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Development of an Enzyme-Linked Immunosorbent Assay for quantification of *Salmonella* Enteritidis-specific antibodies in egg yolk

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■ **Keywords**

Antibodies; egg yolk; ELISA; *Salmonella* Enteritidis.

■ **Abbreviations**

BSA, bovine serum albumin; CFU, colony forming units; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffered saline; SE, *Salmonella* Enteritidis.

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ABSTRACT

The present study aims at developing an indirect ELISA to quantify yolk antibodies specific to all surface proteins of the invasive *Salmonella* Enteritidis (SE), which acquired the 1.8, 14.1, and ~ 50 Kb plasmids. An ELISA checkerboard was used in four different experiments to account for the different parameters included in the preliminary ELISA procedure, and consequently to maximize the difference in Optical Density (OD) values between control positive and negative yolk samples. The first experiment aimed at studying the impact of 5% Bovine Serum Albumin (BSA) dissolved in distilled water as a blocking reagent on a 28 µg/well SE antigen-coated plate, while applying the positive and negative control yolk samples to different concentrations of Phosphate-Buffered Saline (PBS).Conjugate application was maintained constant at a dilution of 1:500 in PBS. The second experiment was similar to the first one, but the positive and negative control yolk samples were diluted in PBS-Tween 20, and the conjugate dilution was changed to 1:1500 in PBS-Tween 20. In the third experiment, the conjugate was diluted at 1:1500 in 5% BSA/PBS-Tween 20 diluent or PBS-Tween 20 diluent with no 5% BSA. The objective of the fourth experiment was to study the impact of four different concentrations of SE-coated antigen levels (28µg/well, 56µg/well, 84µg/well, and 112µg/well), while fixing the blocking step with 5% BSA in distilled water, and the conjugate dilution set at 1:1000 in 5% BSA/PBS–Tween 20, and fixing the control yolk samples dilution at 1% in PBS-Tween 20. This last experimental procedure allowed the highest difference in mean absorbance OD values of the positive control minus the negative control samples, which was equivalent to 0.381. In addition, the final protocol for this ELISA was applied on individual egg yolk samples of two groups of chicken layers: one challenged in the esophagus at 11 days with 5.4 x 10^{10} CFU/ml/bird of SE, and the second group was not challenged. The mean OD values of the egg yolk of antibodies specific against SE of the two groups were significantly different (0.8578 versus 0.5250; $p<0.05$), which indicates the possible application of the developed ELISA for screening SE infection by examining egg yolks produced by commercial layers.

INTRODUCTION

Salmonella Enteritidis (SE) acquiring a high molecular weight plasmid (~ 50 Kb) is reported as a highly invasive pathogen, resulting in significant damage in avian and human hosts (Barbour *et al.*, 1999; Saeed and Nair, 1999). SE was found to be the most common *Salmonella* serotype recovered from human cases in the USA, accounting for 76% of all septicemia cases and 39% of all cases of gastroenteritis (Gruenewald *et al.*, 1999). In poultry, the invasive SE causes more damage in young chicks as compared to adults (Gast and Beard, 1989; Uyttebroek *et al.*,

1991). Natural infection by invasive SE causes the following lesions in young chicks: namely, fibrinous pericarditis, airsacculitis, perihepatitis, peritonitis, cecal cores, and unabsorbed yolk sacs (Gorham *et al.*, 1994). The pathogenesis of invasive SE in adult chicken layers is milder, resulting in slight heterophil infiltration in the ovaries and oviducts (Hoop & Pospischil, 1993).

The classical approach followed in many investigations of SE outbreaks in poultry relies mainly on culturing the egg contents for SE isolation (yolk, yolk vitelline membrane, albumen). This approach is time-consuming, and can be misleading due to the very low frequency of SE- infected eggs (Dadrast *et al.*, 1990). This fact led many researchers around the world to develop indirect ELISA for the identification of SE-specific antibodies in the egg yolk in order to assess the infectivity status of poultry flocks (Nicholas & Cullen, 1991; Furrer *et al.*, 1993; Barrow, 1994; Desmidt *et al.*, 1996; Dera-Tomaszewska *et al.*, 2003). In addition, scientists are targeting the development of the indirect ELISA for the analysis of SE-specific antibodies in the egg yolk instead of serum SE antibodies, thus avoiding the stress induced by handling birds during blood collection, and at the same time, abiding to the biosafety protocols of the farms included in national disease control programs. The nature of the coating of the ELISA plates for SE-specific antibody quantification varies among the scientists due to the difference in the prevalent strain(s) of SE, or due to interests of the scientists in quantification of certain protective antibodies against specific antigens in the SE, such as fimbriae or pilli, flagella, lipopolysaccharide, heat shock protein (HSP 60), or all surface proteins of SE (Dadrast *et al.*, 1990; Nicholas & Cuellen, 1991; Dera-Tomaszewska *et al.*, 2003; Oliveira *et al.*, 2004). The prevalent invasive SE in the poultry of Lebanon is the one that acquired three plasmids, namely, 1.8, 14.1, and ~ 50 Kb (Barbour *et al.*, 1999). Other reports from around the world report the presence of the high molecular weight plasmid of ~ 50Kb, which is responsible for the invasiveness of particular SE strains (Guard-Petter, 1998; Guard-Petter *et al.*, 1999; Van Asten and Van Dijk, 2005).

The purpose of this work was to develop an indirect ELISA to quantify yolk antibodies specific to all surface proteins of the invasive SE that acquired the 1.8, 14.1 and ~ 50Kb.

MATERIALS AND METHODS

Reagents in preliminary ELISA procedure

Salmonella Enteritidis cells were harvested from Nutrient Agar plates, solubilized, and charged by the addition of Sodium Dodecyl Sulphate (SDS) in a w/w ratio of SDS/*Salmonella* Enteritidis proteins equivalent to 1:29.1. The charged antigen stock was incubated at 37°C for 2 hours under continuous stirring. The antigen stock was centrifuged at 1620x g for 15 min, and the supernatant was collected in 1 ml aliquots and stored at –20°C. The diluent used to vary antigen levels was the carbonate/bicarbonate-coating buffer (pH 9.6), and the microtiter plates were Immunolon 1 type (Dynatech Laboratories, Virginia, USA). The coating of 50 µl/well of the antigen was performed at 37°C, overnight. The plate was washed four times with distilled water. The positive and negative control yolk samples were diluted to different concentrations, using different diluents, and added in 100µl/wells. Control yolk samples were incubated at room temperature for 15 minutes. The diluted control yolk samples were discarded, and the wells were washed four times with distilled water. The conjugate used was goat anti-chicken IgG (light + heavy chains), labeled with a peroxidase enzyme (Kirkegaard & Perry Laboratories, Maryland, USA). The lyophilized conjugate was reconstituted in sterile distilled water/glycerol (1:1 ratio), diluted into various concentrations using different diluents, and added at a volume of 50µl per well. The diluted conjugate was incubated at room temperature for a period of 15 minutes, followed by four washings with distilled water. The substrate was added at 50µl/well, and incubated at room temperature for 15 minutes. The substrate was ABTS 1-component containing 2,2'-azino-di (3 ethyl-benzthiazoline-6-sulfonate) at a concentration of 0.3 g/L in a glycine/citric acid buffer(pH 4.0), supplemented with hydrogen peroxide (H_2O_2) at a concentration of 0.02%. Absorbance was read at a wavelength of 450 nm using Organon Teknika reader, model 530 (Organon, West Chester, PA, USA).

Control yolk samples

The negative control yolk samples were collected from eggs of 10 chicken layers kept in isolation, and that were free of *Salmonella* Enteritidis infection, as confirmed by daily culture of the vitelline membrane of the eggs produced from these layers during a one month period, from 27 weeks to 31 weeks of age. An equal aliquot of 50µl of yolk was pooled from 9 eggs

collected during the last day of the 1 month-culture period, and vortexed for one minute with 450µl of sterile saline. The vortexed sample was centrifuged at 3645xg for 30 minutes, and the supernatant was collected and stored in small aliquots at –20°C.

The positive control yolk sample was collected from eggs of 10 chicken layers belonging to the same breed and flock, and kept in isolation. Each of the 10 chicken layers was challenged with $5.4x10^{10}$ CFU of *Salmonella* Enteritidis (SE) at 28 weeks of age, and the vitelline membranes of the eggs produced by the challenged layers were cultured daily for a period of 2 weeks-post challenge, confirming transmission of SE in only one egg at 2 weeks post-challenge. Equal aliquots of the yolk of 8 eggs collected on the 11th day post-challenge were pooled, and vortexed for one minute with an equal amount of sterile saline. The vortexed sample was centrifuged at 3645x g for 30 minutes, and the supernatant was collected and stored in small aliquots at –20°C.

Checkerboard for development of ELISA

The purpose of the ELISA checkerboard was to vary the different parameters included in the preliminary ELISA procedure in order to maximize the difference in the Optical Density (OD) values between positive and negative control yolk samples, targeting a difference greater than 0.350. (Veling *et al.*, 2001; Vandekerchove *et al.*, 2002).

Four different experiments were included in the ELISA checkerboard:

1 - The objective of experiment 1 was to study the impact of 5% Bovine Serum Albumin (BSA) dissolved in distilled water used as a blocking reagent in the step following the washing of the coated SE antigens, while applying different concentrations of positive and negative control yolk samples. In this experiment, the coated SE antigen level was fixed at 28µg/well, the conjugate was fixed at a dilution of 1:500 in Phosphate Buffered Saline (PBS), and the control yolk samples were diluted to 1.0, 1.5, 3.0, and 5.0 % using PBS as a diluent. Each concentration of control yolk samples (positive or negative) was applied in quadruplicate (4 wells). The application of the diluted control yolk samples was either preceded by the blocking step or deprived from it. The volume of the blocking reagent added per well was 100µl, which was incubated at room temperature for a period of 15 minutes, followed by 4 washings with distilled water. Optical Densities (O.D.) at a wavelength of 450 nm of positive and negative control yolk sample in quadruplicate were recorded for each variable parameter.

2 - The objective of experiment 2 was to study the impact of 5 % BSA dissolved in distilled water in the blocking step, following coating with the antigen, and a higher conjugate concentration in a new diluent of PBS-Tween 20, while applying different concentrations of positive and negative control yolk samples diluted in PBS-Tween 20. In this experiment, the coated SE antigen level was fixed at 28µg/well, conjugate dilution was raised to 1:1500 using PBS supplemented with 0.05% of Tween 20. In addition, control yolk samples were diluted to 1.0, 1.5, 3.0, and 5.0 %, using PBS-Tween 20. Each concentration of the control yolk samples was applied in quadruplicate (4 wells). The application of the diluted control yolk samples was either preceded by the blocking step or deprived from it. Optical Densities of positive and negative control yolk samples in quadruplicate were recorded for each variable parameter.

3 - The objective of experiment 3 was to study the impact of 5 % BSA dissolved in distilled water for blocking, and of diluting the conjugate at 1:1500 in either 5% BSA dissolved in PBS-Tween 20 or PBS-Tween 20 diluent deprived of BSA, while applying different concentrations of positive and negative control yolk samples diluted in PBS-Tween 20. In this experiment, coated SE antigen level was fixed at 28µg/well. The control yolk samples were diluted to 1.0. 1.5, 3.0, and 5.0 % using PBS-Tween 20. Each concentration of the control yolk samples was applied in duplicates (2 wells). The application of the diluted control yolk samples was either preceded by the blocking step or deprived from it. Optical Densities (OD) of positive and negative control yolk in duplicate were recorded for each variable parameter.

4 - The objective of experiment 4 was to study the impact of different concentrations of coated SE antigens, while blocking with 5% BSA dissolved in distilled water, and fixing conjugate dilution at 1:1000 using the 5% BSA – PBS-Tween 20 diluent, and fixing control yolk dilution at 1 % in PBS-Tween 20. In this experiment, the different levels of coated SE antigen were 28, 56, 84, and 112µg protein/well. The fixed concentration of

control yolk samples was applied in four sets, with 6 wells/set, where the respective levels of SE antigens in each set were 28, 56, 84, and 112µg protein/well. Optical Densities of positive and negative control yolk in six wells were recorded for each different SE antigen level. Results of experiments 1-4 are presented in Tables 1-4, respectively.

Preliminary application of the developed ELISA

Based on the four experiments above, the final protocol of the developed ELISA was applied on individual egg yolk samples from each of the two groups of chicken layers: one challenged with SE in the esophagus on day 11, and the second group that wass kept in isolation, free from *Salmonella* infection. The respective mean O.D. values of egg yolks obtained from both groups, was statistically analyzed by One-Way ANOVA in order to evaluate the significance ($p<0.05$) of differences in mean O.D. values of yolks obtained from the infected and uninfected layers.

RESULTS AND DISCUSSION

The absence of Tween 20 from the PBS diluent used to dilute the control yolk samples and the conjugate, and the low antigen level used in coating (28µg/well) may have been responsible for the failure in differentiating Optical Density (OD) obtained from the positive or negative control yolk samples (Table 1). A higher difference in the mean absorbance O.D. values of the positive minus negative control yolk samples was obtained when the yolk was diluted at 3.0% in PBS, and the wells were blocked with 5 % BSA in distilled water. However, this difference (0.134) was still lower than the targeted cutoff value of >0.350. It is worth noting that previous tested commercial kits presented a cutoff difference between positive and negative samples equivalent to an average O.D. value lower than 0.350 (Nicholas and Cullen, 1991; Furrer *et al.*, 1993). The inclusion of Tween 20 at 0.05% in the diluent of the control yolk samples and in the conjugate, and lowering conjugate concentration by increasing its dilution from 1:500 to 1:1500 in the presence of blocking by 5% BSA after coating, resulted in higher difference between the positive and the negative control yolk samples (Table 2). More specifically, the 5.0% dilution of the control yolk samples resulted in a wider difference between the positive and the negative controls (0.219), which was still lower than the targeted difference in O.D. value of >0.350. The detergent nature of Tween 20 may have allowed the release of the SE-antibody

Table 1 - Impact of the use of 5% Bovine Serum Albumin dissolved in distilled water as a blocking reagent following antigen coating[a], and using different dilutions of positive and negative control yolk[b] samples diluted in Phosphate Buffered Saline (PBS).

Blocking treatment	Dilutions of control yolk (%)	Mean[c] O.D. values of control yolk		Difference between positive & negative O.D. values
		Positive	Negative	
5% BSA	1.0	2.764	2.735	0.029
	1.5	2.740	2.717	0.023
	3.0	2.897	2.763	0.134
	5.0	2.750	2.933	- 0.183
No BSA	1.0	2.825	2.753	0.072
	1.5	2.760	2.812	- 0.052
	3.0	2.894	2.819	0.075
	5.0	2.825	2.946	- 0.121

a - Coating per well is equivalent to 28 µg of SE protein. b - Yolk was diluted at 1.0-5.0 % in PBS, and the conjugate was diluted at 1:500 in PBS. c - Mean of quadruplicates.

Table 2 - Impact of 5% Bovine Serum Albumin dissolved in distilled water used in the blocking step following coating with antigen[a], and using different dilutions of positive and negative control yolk[b] samples diluted in PBS-Tween 20.

Blocking treatment	Dilutions of control yolk (%)	Mean[c] O.D. values of control yolk		Difference between positive & negative O.D. values
		Positive	Negative	
5% BSA	1.0	0.981	0.881	0.100
	1.5	1.104	0.903	0.201
	3.0	1.140	0.931	0.209
	5.0	1.156	0.937	0.219
No BSA	1.0	0.975	0.886	0.089
	1.5	1.029	0.891	0.138
	3.0	1.037	0.884	0.153
	5.0	1.127	0.929	0.198

a - Coating per well is equivalent to 28 µg of SE protein. b - Yolk was diluted at 1.0-5.0 % in PBS, and the conjugate was diluted at 1:500 in PBS. c - Mean of quadruplicates

component from the remaining yolk components, or else, it could have created an optimal ionic environment the binding of yolk antibodies and conjugate antibodies to the coated SE antigens, and to the captured chicken antibodies, respectively (Lee *et al.*, 2003; Halim *et al.*, 2005).

Results of the comparison of the effect of the nature of the diluent used for the conjugate (5% BSA in PBS-Tween 20 versus PBS-Tween alone) in the presence or absence of blocking by 5% BSA, while using control yolk samples in dilutions between 1.0-5.0% in PBS-Tween 20, are shown in Table 3. The maximum difference in the O.D. values between the positive and the negative yolk samples (0.279) was obtained when the yolk was diluted to 1.0% in PBS-Tween 20, and the conjugate was diluted in 5 % BSA in PBS-Tween 20. It was shown that this improvement in O.D. value differences between positive and negative was due to the inclusion of 5% BSA in the PBS-Tween 20 diluent used for the conjugate. BSA at 5%may have blocked non-specific sites in the microtiter plate well , thereby increasing the specificity of the SE-antibodies present in the positive control yolk samples, or preventing antibodies in the negative control yolk sample that are not specific to SE to bind to the coated antigens (Vandekerchove *et al.*, 2002).

The objective of the assay presented in Table 4 was to obtain a difference between positive and negative control yolk O.D. values higher than 0.350. The variation in this assay was the change in the SE protein level used for coating, which ranged from 28 to 112µg/ well, while yolk dilution was kept constant at 1.0% in PBS-Tween 20, as well as conjugate dilution at 1:1000 in 5% BSA dissolved in PBS-Tween 20. Results in Table 4 show that the maximal difference in O.D. values was 0.381 between the positive and the negative control yolk samples, at an antigen level of 112 µg/ well. This difference was higher than the targeted cutoff of 0.350. The ELISAs developed by other researcher presented O.D. value differences between positive and negative control samples equal or higher than 0.300 (Barrow, 1994; Desmidt *et al.*, 1996; Dera-Tomaszewska *et al.*, 2003; Oliveira *et al.*, 2004).

Interestingly, in spite of achieving the objective of obtaining significant differences in OD values between positive and negative control yolk samples, the negative control yolk samples still presented a high background optical density value of 0.862 (Table 4). Future investigations should target a lower OD value for negative control yolk samples, which could be achieved by increasing yolk sample dilution over 1% (VanderSchalie *et al.*, 1994; Clavijo *et al.*, 1995).

In conclusion, the final protocol of the ELISA for the quantification of SE-specific antibodies in the egg yolk of chickens based on the above data, presented in Tables 1-4, is to coat the microtiter plate wells with 112µg/well of SE proteins, to block them using 5% BSA in distilled water, and to dilute the yolk samples at 1% in PBS-Tween 20, and the conjugate at 1:1000 in 5%BSA dissolved in PBS-Tween 20.

The mean O.D. values of SE-specific antibodies in individual egg yolks, obtained by SE-challenged and

Table 3 - Impact of 5% Bovine Serum Albumin dissolved in distilled water used for blocking after antigen coating[a], and impact of constant conjugate dilution[b] in 5% BSA dissolved in PBS-Tween 20 versus conjugate dissolved in PBS-Tween 20 alone, using different (positive& negative control) yolk dilutions[c] in PBS-Tween 20.

Treatment Blocking	Nature of conjugate diluent		Dilution of control yolk (%)	Mean[d] O.D. values control yolk		Difference between positive & negative O.D. values
	5% BSA in PBS-Tween	20 PBS-Tween 20		Positive	Negative	
5% BSA	+		1.0	1.234	0.955	0.279
	+		1.5	1.208	1.040	0.168
	+		3.0	1.280	1.070	0.210
	+		5.0	1.202	1.134	0.068
		+	1.0	1.270	1.091	0.179
		+	1.5	1.157	1.103	0.054
		+	3.0	1.314	1.053	0.261
		+	5.0	1.288	1.106	0.182
No BSA	+		1.0	1.143	0.978	0.166
	+		1.5	1.239	1.039	0.200
	+		3.0	1.280	1.112	0.168
	+		5.0	1.194	1.115	0.079
		+	1.0	1.145	0.983	0.163
		+	1.5	1.185	0.991	0.194
		+	3.0	1.175	1.007	0.168
		+	5.0	1.124	0.992	0.132

a - Coating per well is equivalent to 28 µg of SE protein. b - Conjugate dilution at 1:1500. c - Control yolk samples were diluted in PBS-Tween 20 at 1.0-5.0 %. d - Mean of duplicates.

control-unchallenged layers, were 0.8578 and 0.5250 ($p<0.05$) (Table 5). Future investigations will evaluate the sensitivity of this developed ELISA for the detection of SE-specific antibodies in chicken yolk at different intervals after a controlled challenge, and for the study the dynamics of the infection titers through the production cycle of infected flocks. In addition, future investigations could implement this developed ELISA to evaluate different eradication or control programs of SE infections in poultry as a prerequisite for their control in food-borne human diseases.

Table 4 - Impact of different concentrations of coated SE proteins while fixing blocking with 5% BSA in distilled water, and fixing conjugate[a] and yolk[b] dilution.

Treatment with different levels of coated SE-antigens (µg protein/well)	Mean[c] O.D. values of control yolk	Difference between positive & negative O.D. values	
		Positive	Negative
28	1.352	1.167	0.186
56	1.355	1.157	0.198
84	1.293	1.097	0.196
112	1.242	0.862	0.381

a - Conjugate was diluted at 1:1000 in 5% BSA dissolved in PBS-Tween 20. b - Yolk dilution was fixed at 1% in PBS-Tween 20. c - Mean of six replicates.

Table 5 - Individual O.D. values of SE-specific antibodies in egg yolks obtained from SE-challenged[1] and control-unchallenged layers.

Number of eggs	O.D. values from layers	
	SE-challenged	Control-unchallenged
1	0.784	0.523
2	0.769	0.566
3	0.921	0.555
4	0.848	0.390
5	0.712	0.426
6	0.846	0.550
7	0.840	0.623
8	0.911	0.567
9	1.089	-
Mean	0.8578[a]	0.5250[b]

1 - Eggs collected on the 11th day post-challenge from layers challenged in the esophagus with SE at 5.4x1010CFU/ml/bird. a-b - Means in the same row followed by different superscripts are significantly different ($p<0.05$).

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