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RESEARCH ARTICLE

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Characterization and Immunologic Response of IgY Against *Leishmania Mexicana*

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ABSTRACT

Avian IgYs are polyclonal antibodies resulting from humoral adaptive immune responses. With perspective for many application, IgYs are considered the most versatile biomolecule, able to be used as alternative in biomedicine and as diagnostic tool. Because they are inducible, these antibodies could be produced to potentiate the recognition of relevant clinical pathogens such as *Leishmania mexicana*. In this investigation anti *Leishmania mexicana* IgYs were obtained by inoculation of inactivated promastigotes in brown Lohmann hens, at different periods. Twenty-four per cent of the eggs were randomly selected for processing. IgYs were precipitated by Ammonium Sulfate and purified by affinity chromatography. Immunoglobulins with 175 kDa, complete structure, and a concentration of 6.3 mg/mL per yolk were obtained. The most reactive antibodies corresponded to week 8 (W8). These IgYs were tested for specific recognition of their target parasite, using structural and proteins viral as GP63 in Western Blot. Also, they identified concentrations of parasite antigen of 5 and 50 µg/mL with maximal antibody dilution of 1:2000 in ELISA. The IgY-mediated immunodetection offers this as solution to the necessity of anti *L. mexicana* antibodies for diagnostics and immunotherapy research.

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IgY Technology, Leishmania, Western Blot, Indirect ELISA

Introduction

Proteins have played an important role in new medicine, because of their functions and applications. The benefits of proteins have been described as safer and more effective medical molecules, in this study we will focus on antibody properties such as antigen binding and immunoreactivity [1]. Antibodies are commonly used to control and neutralize many microorganisms and their toxins, for that reason, thanks to avian immunization, a considerable amount of specific antibodies could be isolated [2]. Since 1996, IgY production has been validated as an efficient alternative to traditional protocols using small mammals [3]. Taking advantage of this, particular antibodies could be obtained, even those complex antigens for mammals [4].

In another way, uncontrolled parasite requires action in certain aspects, referring to diagnostic methods and effective anti-parasitic drugs [5]. Around the word, leishmaniasis is qualified by WHO (World Health Organization) as a neglected disease, reported in 89 countries [6]. Particularly, in South America, an estimated total of 17 countries where this endemic disease has increased the number of clinical cases [7]. In this context, cutaneous leishmaniasis is the most common leishmaniasis, affecting 95% of 12 million cases worldwide [8]. One of the most common Leishmania protozoa is known as *Leishmania mexicana*, causing cutaneous leishmaniasis as well as visceral leishmaniasis in particular cases [8].

However, the early diagnostic test and effective drugs are not available nowadays. Antimonials are prescribed as first-line treatment, but are linked to serious side effects, such as toxicity and resistance [7]. For that reason, the demand of new therapy is emerging, proposing antibodies as prophylactic method and also increasing the diagnostic capacity for future designs like the rapid lateral flow test for cutaneous leishmaniasis, to detect *Leishmania* parasites [9].

Facing this information, the production of anti-parasitic antibody could be easier and more effective by the implementation of IgY technology, providing specific IgY against *Leishmania mexicana.* These polyclonal antibodies could be isolated in high quantities and qualities, furthermore the specificity of recognition could lead to the application of an alternative diagnostic test like Western Blot and ELISA.

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Materials and Methods

Antigen Production

Leishmania mexicana in promastigote state, was cultivated in Drosophila Schneider medium. This parasite culture was provided by Cell Culture Laboratory of Immunology and Virology Laboratory from Universidad de las Fuerzas Armadas ESPE. The pure culture was washed and concentrated by centrifugation at 4500 RPM for 10 min. A promastigote concentration of 1x107 cells/mL was combined with 250 µL of PBS 1x sterile buffer and heat inactivated for 90 minutes. Once done, antigen was combined with Freund complete adjuvant (F5881, Sigma Aldrich) %v/v (1:1) for the first inoculation shot. Afterwards antigen was combined with Freund incomplete adjuvant (F5506, Sigma Aldrich) %v/v (1:1) for the consequently inoculations.

Chicken Inoculation Model

Four 18 weeks old Lohmann Brown animals, were distributed in 1m2 individual cages distributed in control (n=1) and treated animals (n=3). They were provided with chicken feed, disinfected water as needed and cleansing area every day until the end of the experiment to prevent adverse infections. Once they were adapted to the cages, the inoculation procedure started, performing it once a week, using inoculation-latency-inoculation periods. For the first phase, inoculation was structured into five continuous immunization, seven days apart of each dose. The second phase, latency, had no immunization for three continuous weeks. Then a last inoculation to boost the immune reaction, was performed in 9th week, with a final latency state, without immunization dose until the end in 11th week (Figure 1).

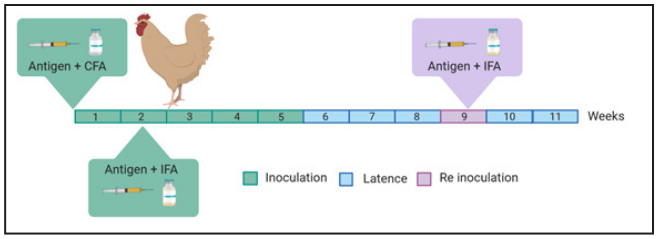


Figure 1: Inoculation model in chickens for IgY production, using inactivated L. mexicana promastigote as

Puncture area was selected as discussed in Oliviera Montini; 2017 [10]. Using intramuscular route, in the upper pectoral region with 1mL syringe and 28g ½ inches needle. For each protocol, muscular area was disinfected with 70% ethanol and during the antigen administration the dose was injected slowly as possible. For protein analysis, all eggs were collected since the start of the chicken inoculation process. Animal welfare was ensured, after, during and in final protocol.

Antibody Isolation and Purification

Random selection was performed, leading to purification of paired egg samples from each relevant week, due to the high total number of eggs collected. Each egg yolks were combined with delipidant solution of apple pectin 0.1% w/v (9000-69-5, Sigma Aldrich) in 1:8 proportion [11]. The solution was homogenized, pH adjusted to 4.5 and centrifuged at 4500 RPM for 1 hour. Aqueous phase was separated from lipid pellet and delipidated again if it required. Aqueous phase was conserved in 4°C, until protein precipitation.

For protein isolation, saturated Ammonium Sulfate (1012175000, Emsure) solution (4.1 M, pH 7.5) was prepared [12]. Aqueous phase was mixed by adding of Ammonium Sulfate solution in 1:1 proportion. The final solution was homogenized and centrifuged at 4500 RPM for 1 hour. Protein pellet was recovered and suspended in 0.02 M and pH 7.5 PB sterile buffer.

For Y immunoglobulin isolation, HiTrap IgY purification affinity column was used (17511101, GE healthcare). Protein pellets were combined with Binding solution (0.02 M PB buffer; (NH4)2SO4 1M; pH 7.5) in 1:5 proportion. Pool protein was filtered through the column and washed with Binding Buffer. Consequently, IgY antibodies were obtained with Elution Buffer (0.02 M PB; pH 7.5), this fraction was retrieved and stored at 4°C. Finally, column was cleaned with Cleaning Buffer (0.02 M PB buffer; 30% isopropanol v/v; pH 7.5) and equilibrated.

IgY obtained from purification was quantified with BCA kit protocol (BCA1 y B9643, Sigma Aldrich) using albumin protein standar from 0 to 1000 µg/mL (9048-46-8, Sigma Aldrich). In 96 well plates, samples were distributed and added BCA working solution. Results were readed by spectrophotometer at 570 nm in Multiskan Fc microplate photometer (51119000, ThermoFisher).

IgY Immunoglobulins Characterization

Western Blot for IgY Purified Protein

For this committed a vertical SDS electrophoresis was performed, using 8% v/v acrylamide-bis acrylamide 19:1 (79-06-1, Sigma Aldrich) loading gels. 50 µg/mL IgY protein concentration samples were combined with loading buffer, then denaturalized by 100°C heat shock for 10 minutes. Electrophoresis conditions were stablished as 110 V and 400 mA for 45 minutes.

As part of this, protein transfer was performed in semi dry Trans Blot Turbo (1704150, Bio Rad), from acrylamide gel to nitrocellulose membranes. Proteins were transferred with three cycles with 25 V and 2.5 A for 8 minutes each.

After proteins had been fixed in nitrocellulose membranes, the blotting was performed by blocking solution with no fat milk 5% w/v (sc-2323, Santa Cruz Biotechnology) in TBS buffer, incubating membrane overnight at 4°C. Membrane was washed three times with Tween 20 0.1% v/v and TBS buffer for 10 minutes each. Membrane was incubated with direct antibody IgG rabbit anti chicken HRP conjugated (A9046, Sigma Aldrich), for one and half hour and washed in the same conditions before mentioned.

To reveal Chemiluminescence, luminol and peroxide (Clarity Western ECL Substrate, 170560, Bio Rad) were mixed in a ratio of 1:1 and applied directed to membrane. Finally, membrane was reveled with C Digit Blot Scanner and the image observed in Image Studio Lite with High sensibility.

Western Blot and ELISA for Parasite Antigen

To evaluate IgY anti *Leishmania mexicana* immune recognition capacity, Western Blot and ELISA were performed using a parasite as antigen as well as *Leishmania braziliensis* and *Leishmania infantum*. Escherichia coli, Salmonella *enteritidis, Staphylococcus aureus* and *Trypanosoma* were also lysed to perform cross reactivity ELISAs.

The Western Blot were carried out using 10% acrylamide-bis acrylamide loading gel, and by loading 25 µg/mL of antigen parasite, parasite membrane protein as the protocol described by Málaga-Machaca, Romero-Ramirez and just for *L. mexicana* pure promastigote parasite [13]. Continuing, SDS electrophoresis

was performed with 110 V, 400 mA for two hours as conditions. Then, nitrocellulose membrane protein transfer was performed as described early and the resulting membrane was run to blot.

First, blocking membrane with no fat milk 5% w/v solution for two hours at room temperature, then the membrane was washed 5 times for 10 minutes each, with Tween 20, 0.1% diluted in TBS buffer solution. Second, the membrane was incubated with IgY anti *Leishmania mexicana* in 1:1000 proportion with no fat milk 2.5% w/v solution as primary antibody. The incubation lasted overnight at 4°C. After, the membrane was washed and incubated with IgG rabbit anti chicken HRP conjugated in a proportion 1:20000 for two hours. Finally, the membrane was washed again and prepared for Chemiluminescence.

To reveal the membrane, luminol and peroxide 1:1 solution was applied directly to the membrane and scanned in C Digit Blotting Scanner with high sensibility.

For the ELISA protocol, a 96 well plates with high binding (CLS3590, Sigma Aldrich) were used. The plate was sensitized with lysed parasites and bacteria respectively, using different concentration gradients. For this commitment, antigens were diluted in 0.1 M Carbonate Bicarbonate buffer pH 9.6 and distributed per well and incubated overnight at 4°C, then washed with Tween 20 0.05% v/v in PBS 1X buffer solution. Subsequently, the plate was blocked with no fat milk solution 5% w/v diluted PBS 1X buffer and incubated for two hours at 37°C. For immunoblotting, IgY anti Leishmania mexicana in concentration gradients were prepared in no fat milk 2.5% w/v in PSB 1X buffer, these were applied following a plate design and incubated for two hours at 37 °C, washed and prepared to the final antibody incubation. As second antibody, IgG rabbit anti chicken HRP conjugated was prepared in 1:30000 proportion in no fat milk 2.5% w/v solution, and incubated for two final hours and washed. To reveal results, ABTS (A9941, Sigma Aldrich) as substrate was applied and incubated for one hour at 37°C and the plate was read with spectrophotometry at 405 nm.

Statistics and Graphics

All tests were analyzed with Graph Pad Prism 6, for two ways ANOVA, multiple comparison with Tukey tests and finally ROC tests.

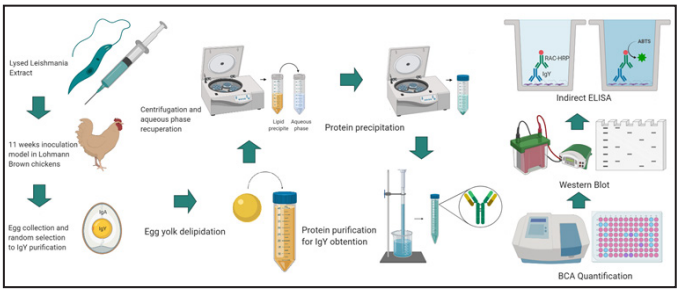
Results

Chicken Inoculation

Eggs production was assessed after each inoculation. Referring to (Figure 3), during two and a half months of inoculation treatment, no difference was detected in animal's normal oviposition. The peak egg production was achieved in weeks three and four. Despite having considerable number of samples, only the 24% by random selection were processed.

Antibody Isolation and Purification

BCA IgY protein quantification provided the following results (Figure 4). IgY increased in the inoculated compared to the control group, achieving average protein concentrations greater than 2mg/mL. During the inoculation phase, from W1 to W5, the IgY increased and was maintained constant. For the latency phase during W7 and W8, still a slight increase was observed. Nevertheless, in W9 with booster immunization, it generated the higher IgY concentration reaching the 6.3 mg/mL. In the final latency phase, the concentration of IgY decreased, even though it wasn't less than 4 mg/mL and maintained constant. Notwithstanding a high concentrations in W9, greater than the rest weeks, no significant difference was detected using Tukey multiple comparisons. With these optimal concentrations, other protocols were assessed, as described in summary in (Figure 2).



antigen with complete (CFA) and incomplete (IFA) Freund adjuvant.

Figure 2: IgY obtention summary and consequent assay use in different protocols

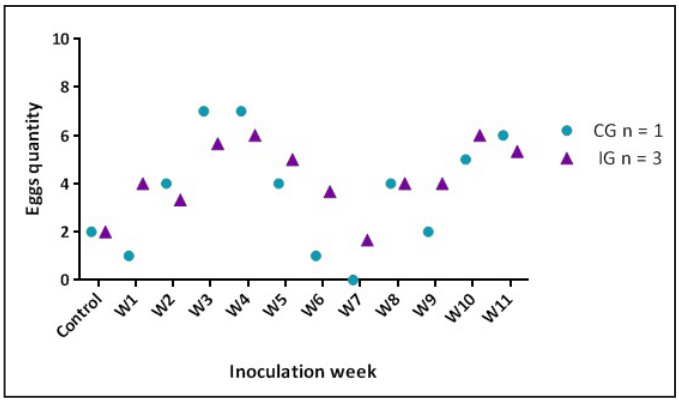


Figure 3: Final egg quantification for each group, control and inoculated chickens, to observe differences before, after and during inoculation. No difference was detected and both groups had similar egg production. Reference: W= week; n= number of animals; CG= control group; IG= Inoculation group.

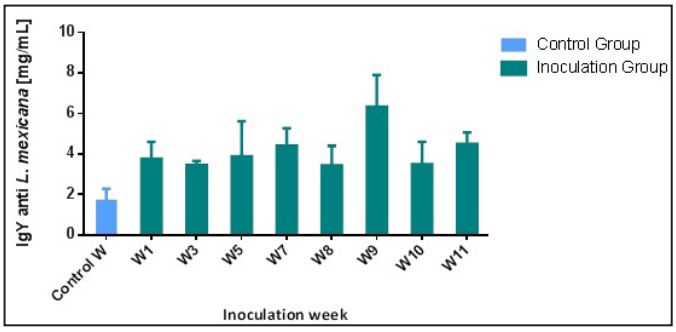


Figure 4: IgY protein quantification by BCA kit after purification classified by weeks

IgY Structure With Direct Western Blot

The resulting images obtained from Chemiluminescence, were analyzed in Image Lab Software 6.0.1. As seen in (Figure 5), IgYs from the most relevant weeks were loaded to evaluation, from one to eleven week. In all samples, complete immunoglobulin structure was observed. First of all, high chains corresponded to 67 kDa. Second, light chains were observed in 35 and 22 kDa as result of denaturalization process. Finally, small IgY fragments were located with 15, 17 and 18 kDa. Therefore total IgY molecular weight had a mean value of 170 and 175 kDa.

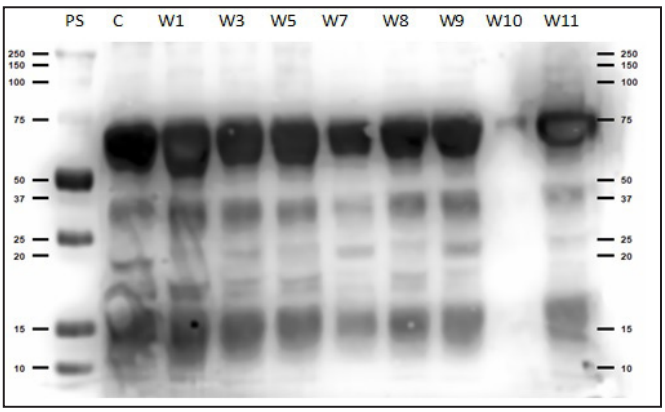


Figure 5: Chemiluminescent direct Western Blot for IgY anti L. mexicana. From left to right, PS: Precision plus protein, all blue pre stained protein stander. C: IgG anti Chicken. W: week from one to eleven.

Indirect Western Blot with IgY anti Leishmania Mexicana as Primary Antibody

IgY anti *L. mexicana* had capacity to recognize its target antigen as well as L. braziliensis and L. infantum. Especially for *L. mexicana,* IgY antibodies recognized many parasitic proteins as shown in (Figure 6), and described in Table 1. At least seven proteins were detected, one of them being linked to GP63, Leishmania virulence related protein. For both samples, sonicated and promastigote parasite, IgY detected the highest amount of antigen. By contrast, if the antigen was just membrane protein, IgYs were specific enough to detect particular epitopes.

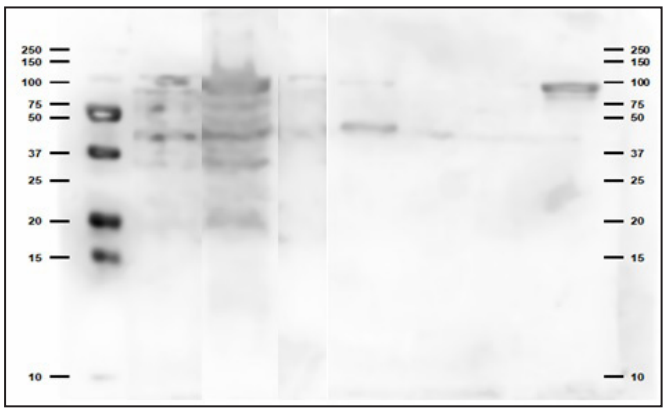


Figure 6: Chemiluminescent indirect Western Blot, detecting L. mexicana with IgY against parasite target. From left to right: 1. Precision plus protein, all blue pre stained protein stander, 2. L mexicana lysed, 3. L mexicana pure promastigote denatured, 4. L mexicana membrane protein, 5. L. braziliensis sonicated, 6. L. braziliensis membrane protein, 7. L. infantum sonicated, 8. L. infantum membrane protein.

However, homologues proteins were identified between the three Leishmania species. The most relevant result was the recognition of the 63 kDa antigen between *L. mexicana* and infantum, but not in *L. braziliensis* sample.

Table 1. Molecular weight analysis of each band detected during indirect Western Blot for L. mexicana, L. braziliensis and L. infantum using IgY anti L. mexicana as primary antibody.

Leishmania mexicana					
Sonicated parasite		Denatured pure promastigote		Membrane protein	
Band	kDa	Band	kDa	Band	kDa
1	105,2	1	98,3	1	107
2	88,6	2	71	2	88,6
3	63,2	3	49	3	43,7
4	48	4	43		
5	42	5	38		
6	30	6	30		
7	20	7	20		

Leishmania braziliensis				Leishmania infantum			
Sonicated parasite		Membrane protein		Sonicated parasite		Membrane protein	
Band	kDa	Ban-da	kDa	Band	kDa	Band	kDa
1	100	1	43,3	1	98,3	1	93,3
2	46,2			2	63,2	2	84,1
				3	41,8	3	41,8

However, homologues proteins were identified between the three Leishmania species. The most relevant result was the recognition of the 63 kDa antigen between L. mexicana and infantum, but not in L. braziliensis sample.

Indirect ELISA for Qualitative L. Mexicana Recognition

As previously mentioned, antigen gradient concentration was evaluated, from the highest to the lowest sonicated parasite concentration. All antibodies evaluated corresponding to different weeks after immunization provided affinity to the target parasite. As result of continuous chicken inoculations, the IgY ability to recognize increased over time, as shown in (Figure 7) where recognition was higher with W11 antibodies, than with those of W1. In this context, the maximum detection capacity was obtained with IgY corresponding to week eight, referring to significant difference results with p value less than 0.05.

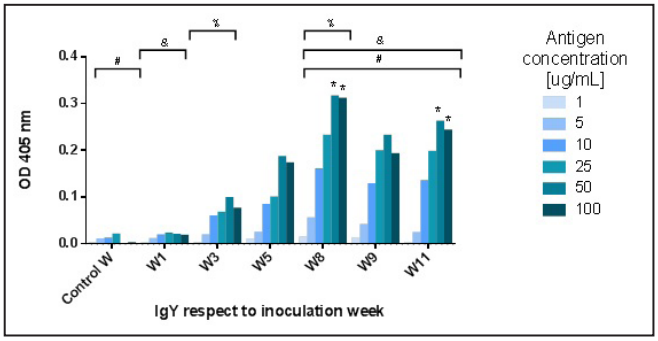


Figure 7: Indirect ELISA results with IgY anti L. mexicana for parasite antigen. Tuckey Test for multiple comparison with p=0.05 resolved: # Significant difference with IgY recognition between Control week and W8, W9 and W11 (p<0.0001; p=0.0032; p=0.0013). & Significant difference with IgY recognition between W1 and W8, W9 and W11 (p<0.0001; p=0.0064; p=0.0026). %

Significant difference with IgY recognition between W3 and W8 (p=0.0028). References: OD: optical density, W: week.

The Tukey Test p=0.05 for a multiple comparison in order to analyze the optimal concentration of antigen showed the best recognition with 50 µg/mL and the lowest with 5 µg/mL of sonicated L. mexicana. The difference was mainly observed with antibodies from W8 (p= 0.013) and from W11 (p=0.029), but graphically observed since IgY in W3 (Figure 7).

This test generated a cutoff value of 0.17 OD an AUC of 0.82 (p value= 0.0015), a sensibility and specificity of 88 and 50% respectively. Finally, a two-way ANOVA, revealed a highly significant influence among time and the IgY antigen detection capacity with a p value less than 0.0001.

For application purposes, the maximum and minimum functional dilution of the antibody were tested, knowing that the most specific antibodies correspond to week 8. Dilutions from 1:50 to 1:5000 with a gradient from 5 to 15 µg/mL of antigen showed the following results in the indirect ELISA test (Figure 8). In comparison, the highest dilutions 1:4000 and 1:5000 had the lowest detection capacity, which were equivalent to the detection of the IgY Control. For that reason IgY can be functional in dilutions from 1:50 to 1:2000. Additionally, between 1:500 and 1:1000 dilution didn't show any significant difference in recognition of L. mexicana, suggesting these concentrations for further applications.

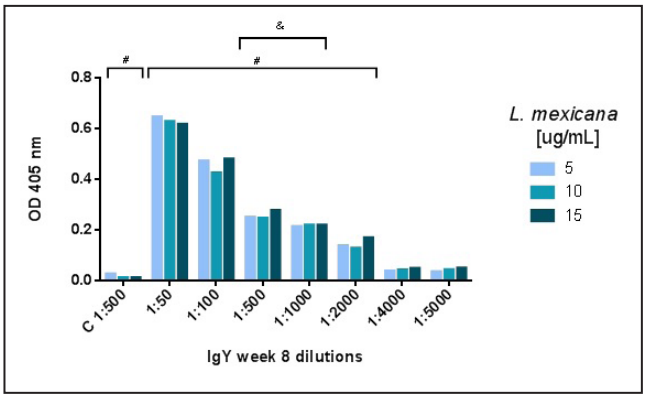


Figure 8: Maximal and minimal functional dilution of IgY anti L. mexicana assayed in indirect ELISA for its antigen. Tukey test for multiple comparison shown: # Significant difference between IgY Control and dilutions from 1:50 1:2000 (p< 0.0001). & No significant difference between IgY 1:500 and IgY 1:1000 dilution.

Indirect ELISA for qualitative recognition of other Leishmania species

L. braziliensis as well as *L. infantum* were detected by IgY anti L. mexicana. Ensuing the previous ELISA results, W8 and W11 IgYs in 1:1000 concentration was used, generating results in (Figure 9) For *L. braziliensis* either IgYs from W8 or W11 were able of detect this species of parasite, but detection was stronger when parasite concentration was higher, providing a significant difference for 15 µg/mL p value <0.0001 (Figure 9A). However, with *L. infantum,* it could be detected with minimum antigen concentration of 10 to 15 µg/mL but recognition was more effective with IgYs from W8 (Figure 9B).

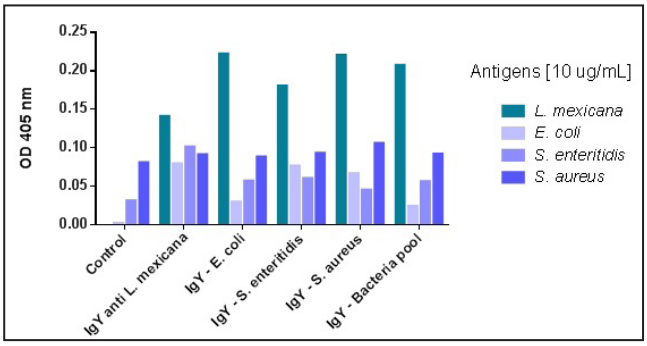


Figure 9: Indirect ELISA results using IgY anti L. mexicana to detect other Leishmania species as L. braziliensis and L. infantum. W: week

For this test threshold value gave 0.02 OD, and an AUC of 1 with p value < 0.0001.

Cross Reactivity of IgY anti L. Mexicana Against Enteric Chicken Bacteria

As result of the characteristics of polyclonal antibodies, its capacity to detect current bacteria in the microbiota was assumed to be similar. Therefore, a prior "blocking process" was applied, where the IgY antibodies were incubated with bacterial antigen and obtained further purified immunoglobulins, remaining the most specific anti L mexicana IgY. The indirect ELISA results are demonstrated in (Figure 10).

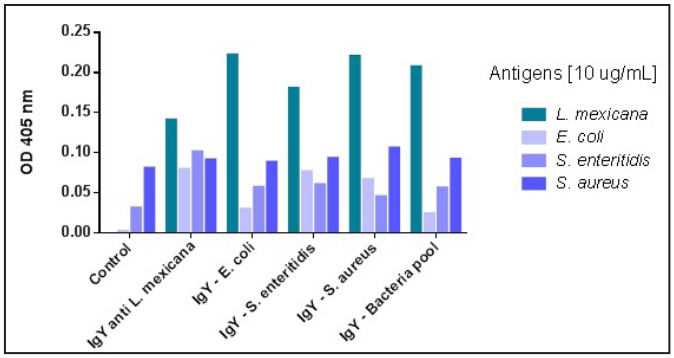


Figure 10: Cross reaction evaluated with indirect ELISA using IgY anti L. mexicana with enteric bacteria as antigen.

Non-blocking IgY detected parasite as well as bacteria accordingly to polyclonal nature. Therefore, after the blocking process, the ability to detect bacteria was decreased, and the specificity of L. mexicana was dramatically increased from 0.1 OD to a maximum 0.22 OD, twice detection specificity.

Cross Reactivity Using IgY anti L. Mexicana for Trypanosoma Parasite

L. mexicana correspond to the *Trypanosomatida* family like *Trypanosoma* parasite. For this reason, IgY anti *L. mexicana* was used in indirect ELISA to identify its immunogenic capacity to detect homologous proteins. In (Figure 11), the results displayed specificity of IgY anti *L. mexicana* to its antigen, due to the low recognition to *Trypanosoma*. In percentage, IgYs had a 26% of cross-reaction to *Trypanosoma* at concentrations above 10 µg/

mL. A significant difference was obtained comparing L. mexicana detection with the three different Trypanosoma concentration 10, 15 and 20 µg/mL, and generating p values of 0.016; 0.015 and 0.036 respectively.

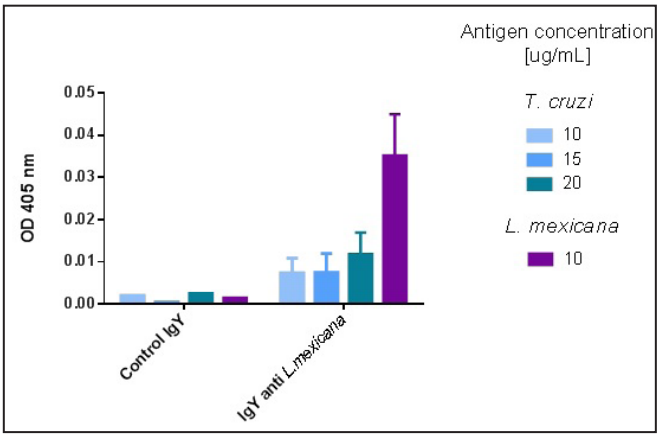


Figure 11: Cross reactivity evaluated with IgY anti L. mexicana for detection capacity of other parasites from Trypanosomatida family as Trypanosoma. * Significant difference between L. mexicana detection with Trypanosoma in 10, 15 and 20 µg/mL concentrations (p value= 0.016; 0.015 and 0.036)

The cutoff value for this test was set 0.021 OD, validating only L. mexicana as a positive detection. Finally, an AUC of 0.92 obtained with a p value of 0.0007.

Discussion

Inoculation of Lohmann Brown hens with a parasitic antigen has proven to be an effective alternative to obtain a continuous source of antibodies [14]. A single hen, ideally, could lay at least 300 eggs during its lifetime and these could be kept for a year at 4°C to preserve IgY concentrations and biological function [15].

Some differences were evident in the posture of the eggs. Low egg production during initial phase may be linked to adaptation of the animals to small areas as Campbell, Lee mentioned [16]. To prevent periods without available samples, a quarantine period has been defined for the control, ensuring the acclimatization of the animal. Before this period, the number of eggs increased and favored the collection of samples (Figure 3).

The adaptation to cages is not only linked to space, but also with recreation objects, food and water dispensers. The closer the feeders, the more the ingested food, guaranteeing correct nutrition during the laying cycles [16]. The water was also considerate, for this reason, it was disinfected weekly with veterinary products of iodine and chlorine, to prevent intestinal infections which could impact on the animals and the results of the investigation.

The sexual maturity in chickens develops during 18 and 24 live weeks, which is important fact to ensure oviposition and IgY concentration in egg yolks [17,18].

The efficient production of antibodies depends of several factors like immunological strengthening, age and physiological state. As Oláh, Nagy said, over-aged chickens may have an ineffective fabricious Bursa to produce enough immunological cells [19]. Animal nutrition is also essential to stabilize daily energy quote in order to maintain immunological reactions, as well as to produce whole eggs with considerable protein concentrations [10]. During the adaptive immune response, nutritional requirement increases and decrease only when adaptive signaling is fully activated [20].

The route of inoculation used in this investigation was intramuscular with temporal frequencies of seven days with different phases. Proceed with a total of six inoculations starting with a complete Freund's adjuvant and succeeding with incomplete Freund's adjuvant. These adjuvants are used to potentiate antigen immunogenicity, releasing antigen continuously in the animal system to ensure the immune responses linked to the titers antibodies in blood [21]. In this way, adjuvants are a relevant factor to the optimal production and to obtaining immunoglobulins [10]. This efficacy has been proven during memory and adaptive immune responses to produce IgYs, as seen in latency phases in (Figure 4).

According to other authors such as: Grando, Baldissera, Esmailnejad, Abdi-Hachesoo and Montini, Fernandes, the intramuscular route has proven effective for this experimentation, considering that subcutaneous and intradermal route could also be attempted, but IgY production could be equivalent in all three [10,17,22]. The characteristics of the antigen are crucial for active immune responses, for parasite and complete cells, the intramuscular route is recommended, which is demonstrated by using hole L. mexicana parasite and heat inactivated [10].

Inoculation frequencies are also a factor in the production of antibodies. IgY has half time in plasma of 4.1 days, for this reason, inoculations were produced every seven days as Schade, Calzado suggests, supporting a continuous production of IgY [3,23].

The adaptive immune responses are generated by two different pathways, one of them the humoral adaptive signal, responsible for the production of immunoglobulins and the other, cellular adaptive signal where macrophages, B and T lymphocytes appear [24]. In this protocol, B lymphocytes were activated, proliferated and stimulated for the production of antibodies in memory response by simply using animal immunizations [25].

Normal IgY concentrations in eggs are 5 mg/mL and these could be concentrated 1.7 times in yolks [26]. According to Grando, Baldissera on average, IgY could be obtained at 8.5 mg/mL per egg yolk and according to Esmailnejad, Abdi-Hachesoo IgY could reach a maximum value of 25 mg/mL. In our results, IgY had a maximum mean titer of 6.5 mg/mL. This determine an increment of three times compared to IgY control, resulting in a higher increase than reported from Murai [17,22,26].

The results presented in this trial agree with the report by Hartle, Magor, where is mentioned that IgY could be obtained in a concentration between 5 and 7 mg/mL and that the IgY in yolk would be proportional to IgY in blood plasma [27]. Amro, Al-Qaisi have also reported the existence of chicken breeds with stronger immune responses to antigens, resulting in larger antibody titers [15].

As Grando, Baldissera IgY production described for Trypanosoma cruzi, Pure tripomastigote in 1x104 parasite concentration was used and it was enough to raise antibodies. Meanwhile, in this study, a parasite concentration of 1x107 promastigote/mL was applied inactivated as an antigen, considering a higher concentration than the used for T. cruzi [22].

Schade, Calzado indicate for a memory-related humoral immune response, it must be developed in time intervals of 10 to 15 days, this evidence responded to our increase in antibodies after the latency phase and the booster inoculation in W9, being sufficient to trigger rapid production of IgY and maintenance up to W11 [3]. The antigen-specific IgYs are found in egg yolks since the fourth or sixth day after the first immunization in accordance with the results presented, because after week 1 and all weeks remaining, antibody concentrations were higher than Control IgY [27, 28].

A combined process of isolation and purification to obtain the maximum concentration of antibodies, was used taking into account the criteria of purity and biological activity [29]. The process started with yolk delipidation with apple pectin which released the most IgYs in the aqueous phase. In combination, natural gum and pH solution guarantee the recovery of antibodies. Pectin interacts with polysaccharides and egg yolk lipoproteins by ionic reactions, hydrophobicity and Hydrogen bonds, for this reason, pH is so important to gelling and precipitating lipids [11].

To precipitate proteins in the aqueous phase, Ammonium sulfate (NH4)2SO4 , has been effective concentrating and maintaining the biological activity of antibodies [29] but it has reduced the purity of IgY, for that reason an affinity chromatographic column was applied [11]. The HiTrap column generates a thiophilic reaction mediated by 2 mercapthopiridine as ligand [30]. In our results, we obtained a recovery 70%, as indicated by the column insert.

In relation to the structure of immunoglobulins, we have identified the mean IgY components like heavy chains, light chains, heavy and light variable regions and CDR regions [4,31]. IgY remains together by disulfide bonds and glycosylation sides [31]. Other bands were observed in Western Blot (Figure 5) with molecular weights of 15, 18 and 17 kDa. These resulted by fragmentation in denaturing process. One could be associated with a heavy and light variable region due to its weigh of 25 kDa in monomeric antibodies [31].

IgY anti L. mexicana presented specificity to its antigen exposing it in different ways such as pure promastigote, sonicate parasite and membrane protein. Although by IgYs polyclonality, L. braziliensis and L. infantum were detected in Western Blot (Figure 6). Just for L. Mexicana, proteins of 48 and 49 kDa were detected possibly corresponding to β tubulin, also observing it fragmented into 20 kDa [32]. The 30 kDa protein band could refer to GAPDH fragment and 38 kDa to the complete structure [32]. In relation with GAPDH, it is obvious detected in sonicated and denatured parasite, because it is a metabolic and no constitutive protein and it doesn't appear in the sample of membrane protein.

A membrane protein study done by Lynn, Marr, specified the existence of a common GP63 membrane protein between L. mexicana and L. infantum expressed in promastigote state [33]. This is relevant for our study, because in these species IgY anti L. mexicana recognized a protein with 63 kDa molecular weight and could be compared to the virulence protein responsible for the evasion of fagolisosoma lysis [33].

The phylogeny reports, present the idea that due to the evolutionary events of expansion, GP63 could be different in three families of parasites. That is the reason why GP63 in L. braziliensis is a distinct protein that the common in L. mexicana and L. infantum [34]. For this reason, no 63 kDa protein was detect in L. braziliensis.

For L. infantum as reported by Brotherton, Racine, proteins of molecular weight greater than 100 kDa could be linked to extracellular membrane receptors, or to 88 kDa with transmembrane proteins like GPI16 [35].

ELISA tests are considered to be one of the protocols for detection and quantification with mayor sensibility in immunodiagnostic [36]. This technique is based in antibody affinity to its antigen, because antibody cannot bind to anything else [37]. As seen in ELISA results, the affinity of the IgYs increased respect to time, observing a stronger recognition reaction in W8, W9 and W11. This underlines the humoral stimulation related with memory and specificity [37].

According to ELISA results in (Figure 7), the ROC test provide an AUC of 0.82 explaining a discrimination capacity of 82%, this value is acceptable between the range as described by Fan, Upadhye [38]. Threshold value proposed by Lardeux, Torrico [39], 0.17 OD determined the positive recognition of L. mexicana with antibodies of weeks 5,8,9, and 11.

For maximum and minimum ELISA detection capacity (Figure 8), as pointed out by Cakir-Koc the dilution of anti-Toxoplasma gondii IgY less than 1:3000, capacity detection of antigens decreased considerably [40]. As we also see in IgY anti L. mexicana and proposed an ideal concentration of 1:500 or 1:1000.

As expected, polyclonal antibodies could interact with homologous proteins for this reason not only L. mexicana is recognized, but also L. infantum, because between them only 26% of proteins are different and the remaining 86% correspond to proteins homologs and could be detected as happened in ELISA (Figure 9) [33].

Referring to the cross-reactive ELISA, enterobacteria as Escherichia coli and Salmonella enteritidis are the most common microorganism found in poultry, which causes many difficulties in this industry, in addition to affinity, the blocking process could reduce bacterial detection and increase the recognition of L. mexicana (Figure 10) [17]. In the end, a strong affinity was observed with Staphylococcus aureus but this is not caused by homology relationship between Leishmania, it could be linked to a previous interaction between animals and bacteria developing memory immunogenicity [41].

Finally but no least important, L. mexicana and T. cruzi belong to the same parasite family: Trypanosomatidae, both responsible for neglected tropical diseases, carried out in a vector – vertebrate host cycle and escape the immune system by different mechanisms

[42]. Leishmania interacts with different major surface proteins like GP63, Lipophosphoglycan LPG or Glycosyl inositol phospholipids GPIs with host cells. In contrast, Trypanosoma interacts with gp82, gp35/50 and GPIs. As a result, IgY anti L. mexicana may be able to recognize homologous proteins, but these are not sufficiently similar to be detected. As shown in (Figure 10), only the recognition of L. mexicana was considered positive and demonstrating the specificity of IgY.

Conclusions

In summary, Leishmania mexicana inactive promastigote was sufficient to be used as an antigen for Lohmann Brown laying chickens, applying it intramuscularly in an eleven weeks model. Antigen developed a successful immunological boost to produce Y immunoglobulins with considerable quantities, effectively taken from those concentrated egg's yolks by maternal transfer and providing eggs as antibody resource.

Maximum concentration of IgY was obtained from week 9, which gave an average of 6.3 mg/mL per yolk, these results were obtained using effective standard methods like pectin delipidation, protein precipitation with Ammonium sulfate and thiophilic purification with affinity liquid chromatography. Demonstrating how the isolation could be effective in both ways, quality and quiantity. For structure analysis, IgY had a final molecular weight of 175 kDa with complete conformation: heavy chains (67 kDa) and Light chains (35 and 22 kDa), indicating the most relevant antibody characteristics.

Immunoglobulins Y had the ability to recognize L. mexicana, with a minimal concentration of 5 to 50 µg/mL. The most effective antibodies were obtained from week 8 and 11, working in solutions until 1:2000 for Western Blots and ELISAs, effectively working because of their antigen specificity. Finally, the antibodies were able to recognize constitutive, metabolic and pathogenic proteins of the parasite like β globulin and GP63. An affinity with other Leishmania species has been observed, mainly with L. infantum because it corresponds to the same evolutionary family and due to policlonal antibody characteristic.

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