

Assessment of Serological Response of Chickens to *Salmonella Gallinarum* and *Salmonella Pullorum* by Elisa

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ABSTRACT

This study was done to assess an enzyme-linked immunosorbent assay (ELISA) to detect chicken serological response against *Salmonella enterica* serotypes Gallinarum and Pullorum. The assays have used soluble bacterial proteins of *Salmonella* Gallinarum strain 9 (AgSG) as detecting antigen and peroxidase and alkaline phosphatase conjugates. According to the results, the antigen, sera and conjugate concentrations were optimized. In addition, the assay using alkaline phosphatase and peroxidase conjugates was helpful to distinguish positive serological reaction to serotypes Gallinarum and Pullorum from Enteritidis.

INTRODUCTION

Following initial studies on the value of the enzyme-linked immunosorbent assay (ELISA) in detecting *Salmonella enterica* subsp *arizonae* in turkeys (Nagaraja *et al.*, 1984, 1986), a number of reports have indicated the value of different indirect ELISA's in the detection of circulating antibodies against the most important *Salmonella* serotypes in poultry, namely Typhimurium (Barrow *et al.*, 1989; Dadrast *et al.*, 1990), Enteritidis (Dadrast *et al.*, 1990; Cooper *et al.*, 1989; Nicholas & Cullen, 1991), Gallinarum and Pullorum (Iba *et al.*, 1991).

Because high concentrations of circulating IgG persist for several months following infection with *Salmonella* (Hassan *et al.*, 1990; Nicholas & Cullen, 1991), the ELISA can overcome the problem of infection detection due to the intermittent faecal excretion of *Salmonella*. IgM and IgA may also be detected, but such classes of antibody do not persist to the same extent as IgG (Hassan *et al.*, 1990, 1991). Because of the persistence of IgG, ELISA's based on this class of antibody have presently a greatest practical potential to indicate the infection status of the poultry flock.

The ELISA based on IgG detection has also some advantages over established serological tests. It has been shown to be more sensitive than both the rapid slide agglutination test, which relies on the use of undiluted serum, and standard tube agglutination; besides, it is at least as sensitive as the microantiglobulin test (Cooper *et al.*, 1989; Nicholas & Cullen, 1991). Unlike the slide agglutination test, developed originally to be used for infections caused by serotypes Pullorum and Gallinarum, ELISAs can also be used to examine eggs, an important aspect to the poultry breeding stock, table egg and food industries (Barrow & Lovell, 1991; Nicholas *et al.*, 1990). The ability of the ELISA to differentiate infections caused by *Salmonella* serotypes has been demonstrated by using several detecting antigens prepared from different serotypes, such as LPS (Cooper *et al.*, 1989; Dadrast *et al.*, 1990; Nicholas & Cullen, 1991) or flagella (Hassan *et al.*, 1990). The ELISA can also be used with serum eluted from blood dried onto absorbent paper, facilitating field sampling (Hassan *et al.*, 1990; Minga & Wray, 1990).



Recent studies have shown the value of ELISA in studying the serological responses of chickens to experimental and natural infection with invasive *Salmonella* including Typhimurium (Hassan *et al.*, 1990), Enteritidis (Nicholas & Cullen, 1991), and Gallinarum-Pullorum serotypes (Barrow *et al.*, 1992; Iba *et al.*, 1991) and has also indicated their value in monitoring field infections such as those with *S. Enteritidis* (Barrow, 1994b) and *S. Gallinarum* and *S. Pullorum* (Berchieri Jr *et al.*, 1995). Most of these assays have used soluble proteins, LPS or flagella as detecting antigens. Barrow *et al.* (1992) showed that either soluble protein or LPS could be used to study experimental infections. The soluble protein was also used successfully in a small field study in which high optical densities were obtained in ELISA evaluation of sera from showing signs of fowl typhoid, but not from uninfected flocks. Afterwards, a larger survey was carried out showing the reliability of the test (Berchieri Jr *et al.*, 1995).

The most effective means of controlling pullorum disease and fowl typhoid is a combination of stringent management procedures and eradication (Snoeyenbos, 1991). Eradication is normally done by identifying infected flocks and eliminating individual reactor birds detected by a serological test. Serology is used because *S. Gallinarum* and *S. Pullorum* are not excreted extensively in the feces, unlike many other *Salmonella* serotypes that are more frequently associated with human food poisoning. For many years, the choice has been the slide agglutination test, which was originally developed by Runnells *et al.* (1927) to test serum and was later adapted by Schaffer *et al.* (1931) to test whole blood using a stained antigen. The test is easy to use and has largely been used as a flock test and helped to eliminate these infections in many countries. However, the test can yield erratic results, which can be dependent on antigen quality. Quantification is also difficult and the test is not applicable to be used with eggs; such a test would be useful for screening breeding flocks (Barrow *et al.*, 1992).

The Brazilian poultry industry is based on the importation of grandparent flocks and the official program of the Government establishes that the flocks must be free of *S. Gallinarum* and *S. Pullorum* and controlled for *S. Enteritidis* and *S. Typhimurium*. In many countries, *S. Enteritidis* was disseminated on poultry farms through vertical transmission (Baumler *et al.*, 2000). In Brazil, dissemination was related to *Salmonella* isolation from imported ostrich flocks (Ribeiro *et al.*, 2003). Environmental conditions facilitated the spreading of these serotypes. In view of the difficulties

in controlling dissemination of *S. Enteritidis*, many commercial flocks of laying hens are vaccinated against it and there is a strong tendency to extend the vaccination program to breeding flocks (Scharr, 2003). In this case, the traditional pullorum test using whole blood will lose be of no value. Studies from Iba *et al.* (1991) and Berchieri Jr. *et al.* (1995) showed that the ELISA using plates coated with soluble protein as antigen and alkaline phosphatase conjugate was able to detect serological response to *S. Pullorum* and *S. Gallinarum*. In addition, the test was supposed to separate positive results of *S. Gallinarum* and *S. Pullorum* from *S. Enteritidis*. The present work was undertaken to assess the ELISA as a tool to search serological response to *S. Gallinarum* and *S. Pullorum*, and also to separate positive reactions of infections caused by *S. Gallinarum* and *S. Pullorum* from *S. Enteritidis*. The ELISA was established using soluble proteins and either alkaline phosphatase or peroxidase conjugates.

MATERIAL AND METHODS

Samples of serum

Reference sera

Adult chickens with no signs of clinical salmonellosis were inoculated intra-muscularly with 0.5mL of inactivated cultures of *Salmonella* (10⁸cfu/mL) to obtain serological response to individual *Salmonella* serotypes. The *Salmonella* serotypes included Enteritidis, Typhimurium, Gallinarum, Pullorum, Infantis, Montevideo, Binza, Livingstone, Anatum, Stanley, Eimsbuettel, Ealing and Virchow. The 10mL broth cultures (Bacto-Brain Heart Infusion Agar - BHI) were inactivated by the addition of 0.2mL of 40% formaldehyde. Negative serum samples to *Salmonella* was also obtained from SPF chickens maintained by Institute for Animal Health, UK. All serum samples were stored at -20°C.

Test sera

Samples of serum from flocks naturally and experimentally infected with 1mL broth culture of *S. Gallinarum* (10⁸cfu/mL) or *S. Pullorum* (10⁸cfu/mL) were tested. In addition, the serological response of layers vaccinated and vaccinated/challenged against *S. Enteritidis* was analyzed.

ELISA Procedure

Peroxidase

The ELISA was conducted as described by Hassan *et al.* (1990) and Barrow *et al.* (1992) with some



modifications. Washed whole cells of a virulent *S. Gallinarum*, strain 9 NaI^r (10⁸cfu/mL) (Barrow *et al.*, 1992), were used to prepare the soluble protein detecting antigen using 8 cycles of sonication (Branson Sonifer 250, USA) at 85 Watts and 30-second intervals. Flexible polyvinyl ELISA microplates (Cliniplate Labsystems, Finland) were used. The assay included five steps, using 50mL of the appropriate reagent in four steps and 100mL in one. After each step, reagents were removed by aspiration, and wells were washed with PBS containing 0.1% Tween 20 (PBST). Firstly, the antigen was diluted in carbonate-bicarbonate buffer pH 9.6 (15mM Na₂CO₃, 35mM NaHCO₃, 0.3mM NaN₃), added to the wells and incubated for 18h/4°C. In the second step, 100mL carbonate-bicarbonate buffer pH 9.6 with 10% skimmed milk (Molico Nestlé, Brazil) was added to each well for 45min/37°C. Test serum samples were diluted in PBST with 10% skimmed milk and added to the plate. Afterwards, it was added in each well rabbit anti-chicken IgG peroxidase conjugate (Sigma, A-9046) diluted 1:2,000 in PBST. Finally, the substrate orthophenylenediamine (10mg/mL) (OPD Sigma, P-8287) was diluted in 25mL of citrate phosphate buffer pH 4.9-5.2 (0.1M C₆H₈O₇, 0.2M NaHPO) and 100mL of hydrogen peroxide. The substrate was then added to the wells and the microplate was incubated for 15 minutes at room temperature. The reaction was stopped by addition of 2N chloride acid (HCl). Absorbance values were read at 490nm using an automatic ELISA reader (Microplate Reader model 550, Bio Rad). Each serum sample was tested in duplicate and the mean was calculated and used in further analysis. All incubations were done in a moist chamber.

Alkaline Phosphatase

The steps of the alkaline phosphatase ELISA were the same as described above for peroxidase ELISA, except for two alterations. In this case, a rabbit anti-chicken IgG alkaline phosphatase conjugate (Sigma A-9171) was diluted 1:1,000 in PBST and the substrate *p*-nitrophenylphosphate (5mg/mL) (pNPP - Sigma N-9389) was diluted in 5mL of diethanolamine buffer pH 9.8 (100mM diethanolamine, 500mM MgCl₂). After the substrate was added to the wells, the microplate was incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 3M sodium hydroxide and absorbance values were read at 405nm.

RESULTS

According to the results, the test should be performed using the antigen diluted at 1:25,000

(5.67mg/mL) for both conjugates and the serum sample diluted at 1:1,000. Results were considered positive when OD=0.800. In this condition, the test is suitable to detect serological response to *S. Gallinarum* and *S. Pullorum*. Additionally, the assay performed with alkaline phosphatase and peroxidase conjugate is able to separate positive reactions to *S. Gallinarum* and *S. Pullorum* from other serotypes, including positive reaction to *S. Enteritidis* (Tables 1 and 2).

DISCUSSION

Unlike avian parathyphic *Salmonella* serotypes, *Salmonella enterica* serotypes Gallinarum and Pullorum are not frequently excreted in chicken feces, but infected birds tend to produce humoral response (Barrow *et al.*, 1992). Breeding flocks must be free of *Salmonella* serotypes Gallinarum and Pullorum, therefore, it is very important to detect them as soon as possible to prevent both disease and dissemination. Among other measures, the pullorum test has been the method of choice to detect birds infected with both serotypes. Besides being a very simple method, since it uses whole blood sample, this test has also been very useful to control and eradicate *S. Pullorum* (Barrow, 1994b). Currently, farmers are allowed to vaccinate breeding flocks against *Salmonella enterica* serotype Enteritidis in many areas in the world, including Brazil. Since they have similar somatic antigens, cross-reactions may occur. Therefore, another serological test would be interesting for flock assessment instead of using only bacteriological examination.

According to Cooper *et al.* (1989), the ELISA is effective to detect serological response in chickens against *Salmonella* Enteritidis and Hassan *et al.* (1990) demonstrated that the ELISA could be performed using soluble protein antigen. This study assessed the ability of an immunoenzymatic assay performed with either peroxidase or alkaline phosphatase conjugates to investigate serological response to a soluble protein antigen from *Salmonella* Gallinarum 9 strain. The antigen was capable to promote reliable serological reaction as suggested by Iba *et al.* (1991) and Barrow *et al.* (1992).

The antigen diluted at 1:25,000 was able to detect IgG in serum samples diluted 1:1,000. According to Barrow (1995), IgG was produced in chickens infected with invasive serotypes like Gallinarum, Pullorum, Typhimurium and Enteritidis and persisted longer than the others immunoglobulins. Thus, the test might be useful for monitoring programs since *S. Gallinarum* and



Table 1 - Enzyme-linked immunosorbent assay (ELISA/AgSG)¹. Determination of single dilution of chicken serum to differentiate serological response to *S. Gallinarum* and *S. Pullorum* from other *Salmonella* serotypes.

Positive sera to <i>Salmonella</i> serotypes	Reciprocal dilution of sample of sera															
	Peroxidase conjugate (serotypes)								Alkaline phosphatase conjugate							
	500	1000	2000	4000	8000	16000	32000	64000	500	1000	2000	4000	8000	16000	32000	64000
Gallinarum (D ₁)	0.273	0.855	0.742	0.738	0.607	0.455	0.338	0.205	1.440	1.285	1.237	0.871	0.732	0.496	0.329	0.254
Ealing (O)	0.066	0.077	0.069	0.062	0.071	0.069	0.076	0.080	0.126	0.093	0.083	0.079	0.082	0.086	0.084	0.075
Livingstone (C ₁)	0.064	0.082	0.069	0.061	0.067	0.067	0.069	0.082	0.184	0.114	0.092	0.083	0.087	0.085	0.091	0.082
Anatum (E ₁)	0.140	0.230	0.138	0.107	0.094	0.207	0.077	0.091	0.272	0.148	0.128	0.099	0.098	0.091	0.086	0.081
Stanley (B)	0.059	0.122	0.084	0.070	0.070	0.053	0.064	0.076	0.206	0.132	0.095	0.091	0.086	0.086	0.082	0.079
Infantis (C ₁)	0.259	0.182	0.117	0.080	0.074	0.067	0.066	0.071	0.448	0.286	0.201	0.131	0.110	0.099	0.086	0.076
Eimbuettel (C ₄)	0.172	0.091	0.069	0.059	0.056	0.061	0.067	0.071	0.263	0.166	0.112	0.097	0.095	0.147	0.084	0.081
Virchow (C ₁)	0.147	0.093	0.063	0.054	0.067	0.063	0.065	0.075	0.214	0.137	0.102	0.091	0.090	0.086	0.093	0.082
Binza (E ₂)	0.173	0.084	0.059	0.053	0.055	0.059	0.067	0.074	0.204	0.123	0.103	0.086	0.092	0.090	0.082	0.079
Typhimurium (B)	0.304	0.114	0.084	0.073	0.073	0.081	0.067	0.080	0.462	0.259	0.160	0.127	0.101	0.092	0.089	0.085
Enteritidis (D ₁)	0.809	0.496	0.514	0.317	0.209	0.141	0.106	0.093	0.292	0.238	0.196	0.158	0.120	0.112	0.090	0.089
Pullorum (D ₁)	1.077	1.152	1.055	1.222	1.113	1.127	0.969	0.784	1.524	1.552	1.425	1.362	1.431	1.228	1.078	0.844
Montevideo (C ₁)	0.105	0.073	0.067	0.055	0.064	0.065	0.068	0.073	0.177	0.114	0.097	0.083	0.083	0.084	0.083	0.078
Negative serum	0.043	0.045	0.043	0.045	0.051	0.051	0.058	0.054	0.067	0.069	0.070	0.073	0.076	0.076	0.071	0.073

1 - AgSG diluted at 1:25,000.

Table 2 - Enzyme-linked immunosorbent assays (ELISA/AgSG). Analysis of serum samples from chickens infected or vaccinated with *S. Gallinarum*, *S. Pullorum* and *S. Enteritidis*.

Conjugate	Positive serum samples to <i>Salmonella</i> serotype		
	Gallinarum ¹	Pullorum ²	Enteritidis ³
Peroxidase	41/41	28/28	0/46 ⁴
Alkaline phosphatase	50/50	41/41	0/46

1 - Serum samples from chickens naturally and experimentally infected with *S. Gallinarum* NaI9; 2 - Serum samples from chickens experimentally infected with *S. Pullorum*; 3 - Serum samples from chickens vaccinated and vaccinated/challenged with *Salmonella* Enteritidis; 4 - positive serum by ELISA/AgSE.

S. Pullorum are not often present in feces. In these conditions, ELISA can be performed with peroxidase or alkaline phosphatase conjugates. However, the test was more specific when using the alkaline phosphatase conjugate (Table 1). Therefore, the alkaline phosphatase ELISA can be used as a serological test for monitoring poultry flocks to search for *Salmonella* specific IgG and could be a useful to examine serum samples, egg yolk and dried blood from any kind of poultry flock (Barrow, 1994a).

In order to specifically detect pullorum disease and fowl typhoid, it would be essential to serologically differentiate these diseases from infections caused by other invasive *Salmonella* serotypes, including Typhimurium and Enteritidis, and some other serotypes in group "D", which might induce cross-reacting circulating IgG following oral infection (Hassan *et al.*, 1990; Barrow *et al.*, 1992). The differentiation between serotype infections was assessed in this work. The test

using either peroxidase conjugate or alkaline phosphatase conjugate was able to detect humoral response to *S. Gallinarum* and *S. Pullorum*. Some intermediary reactions were seen when the ELISA was performed with peroxidase conjugate. However, in such cases, optical density was lower than 0.500. In addition, when it was tested serum samples from commercial flocks infected with *S. Enteritidis* or vaccinated with *S. Enteritidis* bacterin, the peroxidase ELISA did not indicate positive reactions. Despite that serum samples which were positive to *S. Enteritidis* showed OD=0.496 in the ELISA performed with peroxidase conjugate, this test was able to separate positive reactions between *S. Gallinarum* and *S. Pullorum* from *S. Enteritidis*, such as the alkaline phosphatase ELISA. On the other hand, positive results in the peroxidase test exhibited a very strong color, which could be detected with no need of a microplate reader. For this reason, in view of the lack of agreement among results of the experimental serum samples and field serum samples (Table 2), the peroxidase ELISA could be used in monitoring programs in which no discrimination between *S. Gallinarum*, *S. Pullorum*, *S. Enteritidis* and *S. Typhimurium* is needed (Barrow, 1994a). Under experimental conditions, peroxidase and alkaline phosphatase ELISAs were able to identify reactor birds.

Additional work was carried out with serum samples from naturally and experimentally flocks infected with *S. Gallinarum* and *S. Pullorum*. The results corroborated previous researches in which ELISA was useful to detect birds with positive serological response to the agents of fowl typhoid and pullorum disease (Iba *et al.*, 1991; Barrow *et al.*, 1992; Berchieri Jr. *et al.*, 1995).



According to the results of this research, ELISAs with *S. Gallinarum* as soluble antigen and peroxidase or alkaline phosphatase conjugates were able to screen poultry flocks for serological response to *S. Gallinarum* and *S. Pullorum*. The test avoided cross-reaction with serological response to *S. Enteritidis* and *S. Typhimurium*.

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