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Chicken Serologic Response to *Salmonella enterica* Serotype Typhimurium assessed by Elisa

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Chicken, ELISA, enzyme, *Salmonella* sp, serology.

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ABSTRACT

This study evaluated two enzyme-linked immunosorbent assays (ELISA) in the detection of chicken serologic response against *Salmonella enterica* sorotype Typhimurium. The assays have used as detecting antigen the soluble bacterial proteins of a non-flagellated strain of *Salmonella* Typhimurium (AgTM), and antibody conjugated to peroxidase or alkaline phosphatase. According to the results, optimal dilutions of antigen (concentration 5.49 mg/mL) and serum samples in both assays were 1:20,000 and 1:1,000, respectively. In such conditions, the ELISA/AgTM was able to detect serological response to *Salmonella* Typhimurium. Cross-reactions to *Salmonella* serotypes Gallinarum and Pullorum were seen, but not with other serotypes such as Enteritidis.

INTRODUCTION

Breeding flocks must be free of *Salmonella* and early detection is extremely important to prevent the disease and its dissemination (Barrow, 1994). Bacteriological methods are required and have been the traditional means of obtaining such prevention. However, serological methods may be used to detect infection caused by a number of serotypes (Barrow, 1992). After the first enzyme linked immunosorbent assay (ELISA) to detect *Salmonella enterica* subspecies *arizonae* infection has been described in turkeys (Nagaraja *et al.*, 1984, Nagaraja *et al.*, 1986), other reports have indicated the usefulness of different indirect ELISAs to detect circulating antibodies (Barrow, 1991). Since the development of ELISA in the 1980s, they have been increasingly used in poultry flocks in the serological detection of invasive *Salmonella* serotypes associated with human food poisoning, such as *S.* Typhimurium and *S.* Enteritidis (Barrow *et al.*, 1989; Hassan *et al.*, 1990).

Oral infection with invasive serotypes often leads to the production of circulating antibodies, mainly IgG antibodies (Barrow, 1992). The advantages of serology are that *Salmonella* induce the production of high circulating IgG and the titers persist for up to 45 weeks after infection (Hassan *et al.*, 1990; Barrow, 1995). Any degree of cross-reaction between LPS from groups B and D has been demonstrated with sera from chickens infected with *S.* Typhimurium (Barrow, 1992; Barrow *et al.*, 1992). Antigens of variable specificity have been largely used for preliminary screening, including soluble protein antigen produced by sonication of whole cells (Hassan *et al.*, 1990). Few studies have been reported on the use of this antigen in regard to *S.* Typhimurium infections. Barrow *et al.* (1989) examined sera from seven flocks, four of which had bacteriological evidence of *S.* Typhimurium infection. High antibody titers were detected in three flocks by the soluble protein antigen from sonicated whole cells, but in none of the three flocks considered to be non-infected.

The Brazilian poultry industry is based on imported grandparent flocks and the National Health Program establishes that the flocks must be free of *S*. Gallinarum and *S*. Pullorum, whereas *S*. Enteritidis and *S*. Typhimurium must be controlled.

The present research was undertaken to assess an ELISA as a tool to search chicken serological response to *S*. Typhimurium. The ELISA was established using soluble proteins as antigen and antibodies conjugated to either alkaline phosphatase or peroxidase.

MATERIAL AND METHODS

Reference serum samples

Adult laying hens (18 weeks of age) with no signs of clinical salmonellosis and showing negative results in bacteriological inspection and slide agglutination test were given 0.5mL of inactivated cultures of *Salmonella* (10^8CFU/mL) intramuscularly. Serological response was individual for each of the following *Salmonella* serotypes: Enteritidis, Typhimurium, Gallinarum, Pullorum, Infantis, Montevideo, Binza, Livingstone, Anatum, Stanley, Eimsbuettel, Ealing and Virchow. Cultures were prepared in 10mL of Bacto-Brain Heart Infusion Agar and were inactivated with 0.2mL of 40% formaldehyde. Birds were inoculated five times with each *Salmonella* serotype at week intervals. Blood was collected three weeks after the last inoculation and serum samples were stored at -20ºC. Serum samples from SPF chickens negative to *Salmonella* were kindly provided by Dr. Paul A. Barrow (Institute for Animal Health, UK).

ELISA Procedure

1- Peroxidase

The procedure was carried out as described by Hassan *et al*. (1990) and Barrow *et al*. (1992) with some modifications. The soluble protein detecting antigen was prepared by sonication (Branson Sonifer 250 – USA), using 8 cycles of ± 85watts with 30 s-intervals. Washed whole cells of a non-flagellated *S*. Typhimurium strain (10^6CFU/mL) were used. The ELISA included five steps in a humid chamber. Volumes of 50mL were used in all stages, except for blocking (100mL). After the incubation steps, reagents were removed by aspiration and the wells were washed with PBS containing 0.1% Tween 20 (PBST). First, the antigen was diluted in carbonate-bicarbonate buffer pH 9.6 (15mM Na_2CO_3, 35mM $NaHCO_3$, 0.3mM NaN_3) and incubated for 18h at 4ºC in flexible polyvinyl ELISA microplates (Cliniplate – Labsystems - Finland). The blocking buffer was prepared with 10% skimmed milk (Molico, Nestlé, São Paulo, Brazil) in carbonate-bicarbonate buffer. Blocking was performed for 45minutes at 37ºC. Serum samples were diluted in PBST with 10% skimmed milk and added to the plates. It was then added rabbit anti-chicken IgG peroxidase conjugate (Sigma A-9046) diluted to 1:2,000 in PBST. Finally, ortho-phenylenediamine (10mg/mL) (OPD - Sigma P-8287) and 100µL of hydrogen peroxide were diluted in 25mL of citrate phosphate buffer pH 4.9-5.2 (0.1M $C_6H_8O_7$, 0.2M NaHPO). The color reaction was stopped using 2N HCl after 15 min at room temperature. Absorbance was read at 490nm using an ELISA plate reader (Microplate Reader 550, Bio Rad, USA). Each serum sample was tested in duplicate and the mean was calculated for further analysis.

2- Alkaline Phosphatase

The steps in alkaline phosphatase ELISA were similar to those described above for peroxidase ELISA, except for the conjugate and substrate. In this case, it was used a rabbit anti-chicken IgG alkaline phosphatase conjugate (Sigma A-9171) diluted to 1:1,000 in PBST and p-nitrophenyl phosphate (5mg/mL) (pNPP - Sigma N-9389) was used as substrate, diluted in 5mL of diethanolamine buffer pH 9.8 (100mM diethanolamine, 500nM $MgCl_2$). Besides, the color reaction was developed for 30 min at room temperature and then it was stopped with 3M NaOH. Absorbance was read at 405nm.

RESULTS AND DISCUSSION

Salmonella Typhimurium was the most common serotype involved in animal and human foodborne salmonellosis before the outbreaks of *Salmonella* Enteritidis (Barrow, 1992) and it is still frequently isolated (Almeida *et al*., 2000; Zancan *et al*., 2000). Therefore, the first studies to prevent avian salmonellosis based on monitoring programs were focused on searching for serological response to *Salmonella* Typhimurium. Although fecal excretion of enteric *Salmonella* is intermittent, serologic response lasts for several months (Hassan *et al*., 1990) and a serologic test might be useful to demonstrate whether a poultry flock has been or is contaminated by *Salmonella* Typhimurium. The enzyme-linked immunosorbent assay (ELISA) has been used routinely in the poultry industry for monitoring programs of various diseases. Thus, it is not difficult to introduce a new ELISA test for *Salmonella* monitoring. The ELISA can be done using different antigens. Both

LPS and flagellum antigens produce strong reactions. ELISA using LPS as antigen detects IgG produced against LPS in birds infected by *Salmonella* that belong to serogroups B and D (Chart *et al.*, 1990), due to the common antigen O12. Serologic response to flagellum antigen is less persistent and might be detected up to four months (Hassan *et al.*, 1990; Baay & Huis in¢t Veld, 1993). The ELISA using soluble proteins of *Salmonella* Typhimirium as antigen was similar to the assay prepared with LPS as antigen. The results were very comparable; however, soluble protein antigen preparation is less laborious (Hassan *et al.*, 1990; Barrow *et al.*, 1992). According to the present results (Table 1), peroxidase and alkaline phosphatase assays should be performed using the antigen diluted to 1:20,000 (5.49 mg/mL) for both conjugates and the serum sample diluted to 1:1,000. Positivity was considered when readings were higher than, or equal to, 1.0 (OD≥1.00). In this condition the test is suitable to detect serologic response to *Salmonella* Typhimurium. Cross-reaction was observed between *S.* Typhimurium and the serotypes Gallinarum and Pullorum, but not with *S.* Enteritidis whichever conjugate was used. Barrow (1991) and Barrow (1992) reported no cross-reaction with other enterobacteria (*Escherichia coli*, *Klebsiella*, *Proteus* and *Citrobacter*), corroborating our results. Cross-reactions with *Salmonella* serotypes from groups B and D should be of interest, since *Salmonella* of these groups have been isolated from poultry and are of concern to public health (Zancan *et al.*, 2000, Gama *et al.*, 2003; Ribeiro *et al.*, 2003). Therefore, ELISA/AgTM is suitable to indicate evidences of avian salmonellosis and may be used as a screening test for further bacteriological examination.

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Table 1 - Enzyme-linked immunosorbent assays (ELISA/AgTM)[1]. Determination of single dilution of chicken serum to differentiate serological response to *Salmonella* Typhimurium from other *Salmonella* serotypes.

Positive sera to *Salmonella* serotypes	Peroxidase conjugate								Alkaline phosphatase conjugate							
	Reciprocal dilution of sample of sera															
	500	1000	2000	4000	8000	16000	32000	64000	500	1000	2000	4000	8000	16000	32000	64000
Gallinarum (D_1)	1.136*	1.035	0.527	0.428	0.284	0.236	0.156	0.099	1.148	1.124	0.782	0.529	0.432	0.319	0.186	0.136
Ealing (O)	0.092	0.068	0.056	0.051	0.054	0.058	0.067	0.065	0.119	0.084	0.070	0.063	0.070	0.078	0.068	0.061
Livingstone (C_1)	0.141	0.085	0.065	0.054	0.055	0.056	0.062	0.063	0.144	0.088	0.074	0.057	0.086	0.074	0.074	0.059
Anatum (E_1)	0.354	0.199	0.112	0.077	0.065	0.058	0.060	0.062	0.229	0.132	0.098	0.065	0.071	0.068	0.060	0.062
Stanley (B)	0.350	0.195	0.119	0.086	0.069	0.059	0.057	0.061	0.328	0.213	0.142	0.112	0.088	0.076	0.068	0.052
Infantis (C_1)	0.263	0.138	0.086	0.076	0.056	0.053	0.065	0.062	0.246	0.173	0.135	0.100	0.083	0.077	0.063	0.061
Eimsbuettel (C_4)	0.165	0.080	0.061	0.051	0.050	0.048	0.057	0.064	0.191	0.124	0.104	0.083	0.074	0.075	0.061	0.059
Virchow (C_1)	0.189	0.102	0.066	0.054	0.051	0.048	0.056	0.065	0.129	0.095	0.090	0.076	0.076	0.073	0.059	0.057
Binza (E_2)	0.401	0.194	0.112	0.081	0.060	0.052	0.052	0.056	0.257	0.173	0.128	0.099	0.086	0.073	0.061	0.059
Typhimurium (B)	1.231	1.183	0.575	0.356	0.284	0.205	0.130	0.092	1.298	1.018	0.630	0.424	0.268	0.172	0.116	0.085
Enteritidis (D_1)	0.627	0.422	0.290	0.234	0.195	0.080	0.064	0.061	0.285	0.231	0.231	0.167	0.114	0.096	0.085	0.061
Pullorum (D_1)	1.197	1.155	0.490	0.371	0.332	0.218	0.198	0.132	1.927	1.184	0.725	0.498	0.481	0.338	0.234	0.146
Montevideo (C_1)	0.110	0.078	0.055	0.049	0.047	0.052	0.069	0.060	0.154	0.123	0.098	0.083	0.090	0.075	0.062	0.059
Negative serum	0.038	0.035	0.035	0.035	0.038	0.037	0.040	0.041	0.056	0.054	0.059	0.054	0.062	0.060	0.055	0.055

1- AgTM diluted to 1:20,000; *Positive samples: OD ≥ 1.0.

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