Short Communication-**REVISED**

Production of Recombinant *Salmonella* Flagellar Protein, FlgK, and its Uses in Detection of
Anti-*Salmonella* Antibodies in Chickens by Automated Capillary Immunoassay

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Abstract

Conventional immunoblot assays are a useful tool for specific protein identification, but tedious, labor-intensive and time-consuming. A capillary electrophoresis-based immunoblot assay so-called "Simple Western" was developed to enable the protein identification in an automatic manner. This communication describes use of Simple Western for detecting anti-*Salmonella* FlgK antibodies from chicken sera.

Keywords

Salmonella enterica, FlgK, flagellar proteins, zoonoses, Simple Western

Salmonella is a motile, facultative anaerobic, intracellular, non-lactose fermenting, Gram-negative bacterium (Pottinger et al., 2014; Strockbine et al., 2015). This microorganism consists of two species: *Salmonella bongori* and *S. enterica*. Further, *S. enterica* has six subspecies and has more than 2,500 serovars (based on somatic O and flagellar H antigens) (Brenner et al., 2000). *Salmonella* is the leading foodborne pathogen that causes human acute gastroenteritis worldwide (Crim et al., 2015; EFSA, 2015; Johnson et al., 2014; Scallan et al., 2011). Outbreaks of human salmonellosis have been linked to consumption of a variety of contaminated products, including poultry meat and eggs (Jackson et al., 2013; Pires et al., 2014), and result in considerable economic losses to producers as well as medical resources (Mangen et al., 2015; Economic Research Service, USDA, 2015).

In earlier studies, anti-*Salmonella* antibodies in sera could be detected in naturally and experimentally infected poultry flocks (Chart et al., 1990; Gast and Beard, 1990; Gast and Holt, 2001), suggesting that serology may be useful for monitoring *Salmonella* infection in chickens (Marek et al., 2009). Considering the high genetic diversity of *Salmonella*, to find a specific antigen(s) for serology is very challenging. Because more than a thousand of *Salmonella* genomic sequences are available in public domains, we executed a comprehensive search for the sequences (at the nucleotide or amino acid level) that are common among, but unique in *Salmonella*.

In addition for their movement toward or away from various environmental conditions, the bacterial flagella are involved in adherence to establish colonization in hosts, and can induce innate immunity during the course and outcome of infections in hosts (Rossez et al., 2015). The *Salmonella* flagellum, which consists of three distinguished structural regions - the basal body, the hook and the extracellular filaments, has been extensively studied (Macnab, 2003). There are

more than 35 proteins in one complete flagellum. Among them, we are interested in the flagellar hook-filament junction protein at hook (FlgK). The FlgK protein, encoded by the *flgK* gene, is required for flagellar filament formation (Kutsukake and Ide, 1995). Further, FlgK is an important virulence factor that plays a role in intestinal inflammation and is the most immune-reactive flagellar protein (Winter et al., 2009; Kwang and Littledike, 1995). The FlgK protein protrudes from the microorganism so it is reasonable to predict that it is a target for host immune response.

Recently, an automated capillary electrophoresis-based immunoblot assay has been described (Nguyen et al., 2011). The principle of this assay is that samples and reagents are prepared and loaded into designated wells of an assay plate, which has a 25-well format in the Wes system. After capillaries and the assay plate are loaded in a ProteinSimple™ Wes instrument (ProteinSimple, San Jose, CA, USA), proteins are separated through a gel matrix, immobilized to the capillary wall by photoactivation, identified by primary antibody and subsequently detected by a horseradish peroxidase conjugated secondary antibody and a chemiluminescent substrate (ProteinSimple, San Jose, CA, USA) (Nguyen et al., 2011). Except for sample preparation, all operations are automatic and controlled by the software *Compass*. This technology has recently been used by pharmaceutical industries to identify, evaluate and characterize biopharmaceutical products (e.g. Chen et al., 2013; Rustandi et al., 2012; Loughney et al., 2014; Butnev et al., 2015; Hamm et al., 2015; Xu et al., 2015). However, this technology has not been used in clinical diagnosis.

In this communication, we used FlgK as a probe to evaluate whether the automated capillary immunoassay (Simple Western) could be used to detect antibodies against the recombinant *Salmonella* FlgK proteins from chicken sera.

Bacterial strains, culture conditions and genomic DNA isolation: *Salmonella* Heidelberg from our bacterial stock collection were propagated according to the standard microbiological method (Strockbine et al., 2015). Competent *E. coli* for propagation and expression of plasmid DNA and proteins, respectively, were cultured according to the manufacturer's instructions (Lucigen Corp., Middleton, WI, USA). Genomic DNA from *Salmonella* serovars was prepared by lysis of the bacterial pellet in 100 µl of 10 mM Tris-HCl buffer (pH 8.0).

Expression of FlgK in an *E. coli* expression system: The *flgK* gene sequence of *S. Heidelberg* was downloaded from GenBank (accession no. NC_011083) for oligonucleotide primer design. A six-His sequence tag was added to forward primer. The *flgK* gene was amplified by PCR as described previously (Yeh et al., 2013, 2014). The amplified PCR products were ligated and transformed in competent *E. coli* cells using an *Expresso*[®] T7 Cloning and Expression System kit (Lucigen Corp.). The positive clones were confirmed by nucleotide sequencing.

Production and purification of recombinant FlgK protein (rFlgK): The protocol for the production and purification of the recombinant protein was described previously (Yeh et al., 2013, 2014). Briefly, the *E. coli* BL21 (DE3) cells harboring the desired plasmid were cultured in 500 ml of LB broth supplemented with 30 µg/ml of kanamycin, and were induced to express the recombinant 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.

SDS-PAGE: SDS-PAGE was carried out by the method of Laemmli (Laemmli, 1970). The recombinant protein samples were solubilized in 2X Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) and denatured at 98 °C for five min. Electrophoresis was

performed on 10-20% Criterion Tris-HCl-SDS-polyacrylamide precast gels (Bio-Rad). After separation, the proteins in gels were stained with Bio-Safe™ Coomassie G250 Stain (Bio-Rad) according to the manufacturer's instructions. Images were recorded and processed with an AlphaImager HP System (ProteinSimple™, Santa Clara, CA) and its associated software.

Immunoblotting: Immunoblot analyses were performed in two ways: (1) traditional Western blot analysis and (2) automated capillary immunoblot analysis. For traditional immunoblot analysis, the strips were blocked with a solution of 10 mM Tris-HCl (pH 8.0)-0.5 M NaCl-0.5% Tween 20-5% skim milk (TNT-milk solution) at room temperature for two hours, and then incubated with chicken sera at a final 1:1000 dilution in the TNT-milk solution at 4 °C overnight (Yeh et al., 2015; Neal-McKinney et al., 2014). After extensive washes, the strips were incubated with goat anti-chicken IgG antibody conjugated with horseradish peroxidase (1:4,000 dilution) (KPL Inc., Gaithersburg, MD) at room temperature. After one-hour, the strips were extensively washed, followed by incubation with TMB membrane peroxidase substrate (KPL Inc.) at room temperature. To ensure quality transfer of protein, pre-stained molecular markers were included in each protein transfer to polyvinylidene difluoride (PVDF) membranes.

For capillary immunoblot analysis, a ProteinSimple™ Wes system was used according to the manufacturer's instructions (ProteinSimple™, Santa Clara, CA, USA). The default settings were used.

Immunization of chickens with rFlgK: One-day-old chickens were purchased from a local poultry farm, and raised in isolation units in our facility. Food and water were provided *ad libitum*. Chickens were immunized with rFlgK emulsified in Freund's incomplete adjuvant (Sigma-Aldrich Co., St. Louis, MO) at the ages of one and three weeks. For the negative

control, a solution of phosphate buffered saline (PBS) in the same adjuvant was administered to chickens. Sera were withdrawn at the age of 4.5 weeks described as below.

Collection of chicken sera: Chicken sera were withdrawn from clinically healthy six-week-old broilers (Kelly and Alworth, 2013). The sera were stored at -80 °C. Sera from three individual specific pathogen free (SPF) chickens purchased from Charles River (North Franklin, CT) were included as negative controls. These sera have been tested against a panel of poultry pathogens, including *Salmonella Pullorum* and *Salmonella Gallinarum* (by serum plate agglutination assays). They were negative to specific chicken pathogens.

The use of chickens in the 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. The proposal no. was PMSRU-08-2014-C.

When the deduced amino acid sequences of FlgK from 10 *Salmonella* serovars were compared, we noted that the degree of conservation of this protein among serovars is greater than 97% (Supplementary Fig. 1), suggesting that this protein may induce cross-reactive antibodies among *Salmonella* serovars, and have a potential to act as a biomarker for monitoring *Salmonella* infection in animal production.

The FlgK protein was expressed in and purified from recombinant *E. coli* cells. Because a six His tag sequence was added during the expression vector construction, the FlgK protein was purified by nickel-chelating affinity chromatography and had a relative mobility of relevant size and position in SDS-PAGE (Fig. 1A). To further confirm this protein fused with a six His tag, the FlgK protein was electrotransferred to Immobilon membrane. As illustrated in Fig. 1B, one major dense band was detected by nickel-nitrilotriacetic acid conjugated to horseradish

peroxidase on the blot, indicating that the purified FlgK protein is a fusion protein. The calculated molecular mass of rFlgK is 60.9 kDa.

As mentioned above, FlgK is highly conserved among *Salmonella* serovars. Accordingly, we tested whether the FlgK protein had potential as a biomarker for serological assay for diagnosis of *Salmonella* infection. We first used chicken negative sera purchased commercially, and positive sera from broilers immunized with the FlgK protein. Sera from commercial sources and un-immunized chickens did not react to FlgK (Fig. 2A, lanes 5, 10 and 16 - 20). Sera from the FlgK-immunized broilers reacted strongly to the FlgK protein (Fig. 2A, lanes 1 - 4, 6 - 9 and 11 - 15), indicating that FlgK is able to induce hosts to generate antibodies. Multiple minor bands were also observed in the immunoblots. We assumed these proteins were originated from host *E. coli* BL21 (DE3) cells, which were used for rFlgK production. When the chicken sera were pre-adsorbed with the host cells, we observed majority of these protein bands were not detected and the specific antibody reactivity to the recombinant proteins was enhanced (Yeh et al., 2015).

An automated capillary electrophoresis-based immunoblot assay tested chicken sera for reaction with rFlgK protein. The rFlgK protein had a relative mobility of expected size (60.9 kDa) and position in actual electrophoregram by capillary electrophoresis (Fig. 2B). This observation is similar to that in SDS-PAGE (Fig. 1). As expected, the sera from the SPF and PBS chickens did not react to the protein (Fig. 2B, lanes 1 - 9 and 24). Sera from the FlgK immunized chickens reacted relatively strongly to the protein (Fig. 2B, lanes 10 - 23). Several possible reasons for extra bands in Fig. 2B are as follows: (1) The FlgK protein were treated with two different reducing agents – β -mercaptoethanol and dithiothreitol for immunoblot analysis and capillary analysis, respectively. They may have different effects on cysteine status

(reduction or oxidization) in the protein. (2) Because the same dilution factors for chicken sera (primary antibodies) were used in capillary assays (Fig. 2B and Fig. 3), the sera from the immunized chickens had higher concentrations of specific antibodies against FlgK than those from broilers. It is known that the high concentration of the primary antibodies often causes extra bands. (3) The chicken sera used in the experiments were not purified, and impurities of the chicken sera often generate additional bands. Together, these results indicate that the automated immunoblot assay is comparable to the conventional immunoblot assay in specificity, but provides great advantages in easy operation, higher sample throughput and time saving in detection.

Further, we tested 66 individual chicken sera collected from a single flock raised similar to commercial conditions to determine whether this automated immunoblot assay was suitable for detection of anti-*Salmonella* FlgK antibodies from the field chickens. Commercial SPF chicken sera were included in the assay as negative controls (Fig 3, lanes Z0113 and Z0132). We observed 63 out of 66 sera reacted at various degrees to the recombinant FlgK protein (Fig. 3). This result is comparable to that from conventional immunoblot assay.

Recent studies estimated that about 50 bacterial genera were found in chicken gastrointestinal microbiome, including low abundance of *Enterobacteriaceae* (Oakley et al., 2014a, b). We performed phylogenetic analysis to determine the relatedness of the FlgK orthologs among enteric bacteria. The results show that the *Salmonella* FlgK proteins are 79-81%, 79-82% and 73-75% identical to *Escherichia coli*, *Citrobacter* and *Enterobacter*, respectively. This raises an issue of specificity of this rFlgK protein for *Salmonella* serology in chickens. In this study, commercial SPF chickens were tested to be free from *Salmonella*, but may not be free from other enteric bacteria. These SPF chicken sera were negative to the

Salmonella rFlgK protein. Together, although the likelihood of other enteric microorganisms eliciting antibodies that reacted with the *Salmonella* rFlgK protein is unlikely in the experiments of the present study, it is not currently known whether animals infected with other *Enterobacteriaceae* would elicit cross-reactive immunoglobulins. Further studies on the *Salmonella* FlgK epitope mapping and reaction of chicken sera to rFlgK from other *Enterobacteriaceae* are needed.

In summary, the *Salmonella* FlgK protein was successfully expressed in and purified from an *E. coli* expression system. This protein induced strong immune response in chickens. The automated capillary immunoassay was successfully used for detection of antibodies against the recombinant *Salmonella* FlgK protein (as a probe) from chicken sera. These results provide a rational for further evaluation of this automated immunoassay as a tool using this recombinant *Salmonella* FlgK protein in *Salmonella* outbreak investigation.

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Figure Legends

Fig. 1. Representative purification of the recombinant *S. Heidelberg* flagellar proteins analyzed by (A) SDS-PAGE and stained with Bio-Safe Coomassie G250 stain, and (B) immunoblot probed with nickel conjugated with horseradish peroxidase (KPL). (A) Lane 1, molecular mass markers in kDa of 190, 135, 100, 75, 58, 46, 32, 25, 22, 17 and 11 (from top to bottom of the gel) (New England Biolabs, Ipswich, MA); lane 2, FliD; and lane 3, FlgK. (B) Lane 1, FlgK; lane 2, molecular mass markers in kDa as above.

Fig. 2. Analysis of sera from the FlgK immunized chickens to the recombinant *Salmonella* FlgK protein by conventional immunoblot assay (A) and automated capillary electrophoresis based immunoblot assay (B). (A) Conventional immunoblot assay: lanes 1 - 4, 6 - 9 and 11 - 15, sera from individual chickens immunized with the FlgK protein; lanes 17 - 20, sera from the age-matched individual chickens administered with phosphate buffered saline (PBS); lanes 5, 10 and 16, sera from specific pathogen free (SPF) chickens as negative controls; lane H, His tag detected in the recombinant FlgK protein by a Nickel HisDetector kit (KPL); and lanes M, molecular mass markers in kDa as in Fig. 1A. (B) Automated immunoblot assay: lanes 1 - 8, sera from the age-matched individual chickens administered with PBS; lanes 10 - 23, sera from individual chickens immunized with the FlgK protein; lanes 9 and 24, sera from specific pathogen free chickens as negative controls. Molecular mass markers in kDa are indicated in the left column. An asterisk indicates the recombinant FlgK protein.

Fig. 3. Analysis of sera from 66 individual chickens from field reacted to the recombinant *Salmonella* FlgK protein by automated immunoblot assay. Lanes 1 - 66, sera from 66

individual chickens; lanes Z0113 and Z0132, sera from SPF chickens as negative
controls. Molecular mass markers in kDa are indicated in the left column.

Figure 1A

FIGURES

Figure 1B

Figure 2A

Figure 2B

Figure 3-1

Figure 3-2

Figure 3-3