	−	+	10/14
FliH	31.2	+	+	−	+	−	−	−	−	−	−	−	−	−	−	3/14
FliN	11.6	+	−	−	−	−	−	−	−	−	−	−	−	−	−	1/14
FliS	14.8	+	+	−	−	−	−	−	−	−	−	−	−	−	−	2/14
FliY	30.1	+	+	+	+	+	+	+	+	+	+	−	+	−	−	11/14

[a] Flagellar proteins were annotated according to the genome sequence of *Campylobacter jejuni* (GenBank accession no. NC_002163) (Parkhill et al. 2000)

[b] Amino acid sequences were deduced from the nucleotide sequences via www.expasy.ch/Tools/dna.html. Molecular masses of each flagellar protein were calculated via www.ebi.ac.uk/Tools/emboss/

[c] Chicken serum no. indicates sera were collected from individual broilers aged from 5 to 6 weeks

[d] "+" and "−" indicate the sera reacted positively and negatively, respectively, to each recombinant flagellar protein

SDS-PAGE and electrotransferred to PVDF membranes. Immunoblot analysis was carried out with pre-adsorbed sera from 14 chickens. The results of chicken serum reactivities to the recombinant flagellar proteins are summarized in Table 1. Each chicken serum sample reacted to various numbers of the proteins. Among these proteins, the FlgE1, FlgK, FlhF, FliG and FliY proteins were the top five proteins for chicken antibody targets of sera tested so far. These results suggest that these anti-*Campylobacter* antibodies are prevalent in poultry flocks, which acquire this microorganism from the environment during their maturation. Subsequently, these flagellar proteins are expressed and recognized by host immune cells during *Campylobacter* colonization and adhesion in the chicken ceca.

Recent studies have demonstrated that the FlgK (flagellar hook-associated) protein is required for assembly of the flagellar secretory system and involves motility, colonization and invasion of *C. jejuni* in the chicken cecae (Golden and Acheson 2002; Fernando et al. 2007; Neal-McKinney and Konkel 2012). The FlgE (flagellar hook) protein connects the flagellar basal body and filaments and acts as a transmitter that communicates the rotational signals from basal body to filaments (Lüneberg et al. 1998). In addition, the FlgE and FlgK proteins form a hook complex that is required for export and delivery of the *C. jejuni* Cia virulent proteins into the cytosol of host cells (Neal-McKinney and Konkel 2012). Because FlgE and FlgK proteins are flagellar structural components and extend from periplasmic space to the outer surface (Gilbreath et al. 2011; Lertsethtakarn et al. 2011), it is expected that these two proteins are targets for chicken antibody reactivities. There are two flgE genes that encode two distinct hook proteins (FlgE1 and FlgE2) in *C. jejuni*. Both proteins are antigenic in that they induce an antibody response among chickens colonized by *C. jejuni* in the gastrointestinal tract (Shoaf-Sweeney et al. 2008).

On the other hand, the FlhF (flagellar biosynthesis regulator), FliG (flagellar motor switch) and FliY (flagellar motor switch) proteins are embedded in the cytoplasm and inner membrane (Gilbreath et al. 2011; Lertsethtakarn et al. 2011). How the FlhF, FliG and FliY proteins become immune targets and whether subsequently the antibodies to these proteins immobilize *C. jejuni* in chicken ceca are yet to be determined.

In conclusion, 12 recombinant *C. jejuni* flagellar proteins were purified by His tag affinity chromatography, and their antigenicity was assayed using sera from chickens older than 4 weeks of age. The immunoblot results show each serum reacted to various numbers of recombinant flagellar proteins. Among these proteins, sera reacted predominantly to the FlgE1, FlgK, FlhF, FliG and FliY proteins, which are important for flagellar functions so that these proteins may further be evaluated as potential vaccine candidates for broiler chickens to improve human food safety. Because the broiler sera we used in this study were collected locally, the experiments using sera from other geographical locations are underway to determine whether these immunoreactions are universal.

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Conflict of interest The authors claimed no conflict of interest. This manuscript was prepared by the US Department of Agriculture employees as part of their official duties and is therefore in the public domain.

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