ENSAIO IMUNOENZIMÁTICO (ELISA) PARA DETECÇÃO DA RESPOSTA SOROLÓGICA CONTRA *Salmonella enterica* sorotipo Enteritidis EM AVES

Assessment of serologic response of chickens to salmonella enterica serotype enteritidis by Elisa

Gláucia Helaine de Oliveira[1], Ângelo Berchieri Júnior[1], Hélio José Montassier[1]

RESUMO

O presente trabalho foi realizado para analisar a utilização de um Ensaio Imunoenzimático (ELISA) para detecção de resposta sorológica em aves contra *Salmonella* Enteritidis. Para a realização do teste utilizou-se um antígeno que consistia de proteína solúvel obtida após sonicação de cultura de *Salmonella* Enteritidis cepa aflagelar (AgSE – conc. 7,59mg/mL), os conjugados peroxidase e fosfatase alcalina e amostras de soros positivos e negativos para vários sorotipos de *Salmonella*.

Os resultados demonstraram que o antígeno pode ser utilizado diluído a 1:10.000 (AgSE + peroxidase) e 1:5.000 (AgSE + fosfatase alcalina) e as amostras de soros a serem analisadas deverão ser diluídas a 1:1.000 para ambos os conjugados. Nestas condições, o ELISA/AgSE foi capaz de detectar resposta sorológica para *S.* Enteritidis, *S.* Gallinarum, *S.* Pullorum e resposta sorológica em soros provenientes de aves vacinadas contra *S.* Enteritidis.

Palavras-chave: Conjugado; aves; ELISA; *Salmonella* sp.; sorologia.

ABSTRACT

This study was done to assess the enzyme-linked immunosorbent assay (ELISA) for detection chicken serologic response against *Salmonella enterica* serovar Enteritidis. The test was performed using soluble bacterial proteins of *Salmonella* Enteritidis non flagellated strain (AgSE – conc. 7,59mg/mL) as detecting antigen or peroxidase and alkaline phosphatase enzymes. According to the results, the antigen was diluted at 1:5,000 (alkaline phosphatase enzyme) or 1:10,000 (peroxidase enzyme) and the sample of sera at 1:1,000. In this condition the ELISA/AgSE was able to detect serological response to *S.* Enteritidis, *S.* Gallinarum, *S.* Pullorum and serologic response from a flock vaccinated against *Salmonella* Enteritidis with a bacterin.

Keywords: Conjugate; chickens; ELISA; *Salmonella* sp; serology.

INTRODUCTION

The public and professional interest in *Salmonella* Enteritidis and other salmonellas has created an incentive to produce accurate data on the prevalence of *Salmonella*-infected flocks and of *Salmonella*-infected chickens within flocks. Bacteriological methods are required and have been the traditional means of obtaining such data. However, serological methods can be used to detect infection with a number of serotypes. Oral infection with invasive serotypes, such as Enteritidis, Typhimurium, Pullorum and Gallinarum often leads to the production of circulating antibody mainly of IgG class (BARROW, 1992a).

The ELISA for avian salmonellas is indirect, detecting primarily specific IgG in serum or IgG in egg yolk, although the assay can also be adapted to detect specific IgM produced early in infected birds (HASSAN et al., 1990). However, the nature of the IgM response following experimental *Salmonella* infection is unclear. Under field condition IgM was not detected in natural *Salmonella* Enteritidis infected flock, although the time of infection was unknown (CHART et al., 1990). Experimental infection of chickens with other members of the Enterobactereaceae does not induce the production of high titres of cross-reacting antibodies (NICHOLAS; CULLEN, 1991; BARROW et al., 1992; BARROW, 1992b). Organisms tested included avian patho-

[1] Department of Animal Pathology, FCAV, UNESP, Jaboticabal, SP, Brazil. Corresponding author: Via de Acesso Prof Paulo Donato Castellane s/n, 14884-900 – Jaboticabal – SP – Brasil, berchier@fcav.unesp.br, Financially supported by: FAPESP e CNPq.

genic *Escherichia coli* serotypes, and strains of *Citrobacter*, *Klebsiella* and *Proteus*.

The Brazilian poultry industry is based on the importation of grandparent flocks and the official program by the Government establish that the breed flocks must be free of *Salmonella* Gallinarum and *Salmonella* Pullorum and controlled for *Salmonella* Enteritidis and *Salmonella* Typhimurium. In many countries, *Salmonella* Enteritidis was disseminated in poultry farms through vertical transmission (BAUMLER et al., 2000). In Brazil this was related to the imported grandparent flocks (RIBEIRO et al., 2003). The environmental conditions facilitated the spread of this serotype. *S.* Enteritidis does not usually kill birds after two weeks of age but can induce a chronic carrier state (NICHOLAS; CULLEN, 1991). Bacteriological techniques for the isolation of *Salmonella* from clinical and environmental samples are laborious and lengthy. They may not identify all *S.* Enteritidis infected flocks because of the intermittent nature of *Salmonella* excretion and the number of samples that can be processed (WILLIAMS; WHITTEMORE, 1976) while IgG against *S.* Enteritidis last for several months (BARROW, 1994). Samples of serum are taken regularly on poultry farm for serological tests. Thus, the ELISA could be adopted with no any additional management of the birds. The present work was undertaken to assess the ELISA to search serological response to *Salmonella* Enteritidis.

MATERIALS AND METHODS

Samples of serum

1 – Control serum

Adult chickens (18 weeks of age) with no signal of clinical salmonellosis were administered intramuscularly with 0.5mL of inactivated cultures of *Salmonella* (10^8cfu/mL) to obtain serological response to several *Salmonella* serotypes, individually. The *Salmonella* serotypes included were Enteritidis, Typhimurium, Gallinarum, Pullorum, Infantis, Montevideo, Binza, Livingstone, Anatum, Stanley, Eimsbuettel, Ealing and Virchow. The 10mL broth (Bacto-Brain Heart Infusion broth - BHI) cultures were inactivated by adding 0.2mL of a 40% formaldehyde solution before using. The samples of serum were stored at -20ºC. Negative serum samples to *Salmonella* obtained from SPF chickens were provided by Dr. Barrow from Institute for Animal Health, UK.

2 – Additional work

Additional work was carried out with samples of sera from a flock vaccinated against *Salmonella* Enteritidis with a bacterin when it was 17 weeks of age. At any time was collected 24 samples. The samples were taken when the birds were 8 weeks of age (before vaccination), 19 and 42 weeks of age.

ELISA Procedure

1 – Peroxidase

The ELISA 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), with 8 cycles of ± 85watts with 30 seconds of intervals between them. It was used a washed whole cells of *S.* Enteritidis, strain not flagellate (10^6cfu/mL). Flexible polyvinyl microplate ELISA (Cliniplate – Labsystems - Finland) were used. The ELISA included five stages, using 50mL of the appropriate reagent in four stages and one using 100mL. After each stage, the reagents were removed by aspiration, and the wells were washed with PBS containing 0.1% Tween 20 (PBST). First, the antigen diluted in carbonate-bicarbonate buffer pH 9.6 (15mM Na_2CO_3, 35mM $NaHCO_3$, 0,3mM NaN_3) was added into the wells and incubated for 18h/4ºC. Next, 100mL carbonate-bicarbonate buffer pH 9.6 with low fat milk powder 10% (Molico, Nestlé, São Paulo, Brazil) was added to each well for 45minutes at 37ºC. The test serum sample was then added, diluted in PBST with low fat milk powder 10%. After that it was added in each well rabbit anti-chicken IgG peroxidase conjugate (Sigma A-9046) diluted at 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 $C_6H_8O_7$, 0,2M NaHPO) plus 100ìL of hydrogen peroxide. After that the substrate was poured into 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 micro ELISA reader (Microplate Reader 550 Bio Rad). Each serum sample was tested in duplicate and a mean of two values were taken for further analysis. All incubations were done in a moist chamber.

2 – Alkaline Phosphatase

The steps of the alkaline phosphatase ELISA were the same described above to peroxidase ELISA. In this case, the rabbit anti-chicken IgG alkaline phosphatase conjugate (Sigma A-9171) was diluted at 1:1,000 in PBST and the p-nitrophenil phosphatase

(5mg/mL) (pNPP – Sigma N-9389) substrate was diluted in 5mL of diethanolamine buffer pH 9.8 (100mM diethanolamine, 500nM $MgCl_2$). After that the substrate was poured into the wells and the microplate was incubated for 30 minutes at room temperature. The reaction was stopped by addition of 3M sodium hydroxide. Absorbance values were read at 405nm.

RESULTS AND DISCUSSION

The ELISA has been a very useful tool to monitoring *Salmonella* infection in poultry flocks since the faecal excretion is not constant while the serological response persists for several months (BARROW; LOVELL, 1991). Various components of the *Salmonella* are used as antigen in ELISA (COOPER et al., 1989; CHART et al., 1990). Using soluble protein as the antigen for ELISA, Hassan et al. (1990) demonstrated that birds presented serological response to *Salmonella* in the absence of fecal excretion. IgG lasts longer than other immuneglobulins, although the infection by *Salmonella* Enteritidis promotes detectable antibody response to all class of Ig (WITHANAGE et al., 1999). The results of ELISA should be performed using the antigen (conc. - 7,59mg/mL) diluted at 1:10,000 for the peroxidase enzyme and at 1:5,000 for the alkaline phosphatase enzyme and the serum sample diluted at 1:1,000 for both enzymes. Results were considered positive when ODe”1.00 (Table 1). In this condition, the test is appropriate to detect serological response to serotypes Enteritidis, Pullorum and Gallinarum to both conjugated.

The test cannot separate positive reaction to IgG against *S*. Pullorum and *S*. Gallinarum, but differently than the previous works, the ELISA did not show cross-reaction with samples of sera contained IgG against group B *Salmonella* serotypes (MINGA; WRAY, 1990; NICHOLAS; CULLEN, 1991; BARROW et al., 1992). Some tests exhibited OD(s) between 0.400 and 1.000. At field condition, they should be considered suspect. At the beginning of the infection the serological response is weak and increases with the progress of the infection. Thus, test done again few weeks later can eliminate cross-reaction. In addition, the detection of OD between 0.400 and 1.000 suggests the presence of any other serotype, which also is of interest to animal and public health (BARROW, 1994), like Group B *Salmonella* serotype Heidelberg which has been found in poultry breed flocks (ZANCAN et al., 2000).

Similarly, the ELISA was able to detect positive reaction in birds vaccinated against *Salmonella* Enteritidis (Table 2) with both conjugated. The examination of serum samples from vaccinated flock detected at least 6 positive samples in 24 tested (25%) to peroxidase enzyme and 18 (75%) positive samples to alkaline phosphatase enzyme. There is no information available to understand why not all samples yield positive results. However, it shows that the ELISA was able to detect reactors birds. Both enzyme assays were useful. Nevertheless, alkaline phosphatase ELISA was more efficient in any occasion. In conclusion, the ELISA using soluble protein from the *S.* Enteritidis may be a useful tool for monitoring program of *Salmonella* Enteritidis infection in chickens. Nevertheless, additional work at field condition might be done to set it up.

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

Positive sera to	Peroxidase conjugate								Alkaline phosphatase conjugate							
Salmonella	Reciprocal dilution of serum sample															
serotypes	500	1000	2000	4000	8000	16000	32000	64000	500	1000	2000	4000	8000	16000	32000	64000
Gallinarum (D_1)	1.605*	1.629	1.488	1.509	1.258	0.975	0.504	0.411	2.699	2.534	2.503	2.077	1.765	1.327	0.932	0.628
Ealing (O)	0.212	0.153	0.083	0.070	0.064	0.057	0.037	0.037	0.233	0.133	0.099	0.080	0.076	0.084	0.083	0.083
Livingstone (C_1)	0.292	0.145	0.117	0.087	0.068	0.068	0.043	0.041	0.309	0.171	0.118	0.091	0.084	0.084	0.095	0.081
Anatum (E_1)	0.978	0.627	0.340	0.223	0.147	0.116	0.069	0.053	0.682	0.349	0.248	0.135	0.114	0.108	0.101	0.094
Stanley (B)	0.719	0.413	0.237	0.161	0.115	0.087	0.061	0.042	0.653	0.397	0.235	0.163	0.126	0.110	0.094	0.090
Infantis (C_1)	1.057	0.653	0.429	0.245	0.144	0.105	0.067	0.035	1.348	0.840	0.559	0.365	0.229	0.163	0.119	0.100
Eimsbuettel (C_4)	0.558	0.276	0.181	0.131	0.092	0.079	0.064	0.041	0.597	0.353	0.204	0.140	0.117	0.102	0.088	0.086
Virchow (C_1)	0.513	0.261	0.178	0.114	0.089	0.076	0.066	0.046	0.623	0.279	0.185	0.129	0.113	0.103	0.091	0.086
Binza (E_2)	0.445	0.239	0.138	0.098	0.081	0.073	0.064	0.045	0.400	0.196	0.136	0.099	0.091	0.087	0.075	0.078
Typhimurium (B)	0.884	0.559	0.363	0.238	0.145	0.113	0.078	0.047	0.935	0.401	0.214	0.153	0.122	0.106	0.085	0.085
Enteritidis (D_1)	1.289	1.113	0.699	0.503	0.298	0.187	0.112	0.056	1.598	1.250	0.784	0.550	0.338	0.234	0.154	0.124
Pullorum (D_1)	1.815	1.865	1.774	1.770	1.774	1.612	1.443	1.141	2.890	2.815	2.843	2.624	2.312	2.004	1.602	1.175
Montevideo (C_1)	0.317	0.170	0.122	0.089	0.077	0.067	0.059	0.042	0.486	0.257	0.169	0.129	0.104	0.098	0.088	0.086
Negative serum	0.046	0.045	0.046	0.050	0.049	0.047	0.039	0.025	0.077	0.069	0.068	0.065	0.069	0.071	0.067	0.040

[1] AgSE diluted at 1:10,000 (peroxidase conjugate) and 1:5,000 (alkaline phosphatase conjugate).

* OD values.

Table 2. Enzyme-linked immunosorbent assays (ELISA/AgSE)* with samples of sera from a flock vaccinated against *Salmonella* Enteritidis with a bacterin.

Age of chickens	Conjugates					
	Peroxidase			Alkaline phosphatase		
	1**	2**	3**	1	2	3
8 weeks	0/24	0/24	24/24	0/24	0/24	24/24
19 weeks	6/24	14/24	4/24	18/24	6/24	0/24
42 weeks	8/24	2/24	14/24	9/24	9/24	6/24

* AgSE diluted at 1:10,000 (peroxidase conjugate) and 1:5,000 (alkaline phosphatase conjugate).

** 1= positive (OD³1.000); 2= suspect (0.400£OD£1.000); 3= negative (OD£0.400)