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RESEARCH ARTICLE

LPS and Flagellin-Based Models for Serological Screening and Confirmation of *Salmonella* Infections in Chickens [1] [2]

Arzu FINDIK * Özlem BÜYÜKTANIR * Nevzat YURDUSEV *

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* Ondokuz Mayis University, Faculty of Veterinary Medicine, Department of Microbiology, 55139 Samsun - TURKEY

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Summary

Avian salmonellosis due to *Salmonella* B and D serogroups has a great importance because of the public health and the economical losses in poultry industry. For accurate diagnosis of avian salmonellosis, a total of 126 chicken sera evaluated with a commercial *Salmonella* Enteritidis (SE) ELISA kit have been used to develop two indirect ELISA models using lipopolysaccharide (LPS) and purified flagellin (Flg) of SE as diagnostic antigens. After optimization, 154 and 263 sera were tested with indirect LPS- and Flg-ELISA, respectively. The performance of both indirect LPS- and Flg-ELISA was found high and similar for detecting anti-*Salmonella* antibody in comparison with a commercial LPS-ELISA kit. Nevertheless, a limited number of chicken sera were found discordant between Flg- and LPS-ELISAs. Western-blot analyses of the discordant sera demonstrated that they were found reactive with bacterial lysate of SE but only recognized LPS or Flg antigen. It was concluded from this study that the simultaneous use of both LPS- and Flg-ELISAs would allow differential diagnosis of avian specific salmonellosis from zoonotic *Salmonella* infections due to flagellated bacteria and Western blot models can be used as confirmatory serological test.

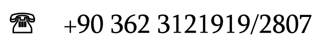
Keywords: *Salmonellosis, ELISA, Flagella, LPS, Western Blot*

Tavuklarda *Salmonella* Enfeksiyonlarının Serolojik Tanısı ve Doğrulanmasına Yönelik LPS ve Flagelline Dayalı Modeller

Özet

Salmonella B ve D serogruplarına ait kanatlı salmonellosis'i halk sağlığı yönünden ve kanatlı endüstrisindeki ekonomik kayıplar nedeniyle büyük öneme sahiptir. Kanatlı salmonellozisinin etkin tanısı amacıyla, SE lipopolisakkarid (LPS) ve saflaştırılmış flagellin (Flg) tanı antijenlerine dayalı iki indirekt ELISA modeli geliştirilmesinde ticari *Salmonella* Enteritidis (SE) ELISA kiti ile değerlendirilmiş toplam 126 tavuk serumu kullanıldı. Optimizasyon sonrası, 154 serum indirekt LPS-ELISA ve 263 serum Flg-ELISA ile test edildi. Ticari LPS-ELISA kiti ile karşılaştırıldığında, indirekt LPS- ve Flg-ELISA'ların anti-*Salmonella* antikor tespit performansları yüksek ve benzer bulundu. Bununla birlikte, sınırlı sayıda serumun Flg- ve LPS-ELISA'da farklılık gösterdiği belirlendi. Bu uyumsuz serumların SE bakteri lizatı ile reaktif olduğu, buna karşın yalnızca LPS veya Flg antijenini tanıdıkları Western Blot analiziyle gösterildi. Bu çalışmadan, LPS- ve Flg-ELISA'nın eşzamanlı kullanımıyla kanatlılara özgü salmonellozisin flagellalı bakteri kökenli zoonotik *Salmonella* enfeksiyonlarından ayırıcı tanısının yapılabileceği ve Western blot modellerinin doğrulayıcı serolojik test olarak kullanılabileceği sonucuna varıldı.

Anahtar sözcükler: *Salmonellozis, Flagella, LPS, ELISA, Western Blot*



İletişim (Correspondence)
+90 362 3121919/2807
yurdusev@omu.edu.tr

INTRODUCTION

Accurate detection of the presence of pathogenic *Salmonella enterica* serotypes such as *S.* Enteritidis (SE) and *S.* Typhimurium (ST) in the poultry industry is of paramount importance for public health [1]. Detection of the presence of these pathogens is generally based on bacteriological and serological tests as well as molecular techniques. However, isolation and identification of the microorganisms are laborious and time-consuming. In addition, false-negative results may be obtained in bacteriological and DNA-based examination due to the intermittent excretion and the rapid quantitative decrease of the microorganisms with age [2-4]. For this reason, several serological tests have been developed and applied for monitoring of the disease status of chicken flocks. Among these tests, enzyme-linked immunosorbent assays (ELISAs) have been largely used and found more sensitive than the agglutination based serological tests. In addition ELISAs are considered as suitable for large scale testing of flocks with more reliability [3,5-9]. The most preferred screening ELISAs are based on the LPS and flagellin antigens. However, the presence of the cross-reactivity between LPS of *Salmonella* B and D serogroups has been demonstrated [1,10,11].

Although cross reactions have also been observed with flagellin-ELISA [1,8], flagella-based ELISAs have been found especially very useful in the discrimination of the infections caused by flagellated and non-flagellated Salmonella in chicken [1,5,8]. The aim of this study was to develop screening and confirmatory immunoassay models based on SE flagellin (Flg) and LPS as diagnostic antigens and discriminate chicken antibody response due to zoonotic *Salmonella* such as *S.* Enteritidis and *S.* Typhimurium from non-zoonotic avian pathogens, i.e. *S.* Pullorum and *S.* Gallinarum.

MATERIAL and METHODS

Bacterial Strain

Salmonella enterica Enteritidis strain (SE/OMU.05.005) from culture collection of Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Microbiology was used. This strain was identified by biochemical tests such as oxidase, O/F, carbohydrate fermentation tests (glucose, sucrose, lactose), H_2S, indol tests and confirmed by slide agglutination tests with mono-specific O4 and O9 as well as Hg and Hm antisera (Denka Seiken, Japan). Its antigenic formula was determined as O1, 9, 12 and gm:-.

Chicken Sera, Antigens and Antibodies

A total of 263 chicken sera were collected from different industrial flocks. *S.* Enteritidis lipopolysaccharide (LPS) (Sigma-Aldrich, USA) and highly purified flagellin were used as diagnostic antigens. In this study, mouse monoclonal *S.* Enteritidis flagella antibody (Flg-Mab) from ViroStat-Monotope (USA) and chicken sera evaluated with a commercial *S.* Enteritidis ELISA kit (BioCheck, CK117) were used as positive or negative controls. All antigens, antibodies and chicken sera were stored at -20°C and -70°C until use.

Preparation of Flagella Rich Fraction

S. Enteritidis strain SE/OMU.05.005 was grown in Brain Heart Infusion Broth (pH 7.2) at 37°C for 18 h. Bacterial cells inactivated by the addition of formaldehyde (0.5% v/v) were harvested by centrifugation at 4000 x g for 20 min and three times washed in physiological saline solution. The bacterial pellet resuspended in lysis buffer (125 mM Tris-Cl, 150 mM NaCl, 2% sodium dodecyl sulfate, pH 6.8) was boiled at 100°C for 15 minutes. The supernatant collected by centrifugation at 25.000 g for 30 minutes was dialyzed initially against deionised water and then 20 mM phosphate buffer containing 150 mM NaCl (pH 7.2) for 24 h at each step. The bacterial lysate was applied to pre-equilibrated Sephacryl S200 gel filtration column in AktaPrime Plus FPLC system (Amersham Biosciences). The protein concentration of the lysate and the fractions was determined by Bichinchoninic acid method (Sigma-Aldrich). One ml of chromatographic fractions was collected at the flow rate of 0.4 ml/min. Immunoreactivity of the chromatographic fractions was analysed by Dot and Western blot assays.

Purification of Flagellin from Polyacrylamide Gels of SDS PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [12] was used for separating the proteins of immunoreactive chromatographic fractions and determining their molecular weight. The protein band corresponding to flagellin (Flg) was excised from the polyacrylamide gel and incubated in the elution buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.5) with shaking in room temperature. Flg diffused into the elution buffer was analyzed by SDS-PAGE and then used as highly purified antigen in ELISA and Western blot assays.

ELISA Procedures

Each well of an ELISA plate coated with 100 µl of the Flg antigen (3 µg/ml) or *S.* Enteritidis LPS (5µg/ml) in carbonate-bicarbonate buffer (0.1 M, pH 9.6) was incubated overnight at 4°C. After washing 3 times with PBST (PBS containing 0.1% Tween 20), blocking step was carried out with 200 µl of PBST-M (1% non-fat milk in

PBST) for 1 h at 37°C. One hundred microliters of Flg-Mab as positive control and the chicken sera diluted 1:250 in PBST-M were added and incubated for 1 h at 37°C. After washing, either goat anti-mouse IgG gamma chain specific or rabbit anti-chicken IgY Alkaline Phosphatase conjugated antibodies (Sigma-Aldrich) diluted 1/30.000 were added and incubated for 1 h at 37°C. Following washing with PBST, 100 µl of pNPP substrate (Sigma-Aldrich) was added and incubated for 1 h at 37°C. The optical density (OD) of the reactions was measured at 405 nm in ELISA reader (Thermo Multiskan). The assays with commercial ELISA kit were performed as described by the manufacturer's instructions. Each assay was carried out at least in duplicate.

Western and Dot Blot Analysis

The antigens separated by SDS-PAGE were electro-transferred to polyvinyldifluoride membranes (Sigma-Aldrich, Immobilon-P) for 1 h at 0.8 mA/cm^2 using a semi-dry transblotter [13]. The membrane strips blocked with PBST-M were incubated at room temperature with either chicken sera or Flg-Mab diluted 1/200 for 60 and 90 min, respectively. After washing, the strips were incubated with AP conjugated rabbit anti-chicken IgY or AP conjugated goat anti-mouse gama chain specific IgG antibody solutions (Sigma-Aldrich) diluted 1/30 000 for 1 h at room temperature. After washing, color reaction was initiated by the addition of BCIP/NBT substrate system (Sigma-Aldrich) and stopped by extensive washing. In Dot Blot analysis, 1 µl of the chromatographic fractions or the target antigens were adsorbed onto nitrocellulose membrane strips. The strips saturated with PBST-M were incubated at room temperature with either chicken sera or Flg-Mab at a dilution of 1:200 for 60 and 90 min, respectively. Other steps of the assay were identical to those of Western Blot assay described above.

Statistical Analysis

All statistical analyses were performed at 95% confidence interval (CI) by using Win Episcope Version 2.0. Cut-off value and diagnostic performance (sensitivity, specificity, positive and negative predictive values, area under curve) of the ELISA models were determined by receiver operating characteristic (ROC) analysis.

RESULTS

Purification and Characterization of Flagella Protein

After gel filtration chromatography separation of the lysate of *S.* Enteritidis, major fractions were first analysed by Dot Blot assay using Flg-MAb. The fractions 50-54 were detected as the most reactive and mixed to be evaluated by Western Blot using Flg-MAb. As seen in Fig 1a, two electrophoretic bands with relative molecular weights of 59 and 53 kDa were found reactive. The major reactive protein band of about 59 kDa was eluted from polyacrylamide gel following SDS-PAGE in elution buffer and evaluated by Western Blot analysis. As seen in Fig 1b, Flg-Mab only reacted with the 59 kDa protein demonstrated that SE flagellin was highly purified.

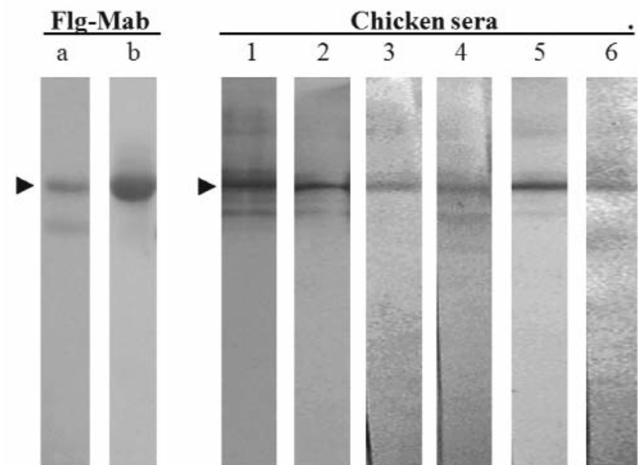


Fig 1. Western blot analysis of purified S. Enteritidis flagellin with Flg-Mab and chicken sera reactive in ELISA

Western blot analysis of (a) pool of gel filtration chromatography fractions 50-54 and (b) the major protein band of 59 kDa (Flg) purified by elution from polyacrylamide gel of SDS-PAGE using monoclonal S. Enteritidis flagella antibody (Flg-Mab). Recognition of highly purified Flg by representative individual chicken serum samples (1 to 6) found reactive in ELISA. Large arrow heads indicate S. Enteritidis flagellin (Flg)

Şekil 1. *S.* Enteritidis'e ait saflaştırılmış flagellin'in Flg-Mab ve ELISA'da reaktif olduğu belirlenen tavuk serumları ile Western blot analizi

(a) Jel filtrasyon kromatografisinden elde edilen fraksiyon 50-54'ün karışımı ve (b) SDS-PAGE poliakrilamid jelden elüe edilen 59 kDa baskın protein bandının monoklonal *S.* Enteritidis flagella antikoru (Flg-Mab) ile Western blot analizi. Yüksek düzeyde saflaştırılmış Flg'nın ELISA'da reaktif olan temsili bireysel tavuk serum örnekleri (1-6) ile tanınması. Geniş ok başları *S.* Enteritidis flagellini (Flg) göstermektedir

The purity of flagella protein was also evaluated by Western blot analysis using the chicken sera previously found positive by ELISAs. Representative results were given in *Fig 1*. While Flg-Mab reacted only with the 59 kDa flagella protein (Fig 1b), chicken sera indicated with 1, 2 and 4 in *Fig 1* weakly reacted with minor electrophoretic bands less than 59 kDa. However, the sera 3, 5 and 6 only recognized the flagella protein of 59 kDa. Although the purified flagellin contained minor bacterial contaminations, they were weakly recognized by some chicken sera but not all. Based on these results, flagellin obtained in this study was considered as highly purified and used as target antigen to detect anti-*S.* Enteritidis flagella antibody.

Development and Evaluation of ELISA Models

A total of 126 chicken sera evaluated with BioCheck SE-ELISA kit have been used to develop and optimize two indirect ELISA models based on *S.* Enteritidis lipopolysaccharide (LPS-ELISA) or 59 kDa flagella protein (Flg-ELISA). After optimization, a total of 154 and 263 sera were tested with indirect LPS-ELISA and Flg-ELISA, respectively. Optimal cut-off values of indirect LPS- and Flg-ELISAs were determined by ROC analysis as 0.450 and 0.500 at OD405, respectively. The results and performance analysis in comparison with commercial ELISA are presented in *Table 1*. The percentage of positive or negative chicken sera was found almost identical with three ELISA models. Although the sensitivity of Flg-ELISA was slightly lower (96.1%), no significant difference was observed with commercial and indirect LPS-ELISAs. In contrast, positive predictive value (PPV) of Flg-ELISA was found higher than those of commercial and LPS-ELISAs *(Table 1)*. Discordant results were observed with only some chicken sera detected as positive in LPS-ELISAs and Flg-ELISA. At 95% confidence interval by ROC analysis, area under curve (AUC) being higher than 99% showed high performance of the ELISA models and their analytical similarity with the commercial ELISA kit *(Table 1)*.

Table 1. *Comparative performance analysis of three types of ELISA*
Tablo 1. *Üç tip ELISA'nın karşılaştırmalı performans analizi*

Test Type [1] (No of Sera)	No and (%) of Sera Evaluated As		Performance Analysis (%)				
	Positive	Negative	SN	SP	PPV	NPV	AUC
Com ELISA (n=126)	12 (9.5)	114 (90.5)	100	98.2	85.7	100	99.78
LPS-ELISA (n=154)	14 (9.1)	140 (90.9)	100	96.6	87.5	100	99.90
Flg-ELISA (n=263)	26 (9.9)	237 (90.1)	96.1	100	100	99.6	99.98

1 *Com ELISA: A commercial LPS-based ELISA kit. Flg-ELISA and LPS-ELISA: Indirect ELISA models developed in our laboratory using highly purified S. Enteritidis flagellin and commercial S. Enteritidis LPS (Sigma-Aldrich), respectively. Test performances were determined by ROC analysis using Win Episcope 2.0 at 95% confidence interval (CI)*

1 *Com ELISA: Ticari LPS'e dayalı ELISA kiti. Flg-ELISA and LPS-ELISA: Sırayla, yüksek düzeyde saflaştırılmış S. Enteritidis flagellin and ticari S. Enteritidis LPS (Sigma-Aldrich) kullanılarak laboratuvarımızda geliştirilen indirekt ELISA modelleri. Test performansları Win Episcope 2.0 programı kullanılarak %95 güven aralığında (CI) ROC analizi ile belirlendi*

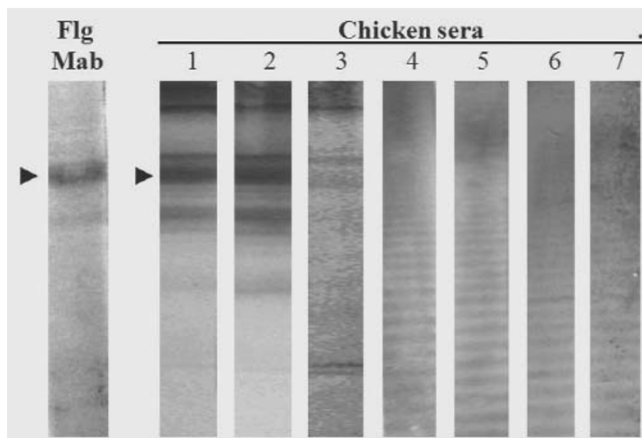


Fig 2. Confirmation of ELISA results by Western blot analysis using *S.* Enteritidis cell lysate and LPS

To confirm discordant results, individual chicken sera reactive in Flg-ELISA but not in LPS-ELISA testing were analyzed by Western blot using *S.* Enteritidis cell lysate (1 to 3) in comparison with Flg-Mab. Confirmation of chicken sera found positive in LPS-ELISA but not in Flg-ELISA by Western blot analysis using *S.* Enteritidis LPS (4 to 7)

Şekil 2. *S.* Enteritidis hücre lizatı ve LPS kullanılan Western blot analizi ile ELISA sonuçlarının doğrulanması

Farklı sonuçların doğrulanmasına yönelik, Flg-ELISA'da reaktif olan fakat LPS-ELISA ile reaksiyon vermeyen bireysel tavuk serumları Western blot ile *S.* Enteritidis hücre lizatı (1-3) kulla-nılarak Flg-Mab ile karşılaştırmalı analiz edildi. LPS-ELISA'da pozitif fakat Flg-ELISA'da negatif bulunan tavuk serumlarının *S.* Enteritidis LPS kullanılarak Western blot analizi ile doğrulanması (4 to 7)

Confirmation of ELISA Results with Western Blot Assay

A total of 7 discordant chicken sera (3 LPS-ELISAs negative but Flg-ELISA positive and 4 LPS-ELISAs positive but Flg-ELISA negative samples) were analyzed by Western blot assay *(Fig 2)*. Lysate and LPS of *S.* Enteritidis were used as target antigens for confirming the ELISA results of the discordant sera. Three sera found negative in LPS-ELISAs but positive in Flg-ELISA were tested with lysate antigen in comparison with Flg-Mab and found reactive with several bands of lysate antigen *(Fig 2*, chicken sera no 1, 2 and 3) in addition to the major band of 59 kDa, which was recognized by Flg-MAb *(Fig 2a)*. In the same manner, four samples detected as positive in LPS-ELISAs but negative in Flg-ELISA reacted with LPS antigen in Western blot assay (*Fig 2*, chicken sera no 4, 5, 6 and 7).

DISCUSSION

The aim of the present study was to develop two indirect ELISA models based on flagellin and lipopolysaccharide of *S.* Enteritidis for screening *Salmonella* antibody in chicken serum. For these purposes, 126 chicken sera evaluated with a commercial LPS-based

ELISA as reference were used to develop and optimize indirect LPS- and Flg-based ELISA models. As seen in Table 1, no difference between commercial and indirect LPS-ELISAs were observed in respect to sensitivity, specificity, positive and negative predictive values and AUC. These results demonstrated that indirect LPS-ELISA developed in this study was found identical to the commercial ELISA.

As *S.* Enteritidis and *S.* Typhimurium cause foodborne diseases, periodic surveillance of commercial flocks of these important zoonotic agents become a priority for the public health purposes. Cross-reactions were frequently observed between *S.* Enteritidis and *S.* Typhimurium infections when LPS fractions were used as diagnostic antigens [10,11]. Although the usual cross-reactions have been encountered, ELISA based on the LPS of *S.* Enteritidis could be used to screen *Salmonella* infections due to microorganisms of serogroups B and D. However, LPS-based ELISA is not capable to discriminate antibody response developed towards non-zoonotic and non-flagellated *S.* Pullorum and *S.* Gallinarum from that developed against flagellated zoonotic microorganisms such as *S.* Enteritidis and *S.* Typhimurium. For this reason, flagellin of *S.* Enteritidis was used as differentiating diagnostic antigen. It was highly purified in two steps; gel filtration chromatography followed by elution from polyacrylamide gel after SDS-PAGE. In this way, native flagellin of about 59 kDa molecular weight was obtained and confirmed by Western blot analysis using Flg-Mab *(Fig 1)*. Previous studies also showed that flagellin of different Salmonella serotypes were determined between 48 and 59 kDa and effectively used to detect the presence of anti-flagella antibody response [14-16].

In this study, a total of 263 chicken sera were tested to evaluate Flg-ELISA model based on highly purified flagellin of *S.* Enteritidis. While the sensitivity of Flg-ELISA was slightly lower (96.1%) than LPS-ELISAs, its specificity was found as the highest (100%). However, the percentage of positive or negative chicken sera found almost identical with three ELISA models indicated their close similarity *(Table 1)*. Although overall performance of Flg-ELISA was found closely similar to that of LPS-ELISAs, a limited number of chicken sera were found discordant between Flg- and LPS-ELISAs. The discordant results obtained by ELISAs were confirmed by Western blot analysis using both lysate and LPS of *S.* Enteritidis *(Fig 2)*. Confirmation of the discordant results by Western blot strongly suggested that some chicken sera contained only detectable level of anti-LPS antibody but not anti-Flg antibody or just the opposite. These results are in accordance with the new standard operating procedures used in the Laboratory of Enteric Pathogens (Health Protection Agency's National Salmonella Reference Centre, Colindale, UK) recommending Western blot analysis based on both LPS and flagella antigens for accurate Salmonella serodiagnosis [17,18].

From the results of this work, it can be concluded that the simultaneous use of both LPS- and Flg-ELISAs would improve serological diagnosis and discrimination of the main zoonotic *Salmonella* infections due to flagellated bacteria such as *S.* Enteritidis and *S.* Typhimurium from avian specific salmonellosis due to *S.* Pullorum and *S.* Gallinarum, and Western-blot models can be used as confirmatory serological test for salmonellosis monitoring of commercial flocks.

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