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Research paper

# Epitope mapping of *Salmonella* flagellar hook-associated protein, FlgK, with mass spectrometry-based immuno-capture proteomics using chicken (*Gallus gallus domesticus*) sera

Hung-Yueh Yeh[a,*], Kyoko Kojima[b,c], James A. Mobley[b,c]

[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] Department of Surgery, University of Alabama at Birmingham, Birmingham, AL 35294, USA
[c] The Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

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ABSTRACT

*Salmonella*, a Gram-negative rod, is the leading foodborne pathogen associated with human acute bacterial gastroenteritis worldwide. The *Salmonella* flagellum is responsible for bacterial movement, colonization and invasion in the host gastrointestinal tract. The flagellum has a complex structure, composed of more than 35 proteins. Among them, we were interested in the flagellar hook-associated protein (FlgK), which is an immunodominant protein in chickens. In this communication, we applied mass spectrometry-based proteomics in conjunction with chicken immunized sera to map the linear immunoepitopes in the FlgK protein, validated the epitopes with peptide ELISA, and determined serum reactivity to the epitopes from commercial chickens. We previously demonstrated the FlgK proteins are highly conserved among *Salmonella* serovars. The rFlgK protein was produced by the recombinant technique, and was able to induce immune response in chickens. Further, this study identified four peptides (AEG, GAQ, TAD and LEI) in the rFlgK protein that were captured by sera from chickens immunized with the rFlgK protein. These four peptides were also reacted to 64 individual serum samples collected from 44 − 52 weeks old chickens, suggesting that these peptides may represent the shared immuno-epitopes on the FlgK protein. The findings of the specific shared linear immuno-epitopes on the FlgK protein 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.

## 1. Introduction

*Salmonella* is the leading foodborne pathogen that causes human acute bacterial gastroenteritis (Crim et al., 2015; European Food Safety Authority, 2015; Johnson et al., 2014; Scallan et al., 2011). A recent World Health Organization report estimates that there were 78 million non-typhoidal *Salmonella* cases causing about 60 thousand deaths worldwide in 2010 (Havelaar et al., 2015). Outbreaks of human salmonellosis have been linked to consumption of *Salmonella* contaminated poultry meat and eggs (Jackson et al., 2013; Pires et al., 2014), resulting in huge economic losses to producers and public health issues to communities (Mangen et al., 2015; Economic Research Service, USDA, 2015).

*Salmonella* is a Gram-negative, motile, intracellular, non-lactose fermenting bacterium with peritrichous flagella (Brenner et al., 2000; Pottinger et al., 2014; Strockbine et al., 2015). The *Salmonella* flagellum has a complex structure and can broadly be divided into five regions – the basal body, the hook, the hook-filament junction, the extracellular filaments and flagellar cap (Minamino, 2014). There are more than 35 proteins in one complete flagellum. Among them, we are interested in the FlgK (hook associated protein 1) protein. This protein locates at the proximal end of the flagellar hook-filament junction, connecting the hook to the filament (Homma and Iino, 1985; Ikeda et al., 1987). The FlgK protein, encoded by the *flgK* gene (previously designated as *flaW*) (Homma et al., 1985; Kutsukake and Ide, 1995), has 553 amino acid with a calculated molecular mass of 59.0 kDa (Homma et al., 1990). This FlgK protein is required for proper formation of flagella (Kutsukake and Ide, 1995; Salehi et al., 2016), and regarded as an important virulence determinant for invasion (Schmitt et al., 2001; Gauger et al., 2007; Winter et al., 2009; Yang et al., 2012). Specifically, the

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

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*Salmonella* flagella lacking this protein have significantly less adherent capacity to broiler skin (Salehi et al., 2016), where is regarded as a major source for contamination of poultry meat during slaughter (Northcutt et al., 2005; Yang et al., 2001). FlgK is one of the most immune-reactive flagellar proteins (Kwang and Littledike, 1995). Our preliminary study showed the anti-FlgK antibodies were prevalent in the poultry flock (Yeh et al., 2016a). These results suggest that the FlgK protein provides us two potential applications: (1) this protein may be used for serological assays (e.g. Yeh et al., 2016a) and (2) the FlgK protein may be used as a vaccine candidate for broilers to control *Salmonella* colonization in the gastrointestinal tracts. However, little information about immune response to this protein is available.

The specific antigen-antibody interaction is based on recognition of an epitope (either linear or conformational) in an antigen by an antigen-binding site in an antibody (Gershoni et al., 2007). Knowing the linear epitopes of an antigen provides us information of the specificity of the host immune responses and enables us to design effective epitope vaccines (Gershoni et al., 2007). Recently, because of sensitivity and accuracy of mass spectrometry in protein characterization and identification, mass spectrometry in conjunction with immunoprecipitation proteomics has been applied for epitope mapping of antigens (e.g. Gourlay et al., 2015; Lassaux et al., 2013; Koehler et al., 2011; Soriani et al., 2010). The principle of this technique is that after enzymatic digestion (often with trypsin) of a protein, the small peptides are captured with an antibody (either monoclonal or polyclonal), and the captured peptides are identified by mass spectrometry (ten Have et al., 2011 for review).

In this communication, mass spectrometry-based proteomics in conjunction with broiler chicken immunized sera was used for mapping the linear immunoepitopes in the FlgK protein, validated the epitopes with peptide ELISA, and determined serum reactivity to the epitopes from commercial chickens.

## 2. Materials and methods

### 2.1. Expression, production and purification of recombinant Salmonella FlgK (rFlgK) protein

The rFlgK protein was expressed, produced and purified as described previously (Yeh et al., 2016a). Briefly, genomic DNA from *Salmonella* Heidelberg was prepared by boiling the colonies in 100 µl of 10 mM Tris-HCl buffer (pH 8.0) at 100 °C for five min (Iwamoto et al., 2003; Yeh et al., 2005). The *flgK* gene was amplified by PCR. The primers for PCR amplification were FlgK-F, 5′-catcatcaccaccatcac TCCAG CTTGATTAATCAC-3′, and FlgK-R, 5′-gtggcggccgctctatta tgcataatctggaacatcatatggata GCGAATAYTCARTAACGCATC-3′. The underlined sequences indicate a six-His tag and a hemagglutinin (HA) tag were included at amino and carboxyl termini, respectively. The amplicon was ligated and transformed in competent *E. coli* cells using an *Expresso*® T7 Cloning and Expression System kit (Lucigen Corp., Middleton, WI, USA) according to the manufacturer's instructions. The *E. coli* BL21 (DE3) cells harboring the *flgK* plasmid were cultured in 500 ml of LB broth supplemented with 30 µg/ml of kanamycin. The cultures were induced to express the rFlgK protein with 1 mM of isopropyl β-*d*-1-thiogalactopyranoside (IPTG). The recombinant protein was purified by affinity chromatography using nickel-chelating resins (Thermo Scientific, Rockford, IL, USA). The purity of the recombinant protein was examined by SDS-PAGE analysis (Laemmli, 1970). The rFlgK was further identified by nano-scale liquid chromatography – electrospray ionization and tandem mass spectrometry (nanoLC-ESI–MS/MS, see below).

### 2.2. Immunization of broiler chicks with rFlgK

One-day-old broilers were purchased from a local poultry hatchery, and were raised in isolation units in our poultry facility according to the standard brooding and growing guidelines (Wilson et al., 2016). Commercial feed and water were provided *ad libitum*. Broilers were given 100 µg of rFlgK emulsified in Freund's incomplete adjuvant (Sigma-Aldrich Co., St. Louis, MO, USA) subcutaneously at one week of age, followed by boosting at three weeks of age with the same antigen and route. A phosphate buffered saline (PBS) solution emulsified in the same adjuvant was administered to broilers as negative controls. Ten days after second immunization, blood samples were collected by venipuncture (Kelly and Alworth, 2013). All sera were aliquoted and stored at −80 °C. To ensure that the commercial broilers were *Salmonella* free, fecal samples were collected each week for bacterial culture as described previously (Stern, 2008). The results showed no *Salmonella* were detected in cultures from fecal samples, indicating the experimental birds were free from *Salmonella* infection. The purpose of our research was to explore the way to reduce the use of antibiotics in commercial poultry production. Such one approach is to develop vaccines. Therefore, commercial broilers were used in our studies.

The use of broiler chickens in our experiments 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, USA. The proposal no. was PMSRU-08-2014-C.

### 2.3. Epitope mapping of rFlgK with chicken polyclonal sera

The protocols for enzymatic mapping of the FlgK epitopes was described previously (Gourlay et al., 2015; Lassaux et al., 2013; Koehler et al., 2011; Soriani et al., 2010). The rFlgK protein was reconstituted in 50 mM ammonium bicarbonate buffer and was digested with trypsin (conjugated to resin) (Sigma-Aldrich Co.) at the ratio of 100:1 at 37 °C with mild rocking. After three-hour incubation, the peptide mixtures were collected by low centrifugation to remove the resin, and the protease inhibitor mix (Sigma-Aldrich Co.) was added. For antibody preparation, the chicken sera were incubated with anti-chicken IgY antibody conjugated resins (GenScript USA Inc., Piscataway, NJ, USA) according to the manufacturer's instructions. After extensive washing of the antibody complex resins, the enzyme digested peptide mixture was added. The mixtures were incubated at room temperature with mild rocking. After one hour, the resins were extensively washed with 50 mM ammonium bicarbonate buffer, and the peptides were eluted with 0.2% trifluoroacetic acid (TFA). The peptides was identified by nanoLC-ESI–MS/MS (below).

### 2.4. NanoLC-ESI-MS/MS analysis of peptides

The peptide identification was performed by nanoLC-ESI–MS/MS at the UAB CCC Mass Spectrometry/Proteomics Shared Facility (MSP-SF) at the University of Alabama at Birmingham, Birmingham, AL, USA according to the protocol described by Ludwig et al. (2016). Briefly, peptide mixtures were injected onto a 1260 Infinity nHPLC stack (Agilent Technologies), and separated using a 75 µm I.D. x 15 cm pulled tip C-18 column (jupiter C-18 300 Å, 5 µm, Phenomenex). This system runs in-line with a Thermo Orbitrap Velos Pro hybrid mass spectrometer equipped with a nano-electrospray source (Thermo Fisher Scientific), and all data were collected in CID mode. The nHPLC was configured with binary mobile phases that include solvent A (0.1% TFA in $ddH_2O$), and solvent B (0.1% TFA in 15% $ddH_2O$/85% ACN), programmed as follows: 10 min at 5% B (3 µl/min load), 90 min at 5–40% B (linear: 0.5 nl/min, analyze), 5 min at 70% B (3 µl/min, wash), 10 min at 5% B (3 µl/min, equilibrate). Following each parent ion scan (300–1200 $m/z$ at 60k resolution), fragmentation data (MS2) were collected on the top most intense 15 ions. For data dependent scans, charge state screening, and dynamic exclusion were enabled with a repeat count of two, repeat duration of 30 s, and exclusion duration of 90 s.

The XCalibur RAW data files were collected in profile mode,