Development and evaluation of an indirect enzyme-linked immunosorbent assay based on a recombinant SifA protein to detect *Salmonella* infection in poultry

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ABSTRACT *Salmonella* is an important zoonotic pathogen that not only endangers food safety and human health, but also causes considerable economic losses to the poultry industry. Therefore, it is essential to establish a rapid, sensitive, and specific diagnostic method for the early detection of *Salmonella* infection in poultry. In this study, we developed a novel enzyme-linked immunosorbent assay (**ELISA**) for the detection of anti-*Salmonella* antibodies using a recombinant SifA protein. Amino acid sequence comparison revealed that SifA is a relatively conserved secretory protein across *Salmonella* serotypes. Therefore, we hypothesized that SifA can serve as a detection antigen for diagnostic testing. The SifA protein was expressed in *Escherichia coli* and used as a coating antigen to establish an SifA-ELISA. Control sera from specific-pathogen-free (**SPF**) chickens infected with *Salmonella* or several other non-*Salmonella* pathogens were then tested using the SifA-ELISA. Specificity testing demonstrated that the SifA-ELISA could detect antibodies against 3 different serotypes of *Salmonella*, whereas antibodies against other non-*Salmonella* pathogens could not be detected. Compared to the SifA-ELISA, the *Salmonella* plate agglutination test (**PAT**) failed to detect antibodies in serum samples from chickens infected with *Salmonella* Typhimurium. This result suggests that our SifA-ELISA may be better than PAT at detecting *Salmonella* infection. Comparing clinical sera, we observed a similar rate of *Salmonella* positivity between SifA-ELISA and PAT (92.6%). In addition, anti-SifA antibodies were continuously detected during *Salmonella* infection of SPF chickens, demonstrating that SifA-ELISA could consistently detect high levels of antibodies for at least 8 wk. Furthermore, the intra-assay and interassay coefficients of variation (**CV**) of the SifA-ELISA were below 10%, which is considered acceptable. In summary, the SifA-ELISA established here is a promising and reliable method for detection of anti-*Salmonella* antibodies in poultry and may contribute to the early diagnosis of *Salmonella* infection.

Key words: *Salmonella*, indirect ELISA, SifA, antibody detection, poultry

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INTRODUCTION

Salmonella is an important pathogenic bacterium worldwide, infecting a broad range of animals and humans. *Salmonella* is categorized under the family of *Enterobacteriaceae*, and 2,659 *Salmonella* serotypes have been identified (Issenhuth-Jeanjean et al., 2014). In general, *Salmonella* Pullorum infection results in reduced hatchability or high chick mortality, whereas adult birds may suffer from a variety of nonspecific signs including diarrhea, inappetence, as well as decreased egg production and fertility (Barrow and Freitas Neto, 2011). Additionally, poultry may carry some *Salmonella* serovars without signs or symptoms of disease (Wibisono et al., 2020). Infected poultry can also be a source of *Salmonella* infection for humans. Surveys have shown that most foodborne outbreaks of *Salmonella* infection are associated with contaminated eggs or meat from chickens (Kimura et al., 2004; Marcus et al., 2007). Therefore, to avoid contamination of poultry and poultry products, early detection of *Salmonella* infection in poultry is crucial and requires a rapid and specific detection method.

The "gold standard" for the isolation and identification of foodborne bacterial pathogens is bacteriological

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culture (Lungu et al., 2012). However, culturing methods for detecting *Salmonella* are typically time-consuming and laborious, and intermittent excretion of *Salmonella* may lead to inaccurate detection (Sommer et al., 2012; Rohde et al., 2017). Thus, there is a need to develop more reliable methods for detecting *Salmonella* infections in poultry.

The main advantage of serological methods for *Salmonella* detection is the ability to rapidly assay a large number of samples at a relatively low cost (Funk et al., 2005). Considering the need for rapid screening of suspected *Salmonella*-positive samples, enzyme-linked immunosorbent assay (**ELISA**) is an optimal candidate for the serological diagnosis of *Salmonella* infection (van Zijderveld et al., 1992; Funk et al., 2005). Several ELISAs for the detection of *Salmonella* antibodies have been reported. These ELISAs rely mainly on several antigens such as lipopolysaccharide (**LPS**), flagellin, outer membrane proteins of *Salmonella* (van Zijderveld et al., 1992; Kuhn et al., 2012). However, indirect ELISA based on LPS or flagellin as coating antigens usually only detects specific *Salmonella* serotypes (Barrow, 1994; Smith et al., 1995; Feld et al., 2000). Moreover, ELISAs based on outer membrane proteins have increased likelihood of cross-reactivity with non-*Salmonella* pathogens (Ma et al., 2018). Hence, a specific method needs to be established for the detection of antibodies raised against a wide range of *Salmonella* serovars while limiting cross-reactivity.

SifA is a *Salmonella* effector protein that plays an important role in *Salmonella* virulence. SifA is translocated into infected cells by the pathogenicity island 2-encoded type 3 secretion system, and is required to maintain the integrity of the *Salmonella*-containing vacuole (**SCV**) (Zhao et al., 2015). In addition, we found that the SifA protein is highly conserved among *Salmonella* serotypes, demonstrating the potential for SifA to be used as a detection antigen. In the present study, the *sifA* gene was cloned into the bacterial expression vector pET28a and expressed in *Escherichia coli*. An indirect ELISA method for detecting *Salmonella* antibodies was then developed using purified SifA protein as a coating antigen. The diagnostic potential of this SifA-ELISA was then evaluated using clinical serum samples of *Salmonella* infected chickens from poultry farms and control serum samples from SPF chickens. Overall, we determine that the SifA-ELISA established here may be used as a diagnostic test that recognizes multiple serovars of *Salmonella*, while exhibiting low cross-reactivity with non-*Salmonella* pathogens.

MATERIALS AND METHODS

Bacterial Strains and Cloning Vector

S. Pullorum SA023, *S.* Enteritidis SA083, *S.* Typhimurium SA014, *E. coli* ACN001, *Bordetella avium* P8, and *Pasteurella multocida* C48-1 were isolated and identified from internal organs of poultry suffering from systemic infections by Veterinary Diagnostic Laboratory, College of Veterinary Medicine, Huazhong Agricultural University. *Haemophilus paragallinarum* CVCC3007 was obtained from the China Veterinary Culture Collection Center (**CVCC**), Beijing, China. The prokaryotic expression plasmid pET28a and *E. coli* strain BL21 (DE3) were used for the expression of SifA protein.

Screening of Indirect ELISA-Coated Antigens

The *Salmonella* effector protein SifA was selected as a coating antigen to develop an indirect ELISA. To assess the conservation of SifA in *Salmonella* serotypes, the amino acid sequences of SifA were compared among *Salmonella* and non-*Salmonella* pathogens by BLASTp (https://www.ncbi.nlm.nih.gov). The amino acid sequences of SifA in prevalent *Salmonella* (Williamson et al., 2018; Yang et al., 2020; Chen et al., 2021; Fernandes et al., 2022) were further compared by the Clustal W method in MEGA 7.0. In addition, phylogenetic tree of amino acid sequences was also constructed using maximum likelihood with 1,000 bootstrap replications in MEGA 7.0 (Kumar et al., 2016).

Expression and Purification of Recombinant SifA Protein

Primers were designed based on the sequence of the *sifA* gene in the *S.* Enteritidis (NCBI Reference Sequence, CP007319.2) and synthesized (Tsingke Biological Technology Co. Ltd., Wuhan, China). *S.* Enteritidis *sifA* was then amplified by polymerase chain reaction (**PCR**) using specific primers: forward Primer: 5'-CCGGAATTCCCGATTACTATAGGGAATGG-3' with an *Eco*RI site (underlined); reverse Primer 5'-CCGCTCGAGTTAGCCGCTTTGTTGTTCT-3' with an *Xho*I site (underlined). The PCR product and the pET28a vector were then both digested by *Eco*RI and *Xho*I, and the PCR fragment was ligated into the corresponding *Eco*RI & *Xho*I sites of pET28a and transformed into *E. coli* BL21 (DE3). The recombinant plasmid was named pET28a-SifA.

E. coli BL21 (DE3) transformed with the recombinant plasmid pET28a-SifA was cultured in Luria-Bertani (**LB**) medium (Oxoid Ltd., Basingstoke, UK) containing kanamycin (50 μg/mL) with constant stirring at 37°C. After the optical density ($\mathbf{OD_{600}}$) of the bacterial culture reached 0.5, expression of SifA was induced by addition of isopropyl β-D-1-thiogalactopyranoside (**IPTG**) at a final concentration of 0.8 mM for 5 h at 37°C. The supernatant and precipitate of lysate from induced bacteria were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**). Recombinant protein SifA was then purified with Ni-NTA affinity chromatography (GE Healthcare Biosciences, Pittsburgh, PA) and presence of SifA was confirmed by Western blot.

SifA-ELISA Procedure

Indirect ELISA was established using recombinant SifA protein as a coating antigen. Five positive serum samples and 3 negative serum samples were used to optimize the antigen concentration. Recombinant SifA protein was diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) to 8 concentrations: 24, 12, 6, 3, 1.5, 0.75, 0.375, and 0.1875 μg/mL. ELISA plates were coated at 100 μL/well of each dilution and incubated at 4°C for 12 h. The plates were then washed 3 times with PBST (phosphate-buffered saline [**PBS**] containing 0.05%, w/v Tween-20) and blocked with 200 μL/well of 5% skim milk at 37°C for 2 h. After washing with PBST another 3 times, 100 μL of each serum sample (diluted at 1:500 in PBST) was added to each well, and the plates were incubated at 37°C for 30 min. After washing 3 times, the plates were incubated at 37°C for 30 min with 100 μL of horseradish peroxidase (**HRP**)-conjugated rabbit antichicken IgG (Jackson Immuno Research Laboratories, West Grove, PA) diluted 1:10,000 in PBST. After washing 3 times, the peroxidase H_2O_2 and the substrate 3,3,5,5-tetramethylbenzidine (**TMB**) were added to each well. After incubation at room temperature (25°C) for 15 min away from light, the reaction was stopped with 50 μL of 0.2 M H_2SO_4 and the OD_{630} was read on a microplate reader (Tecan, San Jose, CA).

Isolation of Serum From Infected and Uninfected Chickens

Three-week-old specific-pathogen-free (**SPF**) White Leghorn (male) chickens were procured from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd. (Beijing, China). Animal experiments were carried out according to the International Guiding Principles for Biomedical Research Involving Animals-2012. Experimental procedures were approved by the Research Ethics Committee of Huazhong Agricultural University (No. HZAUCH-2018-015).

A total of 277 three-wk-old SPF White Leghorn (male) chickens were divided into 8 groups with 30 chickens in each infected groups while 67 chickens in the negative control group. The chickens in Group 1 were orally infected with 10^8 CFU of *S.* Pullorum, while the chickens in Groups 2 to 6 were infected with 10^8 CFU of *S.* Enteritidis, *S.* Typhimurium, *E. coli, A. paragallinarum*, or *B. avium* via injection into the pectoral air sac. Chickens in Group 7 were infected with 10^6 CFU of *P. multocida* via the nostril. Meanwhile, 67 SPF chickens in Group 8 were administered PBS only and served as an uninfected control.

To evaluate SifA-ELISA for the detection of *Salmonella* infection, the production of antibodies against SifA was monitored during infection of chickens with *S.* Pullorum, *S.* Enteritidis, or *S.* Typhimurium. Blood samples were collected from chickens infected with *S.* Pullorum, *S.* Enteritidis, or *S.* Typhimurium, and uninfected chickens on a weekly basis for 8 wk. In the third week of chicken infection, blood samples from chickens infected with *E. coli, B. avium, P. multocida*, or *A. paragallinarum* were used to evaluate the cross-reactivity of SifA-ELISA. Additionally, 67 serum samples were collected from noninfected SPF chickens at the end of the experiment to serve as a negative control. All serum samples were stored at −80°C.

To determine whether chickens were successfully infected, samples from the liver, spleen, and intestine were collected for bacteriological examination (Jouy et al., 2005; Kich et al., 2007) during the third week of infection. After the experimental end point, the chickens in each group were euthanized and necropsied.

Confirmation of the Negative and Positive Thresholds (Cut-Off) for the SifA-ELISA

Fifty negative serum samples from *Salmonella*-free commercial poultry flocks were tested to determine the cut-off value. *Salmonella*-free commercial poultry flocks were determined through bacteriological analysis of fecal samples and environmental swabs (Jouy et al., 2005; Kich et al., 2007). The antibody levels of the samples were indicated by S/P ratios: S/P = (sample OD_{630} − negative-control OD_{630})/(positive-control OD_{630} − negative-control OD_{630}) (Ge et al., 2012). The mean S/P ratio (X) and standard deviations (**SD**) of OD_{630} for 50 negative serum samples were calculated, and the cut-off value was determined to be X + 3 SD. Based on statistical theory, if S/P ≥ X + 3 SD, the poultry sera were classified as seropositive; if S/P < X + 3 SD, the poultry sera were classified as seronegative (Poolperm et al., 2017).

Cross-Reactivity of the SifA-ELISA

Confirmed antisera against *S.* Pullorum, *S.* Enteritidis, *S.* Typhimurium, *E. coli, B. avium, P. multocida*, and *A. paragallinarum* respectively were used to evaluate SifA-ELISA antigenic cross-reactivity. Five different sera were selected from each group, and 5 negative sera were used as a control. The S/P ratio of the test samples was calculated to determine which sample met the criteria for containing anti-SifA antibodies.

Reproducibility of the SifA-ELISA

Sixteen positive serum samples from chicken farms were used to determine the reproducibility of the SifA-ELISA. Reproducibility is usually assessed by determining the level of intra-assay and interassay variation (Shang et al., 2008; van Gageldonk et al., 2008; Ge et al., 2012). The intra-assay coefficient of variation (**CV**) was an average value calculated from the individual CVs for all duplicates, while the interassay CV was calculated from the mean of the high and low controls on each plate. Each sample was tested in triplicate, and the mean S/P ratio, SD, and CV were calculated.

Comparison of SifA-ELISA With the Plate Agglutination Test

Serum samples were collected from 137 SPF chickens. Of these, 29 were *S.* Pullorum antisera, 22 were *S.* Enteritidis antisera, 19 were *S.* Typhimurium antisera, and 67 were negative control sera from uninfected SPF chickens. Besides, 582 serum samples were collected from chickens on poultry farms with clinical manifestations of *Salmonella* infection. These sera were tested using SifA-ELISA and plate agglutination test (**PAT**).

RESULTS

Production of the Recombinant SifA Protein

Protein Basic Local Alignment Search Tool (**BLASTp**) analysis of the *S.* Enteritidis SifA amino acid sequence revealed that the identity between SifAs in prevalent *Salmonella* serotypes was between 89.88 and 100%, while the identity between SifA and proteins in other common non-*Salmonella* strains was below 41.98% (Table S1A). Multisequence alignment of the amino acid sequence of SifA indicated that this protein was highly conserved in *Salmonella* (Table S1B). The phylogenetic tree showed that SifA of *Salmonella* had a very close genetic relationship (Figure S1). These analyses suggested that SifA could be a promising antigen for the detection of *Salmonella* infection.

Therefore, we sought to purify *Salmonella* SifA as a coating agent for the establishment of an indirect ELISA to detect *Salmonella* infection. The *S.* Enteritidis *sifA* gene was successfully cloned into a pET28a expression vector and transformed into *E. coli* BL21 (DE3) cells. Expression of recombinant SifA protein was induced by addition of 0.8 mM IPTG, and SifA was successfully isolated from lysed *E. coli*. The molecular weight of the recombinant SifA protein was approximately 38 kDa as determined by SDS-PAGE (Figure 1A). The His-tagged SifA was expressed in the supernatant and successfully purified by Ni-NTA affinity chromatography. Western blot analysis with mouse anti-His tag monoclonal antibody indicated that the expressed SifA protein was a His-tagged protein of the appropriate molecular weight (Figure 1B).

Development and Optimization of the SifA-ELISA Procedure

The purified SifA antigen was titrated in an ELISA format to determine optimal coating concentrations. The optimal coating concentration is defined here as the minimum concentration of antigen yielding discrimination between the positive- and negative-control sera (Bradshaw et al., 2017). Here we determined the optimal coating concentration of SifA protein for the ELISA as ~6 μg/mL (Figure 2). Then 50 negative serum samples were tested by the above optimized ELISA method, and the mean S/P ratio (X) and SD were calculated as 0.073 and 0.065, respectively. Therefore, we determined our cut-off value for considering a sample seropositive as $S/P \geq 0.268$ (X + 3 SD), and our cut-off value for considering a sample seronegative as $S/P < 0.268$ (Figure 3).

Cross-Reactivity Analysis of the SifA-ELISA

SifA-ELISA demonstrated that all known *Salmonella*-positive serum samples were above the designated cut-off value. This indicates that SifA-ELISA can detect antibodies against *Salmonella* (*S.* Pullorum, *S.* Enteritidis, and *S.* Typhimurium). The S/P ratio of other positive serum samples against non-*Salmonella* pathogens (*E. coli, B. avium, P. multocida*, and *A. paragallinarum*) were below the cut-off value, indicating that SifA-

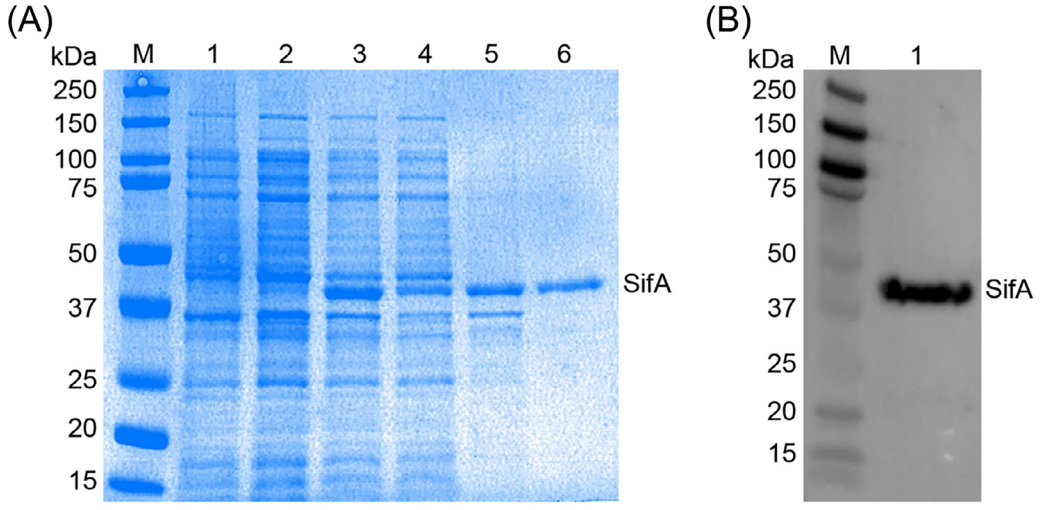

Figure 1. Expression, purification, and identification of the SifA protein. (A) Expression and purification of recombinant protein SifA. Lane M: Precision Plus Protein Dual Color Protein Marker (Bio-Rad Laboratories Inc., Hercules, CA); Lane 1: the lysate of *E. coli* BL21 transformed with empty vector pET28a; Lane 2: the lysate from noninduced cells containing pET28a-SifA; Lane 3: the lysate from IPTG-induced cells containing pET28a-SifA; Lane 4: supernatant of cells containing pET28a-SifA after sonication; Lane 5: sediments of cells containing pET28a-SifA after sonication; Lane 6: Ni-NTA affinity chromatography purified recombinant SifA protein. (B) Western blot analysis of recombinant SifA protein. Lane M: Precision Plus Protein Dual Color Protein Marker (BioRad); Lane 1: the purified SifA protein.

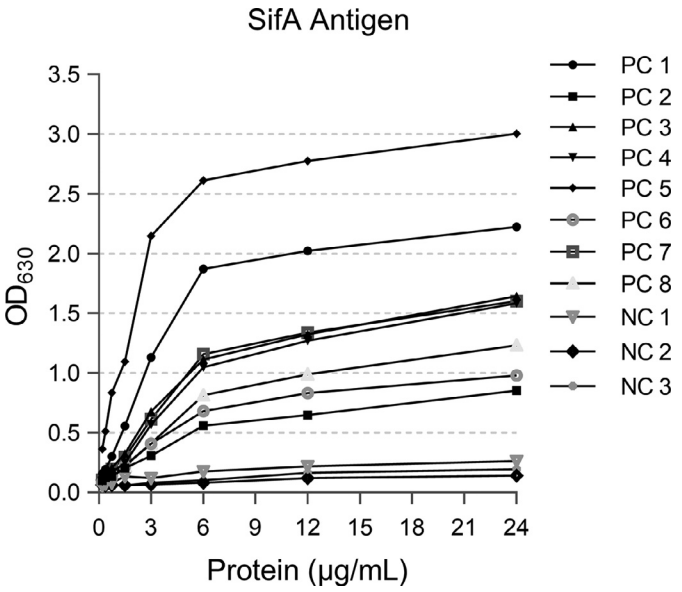

Figure 2. Determination of the optimal concentration of SifA antigen coating. Abbreviations: NC, negative control; PC, positive control. The optimal antigen coating concentration was determined to be ~6 μg/mL based on discrimination of *Salmonella* positive or negative sera.

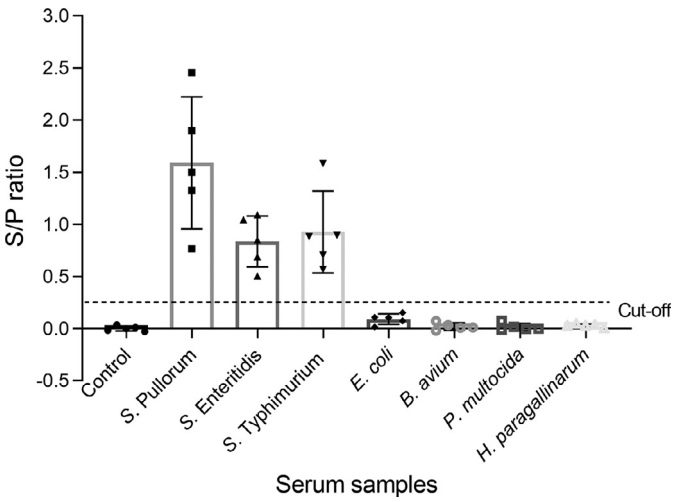

Figure 4. Cross-reactivity of SifA-ELISA. Five different serum samples were tested in each group. Negative sera were tested as a control.

ELISA is highly specific for antibodies raised against *Salmonella* (Figure 4).

Reproducibility of the SifA-ELISA

The reproducibility of the SifA-ELISA was assessed by calculating intra-assay and interassay CVs (Table 1). The results showed that the intra-assay CV of serum samples ranged from 0.47 to 7.80%, while the interassay CV of serum samples ranged from 2.61 to 9.29%. All CV values were below 10%, indicating that the SifA-ELISA is highly reproducible and stable (Jacobson, 1998).

The Detection of Anti-SifA Antibodies in Salmonella-Infected Chickens

SifA-ELISA was used to detect the changes in levels of anti-SifA antibodies in the antiserum of *Salmonella*-infected chickens for 8 consecutive weeks. The results showed that anti-SifA antibodies increased after *Salmonella* (*S.* Pullorum, *S.* Enteritidis, or *S.* Typhimurium) infection and peaked between week 2 and week 3, before anti-SifA antibodies gradually decreased. However, anti-SifA antibodies from antiserum of *Salmonella*-infected chickens remained above the cut-off value through the 8 wk (Figure 5).

Comparison of SifA-ELISA With Plate Agglutination Test

Control sera and clinical serum samples were tested using SifA-ELISA and PAT. The results showed that SifA-ELISA could detect antiserum samples of *S.* Pullorum, *S.* Enteritidis, or *S.* Typhimurium, while anti-SifA antibodies from antiserum remained below the positive cut-off value for all negative control serum samples. In contrast, the PAT could not detect antiserum samples of *Salmonella* Typhimurium (Table 2). As shown in Table 3, the agreement rate of clinical serum samples detected by the SifA-ELISA and PAT was 92.6%.

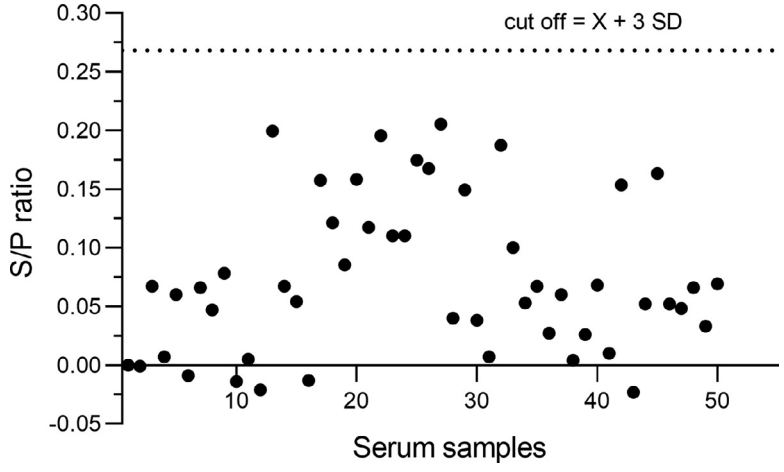

Figure 3. Confirmation of negative-positive cut-off. The dashed line indicates the cut-off value (0.268).

Table 1. Reproducibility of SifA-ELISA.

	Intra-assay test			Interassay test		
Serum samples	Mean	SD	CV%	Mean	SD	CV%
1	0.914	0.043	4.73	0.886	0.057	6.41
2	1.097	0.007	0.64	0.930	0.068	7.33
3	0.683	0.030	4.33	0.804	0.070	8.68
4	0.822	0.020	2.40	0.874	0.049	5.63
5	0.814	0.005	0.60	0.756	0.034	4.44
6	0.435	0.032	7.28	0.425	0.026	6.21
7	0.922	0.023	2.48	0.870	0.069	7.92
8	0.451	0.018	3.91	0.512	0.048	9.29
9	0.940	0.017	1.76	0.930	0.036	3.89
10	0.455	0.014	3.11	0.365	0.022	6.15
11	0.479	0.002	0.47	0.530	0.025	4.81
12	0.469	0.037	7.80	0.462	0.024	5.14
13	0.621	0.005	0.84	0.610	0.051	8.28
14	0.533	0.013	2.40	0.565	0.022	3.84
15	0.672	0.030	4.43	0.689	0.027	3.97
16	0.564	0.012	2.11	0.545	0.014	2.61

Abbreviation: CV, coefficient of variation.

DISCUSSION

In the present study, we developed an indirect ELISA method to detect antibodies against SifA protein to identify *Salmonella* infections in poultry. The SifA protein is highly conserved among various serotypes of *Salmonella*, and amino acid alignment demonstrated high homology of SifA between *Salmonella* serotypes (Table S1). SifA was then established as the coating antigen of an indirect ELISA for the detection of anti-*Salmonella* antibodies. Recombinant SifA protein was expressed in *E. coli* BL21 (DE3) and purified by Ni-NTA affinity chromatography. SDS-PAGE and Western blot showed that recombinant SifA protein was successfully expressed and purified (Figure 1). To distinguish between *Salmonella*-positive and *Salmonella*-negative sera, the optimal antigen concentration for ELISA was determined to be 6 μg/mL (Figure 2). The sera of commercial chickens were then used to validate the cut-off values. A wider range of antigen exposure, including vaccination against other pathogens, may have contributed to higher cut-off values than SPF sera (Barrow, 1994). The antibody level of samples was determined by calculating the S/P ratio using the absorbance value, which is more stable than other parameters (Wang et al., 2009). The SifA-ELISA was indeed reproducible, as demonstrated by intra-assay and interassay CVs below 10% (Table 1).

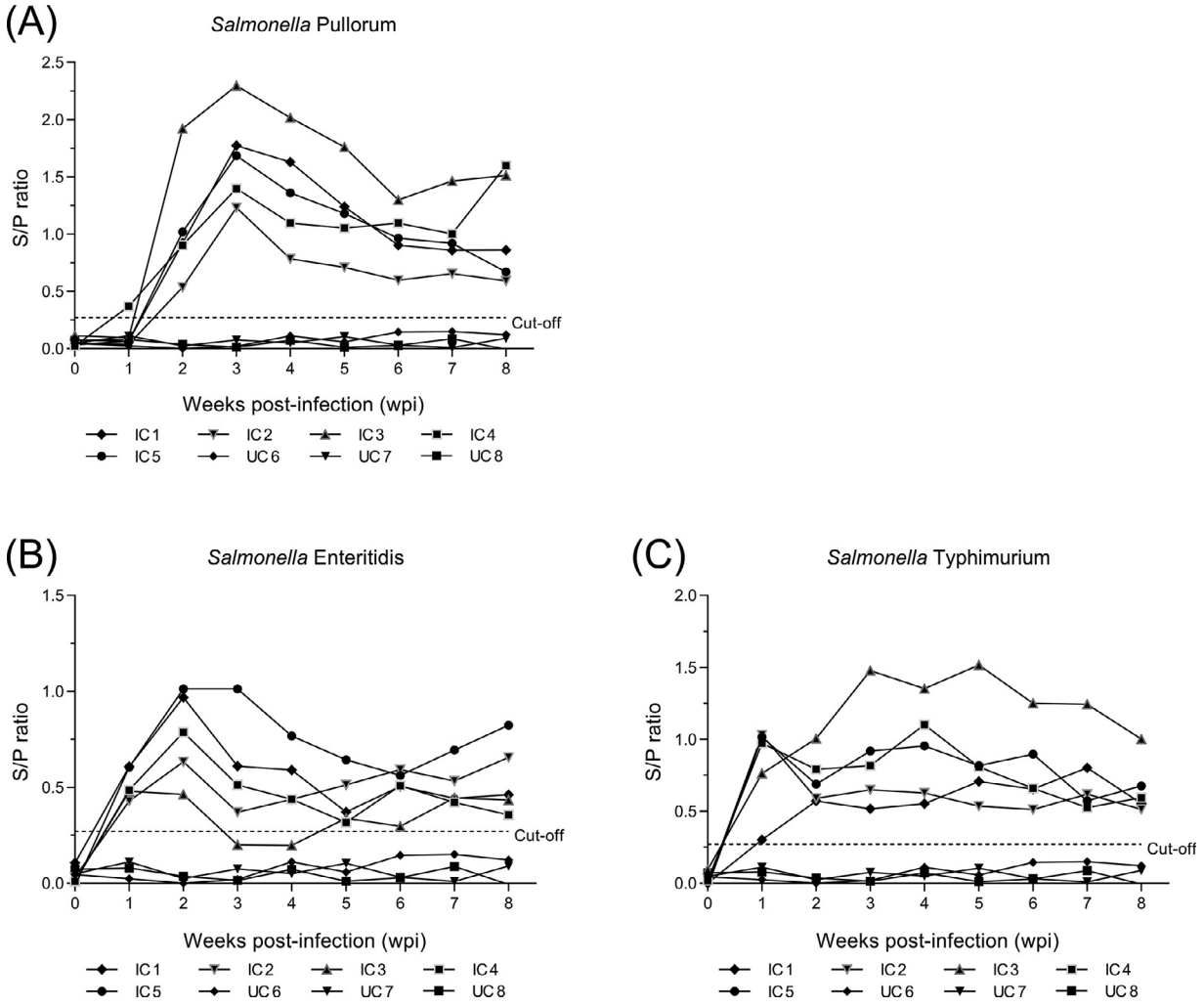

Figure 5. Changes in levels of antibodies against SifA in antiserum from *Salmonella*-infected chickens (*S.* Pullorum, *S.* Enteritis, or *S.* Typhimurium). Five sera from each infected group were collected during the period of 1 to 8 wk after infection. Antibodies against SifA were detected using the indirect ELISA established here. (A) *S.* Pullorum infection group; (B) *S.* Enteritis infection group; (C) *S.* Typhimurium infection group. Abbreviations: IC, infected chicken; UC, uninfected chicken.

Table 2. Comparison of plate agglutination test and SifA-ELISA results in the detection of control serum samples.

Control sera	Total no. of serum samples	Plate agglutination test		SifA-ELISA	
		Positive	Negative	Positive	Negative
Serum against *S.* Pullorum	29	29	0	29	0
Serum against *S.* Enteritidis	22	22	0	22	0
Serum against *S.* Typhimurium	19	0	19	19	0
Negative serum	67	0	67	0	67

To evaluate the diagnostic potential of SifA-ELISA, sera from *Salmonella* and non-*Salmonella* infected SPF chickens were collected. Here we show that the established SifA-ELISA could react specifically with antisera from *Salmonella* (*S.* Pullorum, *S.* Enteritidis, and *S.* Typhimurium) infected chickens, but not with antisera from chickens infected with *E. coli, B. avium, P. multocida*, or *A. paragallinarum* (Figure 4). Subsequently, the SifA-ELISA demonstrated that SPF chickens infected with *Salmonella* (*S.* Pullorum, *S.* Enteritidis, or *S.* Typhimurium) could produce high titers of anti-SifA antibodies, which persisted for at least 8 wk (Figure 5). After infection, anti-SifA antibodies in the serum increased until reaching a maximum before slowly decreasing. However, antibody levels remained above the cut-off value of the SifA-ELISA over the 8-wk period. The kinetics of antibodies against *Salmonella* in this study were also similar to a previous study (Thorns et al., 1996). Antibodies against *S.* Enteritidis or *S.* Typhimurium were detected at 1-wk postinfection. Antibodies against *S.* Pullorum were first observed at 2-wk postinfection, which may be due to slower production of antibodies after oral infection in SPF chickens.

Although many serological methods for the detection of nontyphoid *Salmonella* serotypes have been developed, the PAT is still the most common method for detecting chickens infected with *Salmonella*. However, the interpretation PAT results is somewhat subjective and prone to errors (Ma et al., 2018; Yang et al., 2019). Our results showed that SifA-ELISA could detect antibodies against *Salmonella* (*S.* Pullorum, *S.* Enteritidis, and *S.* Typhimurium) that frequently infect chickens (Wang et al., 2020; Zhao et al., 2020), while the PAT could not detect the antibodies against *S.* Typhimurium (Table 2). This may be explained by the fact that the antigen for the PAT was based on standard (O_1, O_9, O_{121}, and O_{123}) and variant (O_1, O_9, O_{121}, and O_{122}) strains (Proux et al., 2002). *S.* Pullorum and *S.* Enteritidis both have 3 antigens O_1, O_9, and O_{12}, while the O_4 antigen on the surface of *S.* Typhimurium was not found in *S.* Pullorum or *S.* Enteritidis.

Table 3. Comparison of plate agglutination test and SifA-ELISA results in the detection of clinical serum samples.

Plate agglutination test	SifA-ELISA			
	Positive	Negative	Total	% Agreement
Positive	135	16	151	
Negative	27	404	431	
Total	162	420	582	92.6

Clinical serum samples from poultry were tested using SifA-ELISA and PAT (Table 3). Twenty-seven serum samples were positive by SifA-ELISA that were negative with PAT, possibly because SifA-ELISA could detect more serotypes of *Salmonella*, whereas PAT could only detect *Salmonella* serotypes with certain O antigens. Sixteen serum samples were positive by using PAT whereas they were negative by SifA-ELISA, the results of which could be false positive reaction due to cross-reactivity of the agglutination antigen with other gram-negative bacteria (Proux et al., 2002; Yang et al., 2019).

These results indicate that the SifA-ELISA method established in this study is better than currently available tests for the detection of multiple *Salmonella* serotypes during their infection of poultry. However, we acknowledge certain limitations of this study. First, the SifA-ELISA needs to be validated by comparing with commercial ELISA kits or antibody assays. Additionally, further optimization and evaluation is needed by testing more serum samples from more poultry farms and other *Salmonella* serotypes.

In conclusion, an indirect ELISA method based on recombinant protein SifA was developed for the first time, which can be used for the serological detection of *Salmonella* infection in chickens. This method has excellent specificity and reproducibility and could detect multiple serotypes of *Salmonella*. The SifA-ELISA developed here may be applied to the clinical diagnosis of chicken *Salmonellosis* and provides a new method for monitoring *Salmonella* infection on poultry farms.

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DISCLOSURES

The authors declare that they have no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2023.102513.

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