

## Generation and Characterization of IgY antibodies from Lohmann Brown Hens Immunized with *Salmonella spp.* for their Subsequent Application in Nanotherapy

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### Abstract

Salmonellosis is a common type of food poisoning caused by the *Salmonella enterica* bacterium. This disease is common in Ecuador and it is propagated through poultry. A new alternative for controlling the infection in humans and animals is through passive immunity.

Consequently, specific antibodies can be developed as therapeutic agents based on biodegradable nanoparticles liposomes. Specific antibodies are produced by inoculation of hens with specific antigens, followed by retrieving the antibodies accumulated in yolk. The objective of this work was to purify specific IgY antibodies against *Salmonella spp.* from Lohmann Brown hens. A group of hens was immunized with a pool of *Salmonella enterica* subsp *enterica* serovar Enteritidis and *Salmonella enterica* subsp *enterica* serovar Infantis. These two pools of bacteria were previously inactivated with a treatment (T1) by formaldehyde or with a treatment (T2) by heat. Lipids were removed using 0.1% pectin, the protein pelleted with ammonium sulfate (35% w/v) is re-suspended in PB buffer 0.025M pH 8. The Total IgY antibodies were purified by DEAE cellulose. The highest yield of total IgY was 5.5 mg IgY/mL yolk following the (T2) treatment at 63 days post-immunization. The characterization was performed with ELISA and MABA techniques and the immunoreactivity was evaluated by Western blot in total antisera and purified protein, hybridized with commercial anti-chicken antibodies from SIGMA. The heavy chain 67 kDa and the light chain 25 kDa were visualized. Such antibodies could be able to ensure quick and immediate protection against several biotargets, the new IgY has potential for nanotherapy against Salmonellosis diseases.

**Keywords:** Nanotherapy; IgY; Antibodies; Salmonella

### Introduction

Enterobacteria are microorganisms responsible for most cases reported for food and water-borne infections in humans. One of the main causes of zoonotic disease is caused by bacteria of the genus *Salmonella* that have a great economic interest worldwide in the poultry sector. There have been about 250 serovars of *Salmonella enterica* isolated from poultry, that colonize the bird in the period of growth, this contaminate meat constitute a mayor means of pathogen transport to humans [1]. In Ecuador around 9,908 cases of salmonellosis were reported in 1990, this cases number increased dramatically in 2001 to reach 18,772 cases, in 2008 due to prevention system this number was reduced to 3,286. The two provinces with the largest outbreaks were Manabí and Guayas with 95 and 156 cases of salmonellosis respectively [2].

In 1893, Klemperer first demonstrated that the immunization of a hen resulted in the transfer of specific antibodies from the serum to the egg yolk. For over a hundred years there was no scientific application for this knowledge. But when the animal welfare became a matter of serious ethical concern for the scientific community, the results of Klemperer have attracted a great attention, particularly since the 1980s [3].

This new technology of IgY reduces the production costs at large scale and it is a useful tool in diagnosis and offer more advantages than the analogous IgG since its extraction is performed by non-invasive methods and fewer animals were used to obtain large amounts of antibody from egg yolk [4]. As IgY are lacking the binding sites in the Fc region of the heavy chain reactions, there is no crossing with The rheumatoid factor IgY, in opposite with IgG which reduce the false positives [5]. In addition, the bird's antibodies not activate the complement system of mammals [6] and oral administration of immunoglobulins doesn't induce bacterial resistance so it can be supplied specifically against different microbial agents, and doesn't interfere with the normal flora of the animal and can be administered in combination with probiotics [7]. Historically, IgY, the major immunoglobulin class present in avian serum and egg yolk was called IgG, due to its function and serum concentration in comparison with the mammalian IgG. However, it has become clear that this is inappropriate, especially because of fundamental structural differences between IgG and IgY molecules [3].

IgY makes up about 75% of the total immunoglobulin pool. The serum concentrations of IgY, IgA, and IgM have been reported to be 5.0, 1.25, and 0.61 mg.ml<sup>-1</sup>, respectively [8].

In this study, the specific antigens induce an immune response leading to high level of hens IgY antibodies production in the egg yolk. The continuous immunizations with *Salmonella enterica* serotypes S.

*enteritidis* and *S. infantis* responsible for most of human pathologies were performed in order to maintain high levels and to ensure the transfer of the antibody. In this sense, the objective of this research was to purify and identify IgY antibodies from Lohmann Brown chickens immunized with *Salmonella* spp. As a low cost alternative that can be used in passive immunity in humans and animals.

## Materials and Methods

### Materials

Lohmann Brown hens eggs immunized with *Salmonella* spp. was used. Poultry are obtained from IASA I of the Universidad de las Fuerzas Armadas and eggs stored at 4°C.

At 18 week of age, the hens were intramuscularly injected with 0.25 mL of the emulsion at 4 sites in the breast muscle (total 1 mL/bird). Booster immunizations were given at 2, 4, and 6 weeks after first immunization with Freund's incomplete adjuvant (Sigma) emulsified with  $5 \times 10^6$  CFU/mL was used [9].

### Antigen preparation

The two pool of bacteria *Salmonella enteritidis* and *Infantis* were grown on XLD agar at 37°C. One group was inactivated by fixation with formaldehyde (T1) and other by heat (T2). To do this 30 mL of a bacterial culture of *S. enteritidis* and *S. infantis* incubated for 2 hours under stirring at 37°C. The colonies number/mL of each culture was determined by spectrophotometer at 600 nm, the dilutions were made to have a total of  $5 \times 10^6$  bacteria CFU/mL. Once obtained 2 tubes with 500  $\mu$ L of each bacteria (*S. enteritidis* and *S. infantis*) were inactivated with (T1) or (T2) [10].

### Immunization of hens Lohmann Brown

The first immunization with the antigen was performed in combination with incomplete Freund's adjuvant in ratio 1/1, mixed in a syringe by passing the liquid through it until a homogeneous emulsion. With the Antigen of the second, third and fourth immunization was added 10  $\mu$ g DNA of each bacterium, and for the fifth immunization was incorporated CpG ODN inoculation 2135 sequence (5'-TCG TCG TCG TTT GTC GTT GTT TT-3') to enhance the immune system of the immunized hens. A dose of 1 mL immunogen was injected by intramuscular [11].

### IgY isolation

The white of egg yolk was removed and placed without the membrane in a 50 ml tube; the yolk obtained was diluted in 60 mL of 0.1% pectin. Lipids was discarded, the ammonium sulfate 35% was added to supernatant and collected in tubes then stirred at 4°C overnight. To precipitate the proteins the solution was centrifuged at 10200 rpm for 25 minutes at 4°C, the supernatant was discarded and the pellet dissolved in 2 mL of 0.025M PB buffer at pH 8.

### IgY purification

The purification protocol was based on the work of Akita and Nakai [12]. A column of 13 mL was armed with a reconstituted resin (DEAE cellulose) and suspended in buffer PB 0.025 M pH 8. The purification process was performed at a flow rate of 1 mL/min.

### IgY antibody identification

The 96-well polystyrene Elisa plates were coated with antigens lysates from *Salmonella enteritidis* and *Salmonella Infantis* at a concentration 30  $\mu$ g/mL overnight at 4°C. The plate was washed 5 times with PBS-T (0.1% Tween20) and blocked with 5% skim milk for 1 hour at 37°C. Subsequently the plate was washed 5 times with PBS-T and incubated for 1 h at 37°C with anti-IgY (1/500, 1/250 dilution) from *Salmonella* spp. inactivated (T1) or (T2), along with a negative control (1:50 dilution bovine serum) and positive controls (1:50 dilution of serum immunized) chicken. After the incubation time the 96 well plate was washed 5 times, 100  $\mu$ L /well of anti-chicken IgG (IgY) peroxidase conjugate 1 was added and incubated for 1hour at 37°C. The plate was washed 5 times and the peroxidase activity was revealed with tetramethyl benzidine diluted in dimethylsulfoxide, phosphate-citrate buffer 0.05M and 1% H<sub>2</sub>O<sub>2</sub>. The reaction was stopped with 50  $\mu$ L/ well of 2M sulfuric acid and the reading of the colorimetric reaction was performed in a microplate reader iMark Elisa Reader at 450 nm.

### Western blot

**SDS-PAGE:** The Antigens proteins lysates from *S. enteritidis* and *S. infantis* were analyzed by SDS-PAGE using a Mini Protean Tetra-cell (Bio-Rad), acrylamide gels/liquid bisacrylamide 30% under reducing and denaturing conditions. gel concentration was made at 4% (0.5M Tris-HCl pH 6.8) and a separating gel 8% (1.5M Tris-HCl pH 8.8), the protein concentration of *S. enteritidis* and *S. infantis* was determined BCA Protein Assay Kit and dilution was performed in PBS to give final concentrations of 300 mg/mL, 200  $\mu$ g/mL, 100  $\mu$ g/mL and 50  $\mu$ g/mL each antigen with loading buffer (glycerol, 0.5M Tris pH 6.8, 10% SDS, bromophenol blue, weight marker of 10-230 kDa (BioLabs), samples with 2% mercaptoethanol).

The SDS-PAGE performed to detect chains IgY for (T1) (T2) was performed under the same above conditions. Samples with IgY (10, 20, 38, 59 and 63 days) were diluted to 500  $\mu$ g/mL bovine serum was placed as a negative control (1:50) and commercial IgG (1:500). The electrophoretic run for both cases was 120V for 1 hour.

**Transfer and immunolabeling:** The Proteins were transferred to nitrocellulose membranes at 70V for 90 min. To check the transfer from the gel to the membrane with Ponceau red staining was performed for 10 min and washed with distilled water to remove all dye.

## Results

### Immunizing hens Lohmann Brown

Growth curves of *S. enteritidis* and *S. infantis* was made, yielding an exponential growth around two hours, this allowed quantify the bacterial population and dilutions for an antigen with  $5 \times 10^6$  UFC.

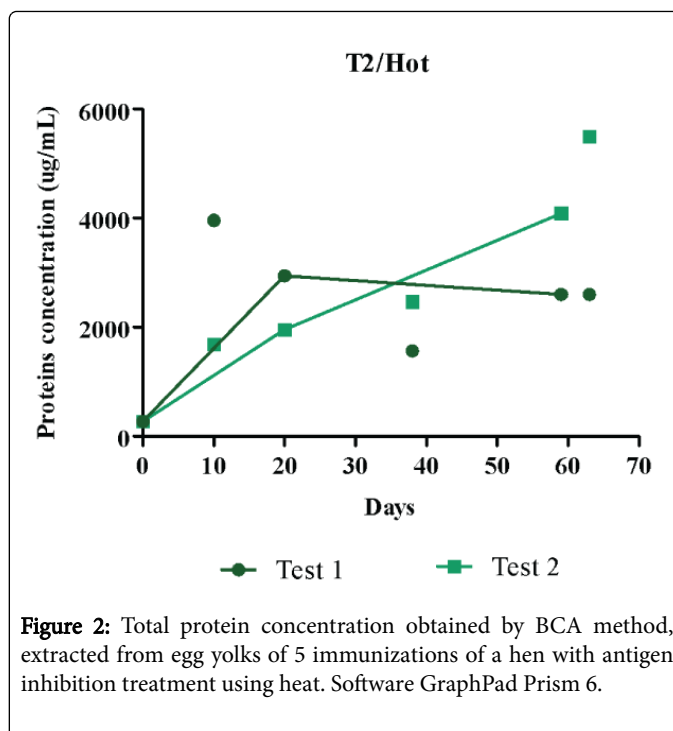
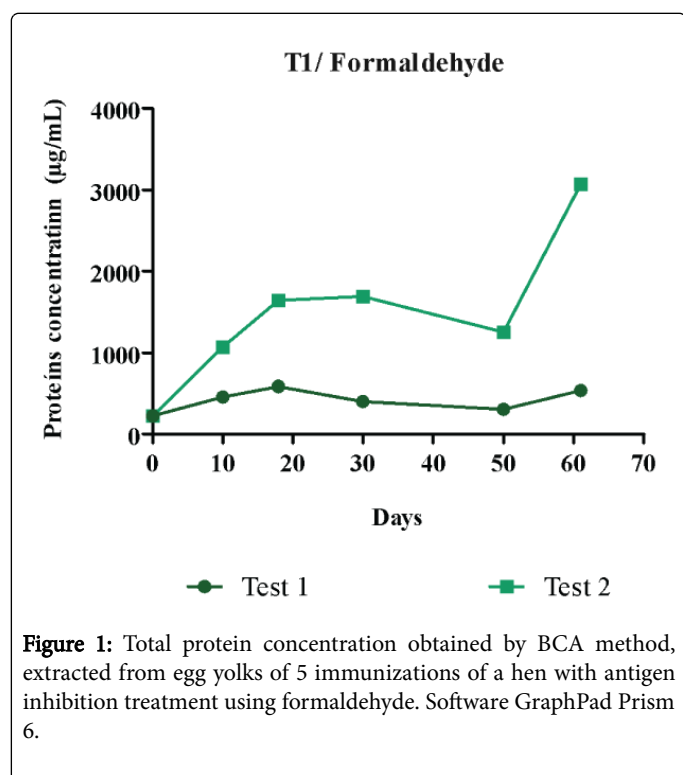
Using heat or fixed with formaldehyde inactivated antigen in combination with Freund's adjuvant in two hens Lohmann Brown. The hens were alimented with a diet based on balanced and supplemented with vitamins and spirulina, each hen immunized with a dose of 500  $\mu\text{L}$  of *Salmonella spp.* immunogen the inoculation was performed by intramuscularly injection. An average of 6 eggs were collected per week during the immunization period, and stored at 4°C.

### IgY antibodies extraction

The yolk was diluted in water to separate the protein from the lipid fraction. The pectin was used at 0.1% that allows lipids precipitation and recover a clear supernatant containing the protein of interest. The protein precipitation was performed with ammonium sulfate at 35% and a pellet was obtained as the product of the extraction and total immunoglobulin from egg yolks from hens immunized with *Salmonella spp.* This pellet was resuspended in buffer 0.025M pH 8.

### IgY antibody purification and quantification by BCA

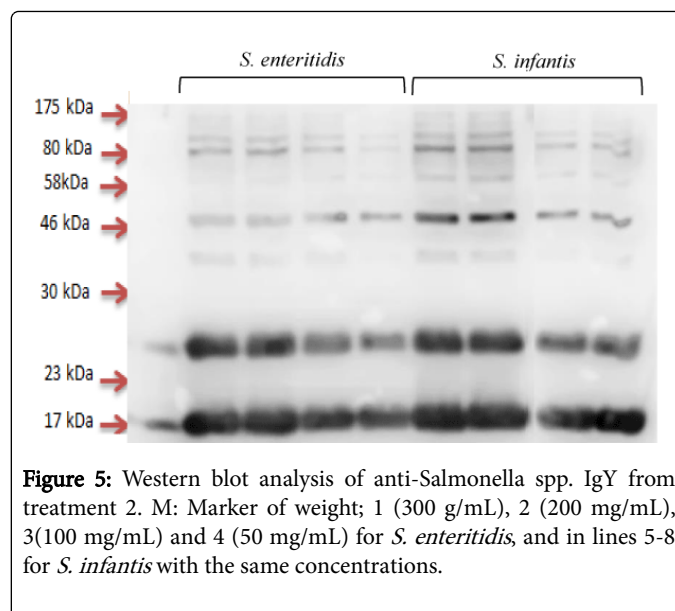
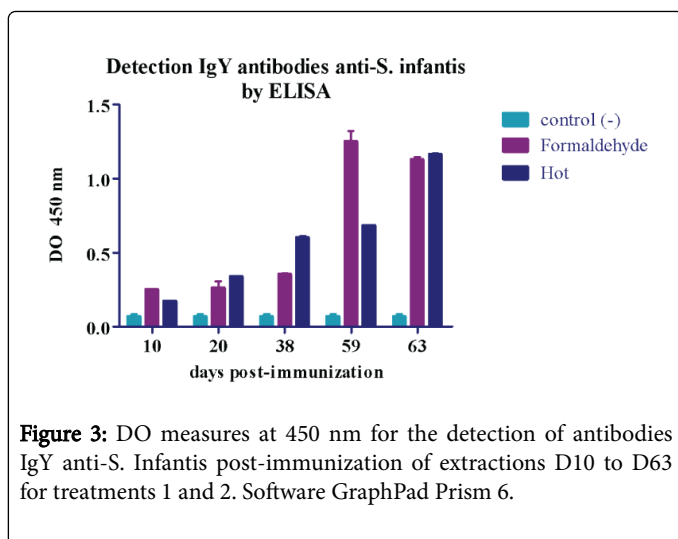
We found a different quantity of IgY after different immunizations, an increase in the production of whole antibodies from the first week after starting the immunization period, Figure 1 shows antibody production obtained from egg yolks after (T1). Figure 2 shows antibody production obtained from egg yolks after (T2).



The concentration of IgY becomes increasing from day 10, then there is a slight decrease days after making the fourth immunization, but after the last inoculation there is an increase in production reaching a peak of 3067.14 mg IgY/ml egg on day 63 after starting the immunization period.

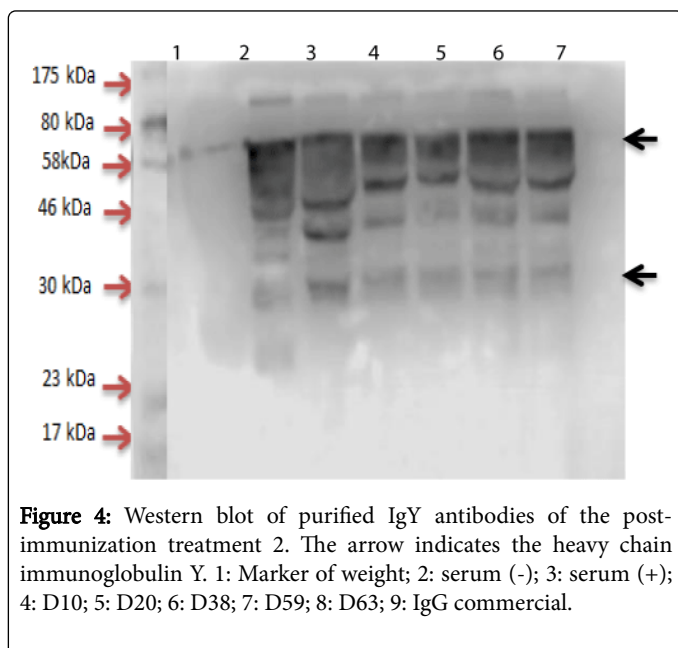
### IgY antibody identification

ELISA for detection of antibodies specific IgY anti-*Salmonella spp.* The specificity of the antibodies IgY anti-*Salmonella spp.* was evaluated for one concentration of 30  $\mu\text{g/mL}$ , to which to IgY antibody dilutions (1/250 and 1/500) for each extracted and purified sample were standardized corresponding to the 10, 20, 38, 59 and 63 days. The best dilution of the antibodies was obtained at 1/500. The levels of antibodies against *S. enteritidis* increased from the second week after starting the immunization protocol reaching higher production in T1 (antigen formaldehyde-inactivated) relative to T2 corresponding to day 10. From the fourth booster immunization there is an increase of antibody levels in both treatments and after the last immunization at the day 63 a greater increase for the T1 on day 59 and day 63 for T2 is obtained, by this time T1 starts to decrease. Figure 3 represent the levels of IgY antibodies anti-*S. infantis* that began to rise from the fourth and fifth booster immunization, with high levels of antibodies production to T 1 on day 59 and for T2 on day 63, at which time the levels of IgY T1 begins to decrease.



### Western blot

The detection of IgY was performed by Western Blot test. Each IgY extracted (10, 20, 38, 59 and 63 days). Was conjugated to secondary antibody anti-chicken IgG (IgY) for both the T1 (Figure 4) and T2 (Figure 5).



The Immunodetection of *Salmonella spp.* antigens lysates by IgY purified antibodies was performed by Western blot after the fourth immunization (day 59) for the treatment 2, which had the most quantity of total protein IgY antibodies determined by BCA.

Figure 5 represents the immunodetection of bands of about 90, 80, 50, 40, 25 and 17 kDa for both *S. enteritidis* and *S. infantis* at the antibody dilution 1/1000.

We observe an increase in the color intensity of the channels which indicated greater recognition of the lysates of *S. enteritidis* antigens at a concentration of 40 µg/mL. More coloring channels (6, 7 and 8) indicate the presence of antibodies IgY anti-Salmonella spp. in sera of chickens of both treatments. The reactivity of the purified antibodies was specific for the detection of antigens of *Salmonella spp.* no cross-react with antigens to confront lysates K12 *E. coli* strain.

### Discussion

#### IgY antibody identification

The two hens showed an immune response regardless of treatment applied, for the ones that was inoculated with  $5 \times 10^6$  CFU of a pool of *Salmonella spp.* bacteria. It was sufficient to induce the development of specific IgY antibodies. The dose used was highly immunogenic to raise antibodies in chickens. According Schade et al. [13] the immunogen directly influences the immune response in animals and in the title of specific antibodies [9].

The immunization used during a period of 44 days, the long period of immunization handled in this study can induce the production of antibodies against other molecules that form part of adjuvant which was combined antigen. One of the advantages of using this adjuvant is the production of highest specific antibody titer IgY from hens [14]. The increase in antibody production in this research was favored with the use of modulators (adjuvant) and the sequence CpG-ODN 2135 used to increase the number of B cells and therefore the ability of the hens to produce more antibodies, similar results have been shown in the work of Krieg et al. [15] and Krieg et al. [16].

Akita and Nakai [12], showed that the pH 5.2 allows to recover about 94% of IgY soluble protein fraction in water (WSF) of egg yolk and generally when working at acidic pH in a range of 4.6 to 5.2 allows obtaining uncontaminated supernatants lipid. The incubation with pectin at pH values about 4 and increased stirring time at pH 5.2 before centrifugation favorise the obtention of supernatant without lipids.

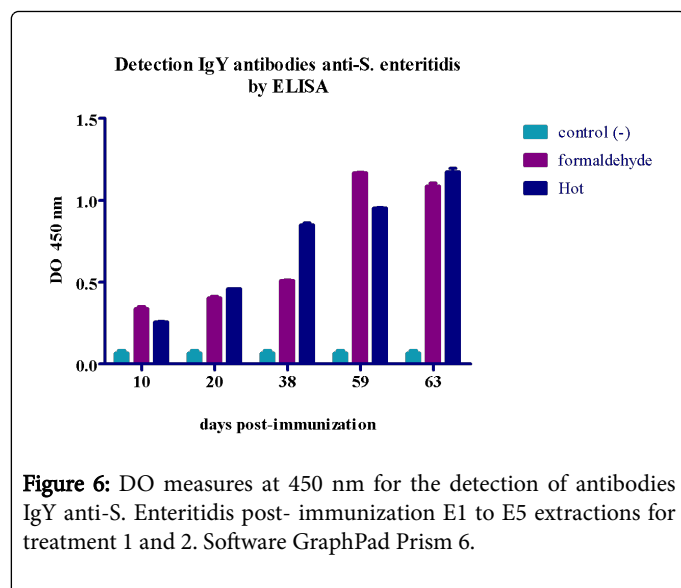


The proteins have the ability to form hydrogen bonds with water, so by adding salt hydrogen, the bonds are broken causing precipitation and loss of solubility. Ammonium sulfate was added to precipitate proteins; one of the most soluble salts only allows precipitation of immunoglobulins. According to Akita and Nakai [12], it is generally employed in 30 and 36% saturation, this research using ammonium sulfate to 35% saturation allowed precipitation of total IgY, however in this method is not possible to obtain a completely purified protein, being necessary to perform some type of chromatography.

For purification step in this assay, ion exchange chromatography with DEAE cellulose was used to separate the immunoglobulin from other water-soluble proteins such as  $\alpha$ ,  $\beta$ , livetins and the LDL fraction [17]. The IgY was eluted with PB buffer 0.250M pH 8 [18] and managed to obtain two product elution fractions, according Akita and Nakai (1992) the use of such columns can recover about 95% IgY. The IgY purified remained stable at 4°C at pH about 8, maintaining the antibodies between 4 and 37°C at pH 4 to 10 are suitable for the stability of the antibodies. Chromatography after removing small molecular weight proteins was performed with membrane filter tubes 100 kDa and IgY concentration was quantified by BCA Protein Assay Kit.

### ELISA for detection of antibodies specific IgY anti-*Salmonella* spp.

The identification of IgY antibodies against *Salmonella* spp. was performed by an ELISA assay in which a titer of IgY antibodies 500 was obtained. The specificity of IgY antibodies purified from treatment 1 (Figure 6) was observed from the second week post-immunization, a studies by Sunwoo et al. [19] in laying hens showed that the transfer of IgY from serum to the egg yolk may take about 2 weeks. From this point, the levels of anti-*Salmonella* spp antibodies began to increase on day 10 post-immunization showed higher specificity for protein lysate antigen from *Salmonella enteritidis* in comparison of antigen from *Salmonella infantis*, reaching higher levels on day 59 after the fifth immunization. High titers of antibodies IgY not able to maintain as from day 63 these levels started to decrease. Immunogenicity in chickens caused largely depends on the way of inactivating the antigen. At high concentrations of formaldehyde antigen stability decreases.



In treatment 2, the anti-*Salmonella* spp. Antibodies began to increase from the second week as in treatment 1, with the difference that the levels of specific antibodies remained after the fifth immunization (day 44), i.e, on day 63 the amount of antibodies IgY anti-*Salmonella* spp. continued to rise. The immunogenicity of heat inactivated antigens can be explained by the thermal stability of somatic antigens of *Salmonella* spp. which prevents denaturation also avoiding loss polysaccharide antigens [20].

### Western blot

Under reducing conditions, the disulfide bridges break and cause the separation of heavy and livid antibody chain, so to confirm this activity, we did a Western blot assay where it was observed (Figures 5 and 6) the immunoreactivity of the heavy chain 67 kDa and light chain 25 kDa of each extractions (E1-E5) in both treatments and the positive serum. Hanses et al. [21] reported that the heavy chain of the IgY represents 75% and 25% light chain.

The recognition of the same size protein indicated that serotypes studied share most of the antigenic proteins found in the outer membrane (OMP) of bacteria [22], but different antigen O and H that may be represented by *S. infantis* additional band of 58 kDa. Similar results were described by Kudrna et al. [23] in which they found a common proteins from outer membrane (OMP) in *S. enteritidis* and *S. infantis* by densitometry. In a range of 34 to 19 kDa in which the number of common antigenic proteins of both serotypes They were significantly higher, and in a range of 19 kDa to 12 kDa as many antigenic proteins were found in lysates of *S. enteritidis*. Sahar and Nagwa [24] reported similar results with immunodetection of protein bands of *S. enteritidis* of 17 to 31 kDa *S. infantis* common antigenic protein which corresponded to the outer membrane of bacteria (OMP). Such proteins are easily recognized by the immune system to be exposed on the bacterial surface so have been identified as major immunogens system.

The presence of higher molecular weight bands at 67 kDa could be related to residues of toxins in *Salmonella* [24].

The immunoreactivity of anti-IgY antibodies *Salmonella* spp. From the extraction 4 and corresponding to treatment 2 was checked by western blotting assay. The results showed that the IgY antibodies obtained were able to recognize antigens lysates *S. infantis*, *S. enteritidis* and diluted to final concentrations of 300, 200, 100 and 50  $\mu$ g/mL. six immunoreactive bands for *S. enteritidis* and seven for *S. infantis*, the which are about 90, 80, 50, 40, 25, 17 kDa and an additional band recognized in the lysate of *S. infantis* of 58 kDa.

### Conclusion

It was achieved antigens from pure cultures of *S. enteritidis* and *S. infantis* for the immunization process in Lohmann Brown hens, obtaining polyclonal IgY antibodies anti-*Salmonella* spp. The use of pectin 0.1% allowed complete separation of lipids and together with the precipitation with ammonium (35%) and ion exchange chromatography with DEAE cellulose sulfate is achieved the separation of IgY with an efficient method that maintained the specificity of antibody and reached a peak of 5.5 mg IgY/mL of egg yolk with treatment 2 (antigen heat inactivated).

The specificity of the antibodies IgY anti-*Salmonella* spp. It was assessed by ELISA at a dilution 1/500 antibodies were able to recognize both antigens lysates of *S. enteritidis* *S. infantis* as at a concentration of

30 µg/mL. Similar results were obtained with the Western Blot technique, that the heavy chain of the IgY was at 67 kDa and the light chain at 25 kDa. For both treatments (heating and fixing formaldehyde) increased production of IgY antibodies for antigens of *S. enteritidis* was determined. A total of 15 studies demonstrating the effect of IgY against diarrheagenic pathogens in poultry were selected. Currently, the oral passive immunization using chicken IgY has been applied as an alternative to antibiotics for the treatment and control of diarrhea in animals and humans [25]. Passive immunization is one of those strategies. Egg yolk antibody prophylaxis and nanotherapy have a natural place in poultry industry. In poultry, maternal antibodies are transmitted to the offspring via the yolk of the egg.

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