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Production and Evaluation of Immunoglobulin Y Anti-*Brucella abortus* (Vaccinal Strain B19)

Pollyanna Mafra Soares, Mayara Mafra Soares, Mariane Pacheco dos Santos Lourencetti, Muriell Ribeiro Ganda, Mariana Assunção de Souza, Tatiane Cristina Fernandes Tavares, Álvaro Ferreira Júnior & Anna Monteiro Correia Lima

ABSTRACT

***Background*:** The immunoglobulin Y is a principal antibody current in the blood of hens, which are transferred from the maternal blood serum to the egg yolk. The extraction of IgY from the egg yolk apply animal welfare when compared to the extraction of IgG, reducing the number of animals and prevent a bleeding of hens through the extraction of the IgY from eggs, besides that IgY presenting high specificity for antigenic binding. The objective of this study was to produce specific polyclonal IgY antibodies anti-*Brucella abortus* by immunizing hens with B19 vaccine and evaluate their reactivity through Buffered Acidified Plate Antigen (BAPA), 2-Mercaptoethanol (2-ME) and indirect ELISA diagnostic tests.
***Materials, Methods & Results*:** Four 25-week-old White Leghorn hens were immunized, two of them comprising the control group (Group 1) with phosphate-buffered saline (PBS) with adjuvant, and the others two immunized with B19 vaccine (*Brucella abortus* vaccine strain B19), representing the Group 2. The immunizations occurred six times with a 15-day interval between each. Blood samples were taken biweekly (seven times); and daily, the eggs were collected for 13 weeks, the first collection of blood and eggs, performed one week before the first immunization of each group. The IgY was purified from egg yolk, using the method of dilution in acid water and precipitation with ammonium sulfate for delipidation. BAPA, 2-ME and ELISA tests performed to verify the specificity of IgY confirmed the reactivity of polyclonal antibodies specific to the antigen used both in blood serum samples and in the purified egg yolks. The hens from the control group did not present reactivity in the diagnostic tests used, which was already expected, since no antigen was used in any of their immunizations. Hens immunized with the *Brucella abortus* B19 vaccine produced detectable reactive antibodies in the three tests used on blood serum and IgY samples extracted from the egg yolk. In Group 2 (vaccine B19), blood serum samples started to react one week after the first inoculation, and the IgY samples extracted from the egg yolk were reagent two weeks after serum IgY appear reactivity, showing the transfer of specific antibodies to the egg yolk, was late.
***Discussion*:** Although the transfer of serum Igy to egg yolk was late when compared to others authors which found that the transovarian passage of immunoglobulin Y occurred in approximately three to six days after IgY being detected in blood serum, the results of this study showed the occurrence of the transfer of blood serum IgY anti-*Brucella abortus* to egg yolk of hens immunized with B19 vaccine, the same found by others researches found the same results with others antigens. Thus, it can be concluded that immunoglobulins Y produced in this study can be used as specific antibodies in diagnostic tests for the detection of the *Brucella abortus* antigen, in addition, this process guarantees the welfare of the animal, since it avoids bleeding and it is possible to obtain high concentrations of antibodies directly from the hen egg, which is a great advantage, because IgY can be easily isolated from the egg yolk by the precipitation technique discarding the need of invasive and painful procedures that involve bloody interventions to obtain the serum antibodies like occur in mammals for extraction of IgG.

Keywords: antibodies, immunodetection, chicken egg.

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Faculty of Veterinary Medicine, Federal University of Uberlandia, Uberlandia, MG, Brazil. CORRESPONDENCE: P.M. Soares [pollymafra@yahoo.com.br - Fax: +55 (34) 32258644]. Faculty of Veterinary Medicine, Federal University of Uberlandia (UFU). Rua Ceará, s/n. Bairro Umuarama. CEP 38405-315 Uberlandia, MG, Brazil.

INTRODUCTION

Antibodies purified from the egg yolks (IgY) of immunized hens, have proved useful in various applications, including immunodiagnostics, immunotherapy and proteomic studies, also serving as a tool for the purification or detection of antigens and as a protective agent in passive immunization [12,17].

Compared to the conventional IgG production technology, several characteristics of IgY make its use as an immunobiological agent advantageous, among them the animal welfare, because IgY technology reduce the painful handling of animals, since collecting eggs is a simple non-invasive method and reduces the number of animals used in the production of antibodies; and its economic viability [12,16]. In addition to producing IgY quickly and in large quantities, hens maintain high levels of antibodies for a long period and IgY are also molecules with high specificity for binding in antigens that can invade the body [12].

Given the numerous advantages of IgY antibodies, the objective of this research was to produce immunoglobulin Y anti- Brucella abortus by immunizing hens with B19 vaccine and evaluating the reactivity of these specific polyclonal antibodies in Buffered Acidified Plate Antigen (BAPA), 2-Mercaptoethanol (2-ME) and indirect ELISA diagnostic tests.

MATERIALS AND METHODS

Animals and immunization protocol

This research was conducted at a location intended for poultry-related experiments in Federal University of Uberlandia. Four 25-week-old White Leghorn laying hens were used. The animals were housed in a facility suitable for animal experimentation, with ad libitum access to food and water. The four hens were divided randomly into two experimental groups of two animals, each group in its own cage. The group 1 was immunizated with PBS plus adjuvant (Freund's adjuvant1) and the group 2 was immunizated with B19 vaccine diluted in PBS plus adjuvant.

The immunization scheme was carried out at 15 day intervals. To carry out the immunizations was used as protocol in the first application, the complete Freund's adjuvant1 composed of a water-oil emulsion containing *Mycobacterium* sp. was used, while in the remaining applications the incomplete Freund's adjuvant1 was used, whose composition did not contain *Mycobacterium* sp., corresponding to 50% of the total volume of the solution [4]. A total volume of 500 µL (250 µL of antigen diluted in PBS and 250 µL of adjuvant) was injected at four different points deep in the pectoral muscle of the chickens, and post-vaccination reactions were monitored. The two groups were vaccinated six times, group 1 was immunized with 250 µL of PBS, and Group 2 with 250 µL of B19 vaccine (smooth sample of *Brucella abortus* vaccine strain B19) diluted in PBS.

A total of seven blood samples of 1 mL were collected from the wing (ulnar vein) at 15-day intervals, the first sample 1 week prior to the first inoculation and the others 1 week after each inoculation. The eggs were collected daily, starting a week prior to the first inoculation (pre-immunization). The collected eggs were separated per week and stored at 4ºC until the delipidation process was performed. The purpose of the first blood and egg collection, a week prior to the first immunization (pre-inoculation), was to check that the hens were non-reactive to the antigen used in this experiment.

Extraction and purification of polyclonal IgY

These process were carried out Laboratory of Infectious and Contagious Diseases of the Federal University of Uberlandia (LADOC/UFU). Egg yolks were carefully separated from the whites and washed with ultrapure water to remove all traces of albumen. The yolk membranes were then broken and the yolks placed in 50 mL conical flasks, which were stored at -20ºC until the moment of purification. At the end of the procedure, a weekly pool of yolks was obtained from each of the groups of immunized hens.

The beginning of the extraction and purification processes of total IgY antibodies took place through delipidation, which is performed to remove the lipid fraction of the yolk, like protocol [1] with some adaptations. The pure egg yolk was diluted with ultrapure water in a ratio of 1:15 (v/v). After homogenization, the pH of the mixture was adjusted to 5.0-5.2 by means of a 0.1N HCl drip, after which the acidified mixture was stored overnight at 4°C. After this period, the solution was centrifuged at 10000 *g* for 15 min at 4°C. The pellet containing the lipid rich fraction was discarded and the supernatant containing the total antibodies was filtered (Millipore filters 0.45 µm and 0.25 µm). The pH of the filtered sample was adjusted to 7.4 with 10X PBS and stored at 4°C. After delipidation, precipitation was carried out with ammonium sulfate[5],

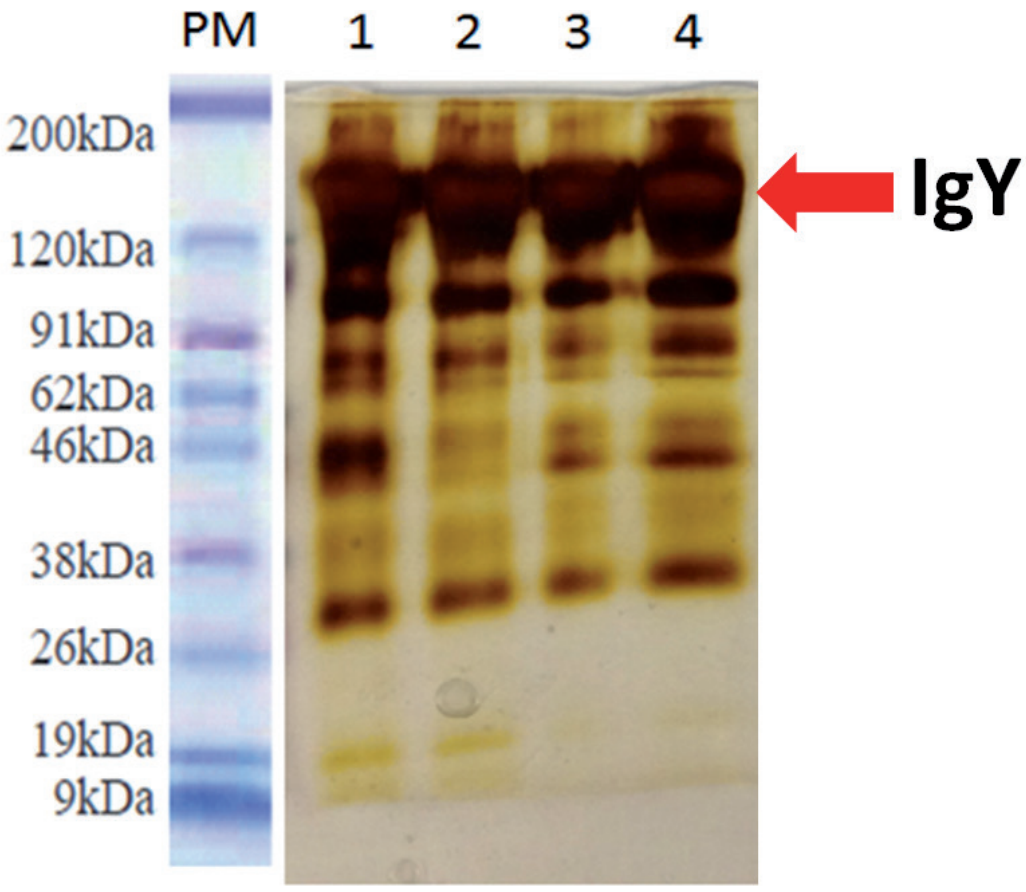

Figure 1. Electrophoretic profile of IgY extracted from egg yolk and purified with ammonium sulfate, using silver stained gel. MW - molecular weight standard (AMRESCO), 1 (10 µg of purified IgY, week 11, Control Group); 2 (10 µg of purified IgY, week 9, Group B19); 3 (10 µg of purified IgY, week 10, Group B19); 4 (10 µg of purified IgY, week 11, Group B19).

with adaptations. A concentration of 20% (w/v) of sulfate was added to the filtered sample and stirred for 30 min at 4°C. The material was then centrifuged at 2000 *g* for 30 min at 4°C, the supernatant was discarded, and the pellet resuspended in 1x PBS, pH 7.4.

The final product of the samples was subjected to dialysis in a centrifuge tube with filter (AMICOM Ultra-15 Centrifugal Filter Unit)[2]. The sample, along with PBS at pH 7.4, was centrifuged at 4000 *g* for 30 min at a temperature of 4ºC; this procedure was performed three times. After concluding the extraction of total IgY, the protein concentrations in all the fractions were quantified. The purity of the preparations was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using 16% w/v polyacrylamide gel and applying 10 µg of sample per well. The average protein level obtained was approximately 18.5 µg/µL. The protein bands were visualized by staining the gel with silver. IgY was extracted and purified from the egg yolks of the hens but not from their blood serum.

Serology of the hens

The humoral immune response of the vaccinated hens was evaluated at LADOC/UFU laboratory based on the official brucellosis diagnostic tests specified by Brazil's National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCE-BT) according by [6], namely, buffered acidified plate antigen (BAPA) and 2-Mercaptoethanol (2- ME); and the indirect ELISA test, with the antigens used for inoculation of the two groups.

For the BAPA test, individual samples of serum were used from the 7 samplings collected from the two immunized groups, as well as from the weekly pooled IgY samples extracted from the yolks of the eggs collected daily from each group for 13 weeks. And for the 2-ME test, only weekly pooled IgY samples extracted from the yolks of the eggs collected daily from each group for 13 weeks were used. The 2-ME test was not performed on the individual samples of blood serum from the hens in each group, because the amount of serum was insufficient for testing.

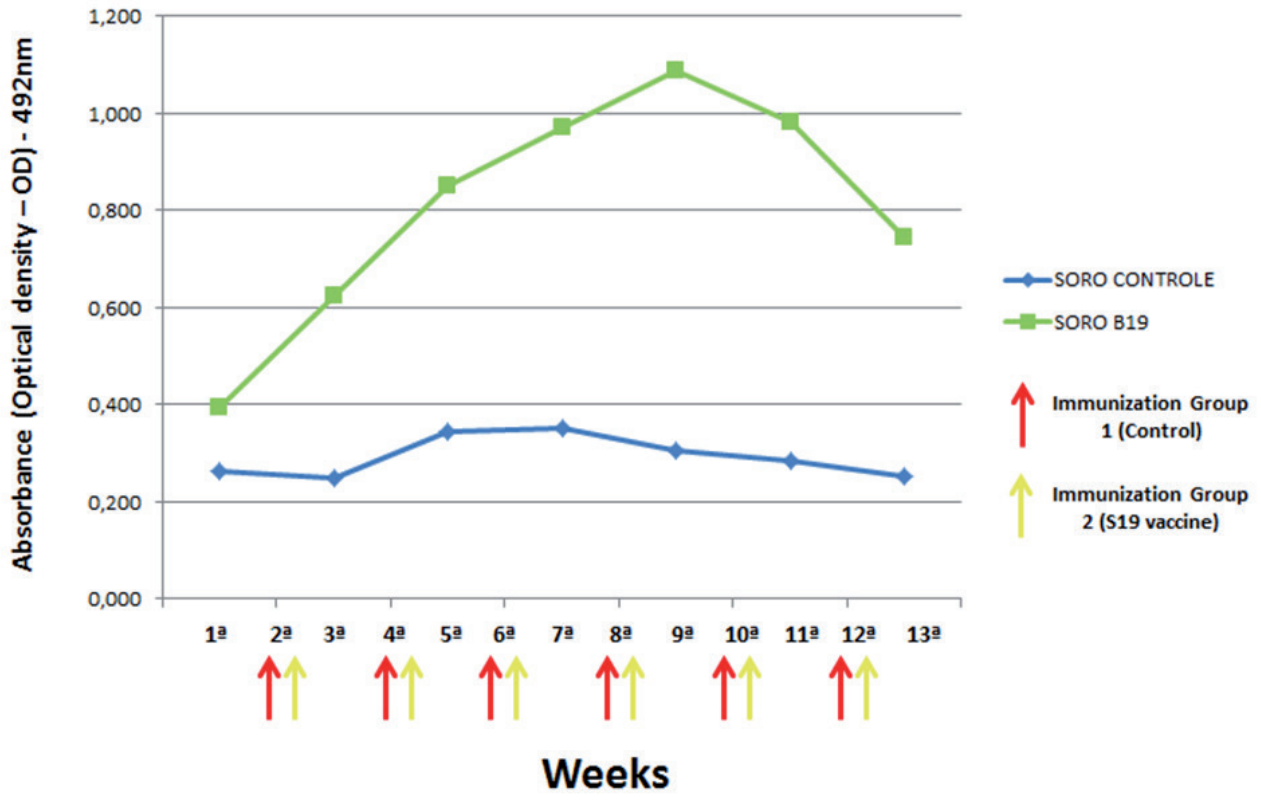

Figure 2. IgY absorbance curve in the samples of blood serum from the chickens of control and B19 groups obtained by indirect ELISA, using B19 as antigen, for 13 weeks.

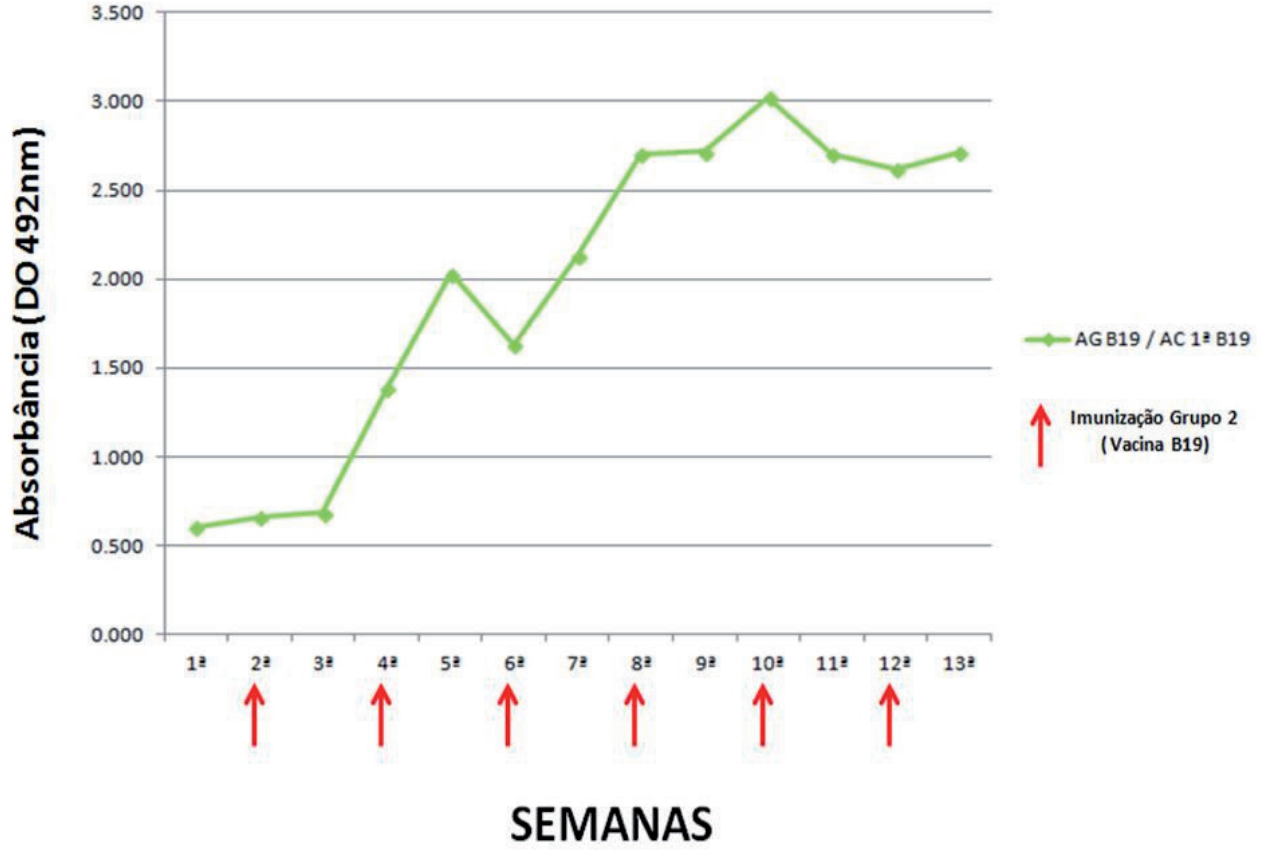

Figure 3. Production kinetics of IgY extracted from egg yolks of chickens immunized with the B19 vaccine antigen obtained by indirect ELISA for 13 weeks.

Indirect ELISA to confirm the specificity of IgY antibodies

Two polystyrene microtiter plates (MaxiSorp)3 were sensitized with 50 µL/well of B19 vaccine diluted 1:100 in carbonate-bicarbonate buffer (NaHCO3 0.1M, pH 8.6) and were incubated overnight at 4ºC for 16 h. The next day, the contents of the plates were discarded, the plates were blocked by adding 300 µL/well of phosphate-buffered saline (0.01 M PBS, pH 7.4) plus 5% skim milk (PBSM 5%), and then incubated for 2 h at 37ºC. After incubation, the microtiter plates were washed three time with PBS and 50 µL/well of primary antibodies previously diluted 1:100 with PBS containing 0.05% Tween and 5% skim milk (PBSTM) were added to the plates, which were then incubated for 1 h at 37ºC. On one of the plates, the primary antibodies sensitized with B19 came from a pool (7 collections) of IgY from the blood serum of hens immunized with B19 vaccine and that of the control group. The other microtiter plate contained antibodies from a pool (13 weeks) of IgY from the egg yolks of hens immunized with B19 vaccine and from the yolks of the control group.

After one hour of incubation, the plates were washed three times with PBS containing 0.05% Tween (PBST 0.05%), followed by the addition of 50 µL/well of peroxidase-labeled antibodies of rabbit IgG conjugated with anti-chicken IgY (Sigma) at dilutions of 1:5000 diluted in PBSTM, and incubated at 37ºC for 1 h. After incubation, the plate was washed three times with 0.05% PBST buffer. To develop the reaction, one o-phenylenediamine tablet (OPD- 15 mg)[4] was diluted in 22.5 mL of MilliQ water and 9.75 µL of hydrogen peroxide. Then, 100 µL of this solution was added to each well of the plate, which was kept away from light for 15 min. The reaction was then stopped with 4N sulfuric acid (25 µL), and a reading was taken at 492 nm on a microplate spectrophotometer (Thermo Plate TP- Reader)5. Antibody levels were analyzed based on the recorded absorbance values.

Statistical analysis

The data obtained in this study were analyzed and compared from the descriptive statistics.

RESULTS

The analysis of the molecular weight (MW) profile of IgY from Groups 1 and 2 through one-dimensional electrophoresis indicated that the protein band of IgY ranged from120 kDa to 200 kDa (Figure 1). The IgY purified from egg yolk (Figure 1) showed that the purification process with 20% ammonium sulfate was not 100% efficient, since other protein bands besides IgY were visible in the electrophoresis gel.

Serological tests such as BAPA, 2-ME and indirect ELISA determined the hen IgY reactivity to the inoculated antigens. The hens of the control group (Group 1) did not reacted to the BAPA test. However, the two hens of the group immunized with B19 vaccine (Group 2) began to react to the BAPA test starting from the 2nd collection (one week after the first inoculation) and continued to be reactive up to the 7th collection (week 13).

In the BAPA and 2-ME tests performed with samples of IgY extracted from the weekly pool of egg yolks, Group 1 (Control) did not respond to BAPA and 2-ME tests, but Group 2 (B19 vaccine) was reactive to the BAPA test from week 5 up to week 13. Moreover, in the 2-ME test, Group 2 was reactive with a titer of 200:200 starting in week 8 up to week 13, although the reaction of this group to the 2-ME test was inconclusive from week 5 to week 7.

In the indirect ELISA performed on serum samples from hens, it was observed that starting from the 1st week post-immunization, Group 2 showed an increase in the titers of anti-B19 specific antibodies, and a higher peak in the concentration of antibodies one week after the 4th immunization (week 9) [Figure 2]. When tested with the B19 antigen in the indirect ELISA, the concentration of antibodies in the control group (Group 1) was low and remained low throughout the study, indicating negligible specific activity, and could therefore be used as a negative control in subsequent studies.

The production kinetics of IgY extracted from egg yolks of Group 2 was also evaluated, as shown in Figure 3, presenting a considerable increase in the absorbance values. However, it should be noted that there was a drop in antibody production after the 10th week.

DISCUSSION

The reports of several authors contain divergent values for the molecular weight (MW) of IgY. Previous studies report a MW of 167.25 kDa for IgY [7,8], of 180 kDa [23,25] and a MW of 190 kDa [9], while [15] report a MW of up to 206 kDa. Because of the disparate MWs, most authors established a molecular weight of about 180kDa [2,23,26]. The data found in this study is close to this value, since the protein band corresponding to IgY showed a MW ranging from 120 to 200 kDa.

The IgY purification methods described comprise two steps: delipidation of the egg yolk, which consists in separating the livetins from the other lipoproteins, and purification of the aqueous extract to separate the IgY from other components [6]. Purification of IgY from the aqueous extract is performed after the delipidation step. There are three methods widely described in the literature, which are performed separately or in combination, namely, salt precipitation, affinity chromatography, and ultrafiltration [3].

The PEG-6000 and 25% ammonium sulfate precipitation methods were analyzed in previous studies, which found that the PEG-6000 method was more effective than the ammonium sulfate method because it was able to extract IgY from egg yolk with a high degree of purity [5]. Those findings are consistent with the data found in this study, in which the ammonium sulfate precipitation method did not prove to be totally efficient, but on the other hand, the results found in this study are not consistent with [3,20], who claim that ammonium sulfate precipitation is the most efficient method of purification.

With regard to the BAPA test performed on serum samples from the hens of Groups 1 and 2, it was expected that those of Group 1 would be non-reactive, since no antigen was used to immunize them. The hens of Group 2 (B19 vaccine) began to react one week after the first immunization and remained reactive until the last sampling after the sixth inoculation. This is consistent with the findings of [14], who reported that eight hens inoculated with a suspension of *Brucella abortus* were reactive to the BAPA test one week after immunization, and that six were sacrificed on day 15, while the remaining two were analyzed for response to the BAPA test up to day 43, and remained positive until the last serum sampling.

Other studies have also observed that hens can produce high-avidity antibodies after the first immunization. This production depends on several variables, including type of antigen (dose and molecular weight), the adjuvant, the route of administration, the animal genetics and type of animal husbandry [21]. The results demonstrated that the variables used on Group 2 were suitable for the production of antibodies in the week after the first immunization.

The hens response of Group 2 was similar to that of cattle one week after vaccination. The humoral immune response of cattle vaccinated with a B19 sample is characterized by the presence of four major immunoglobulin isotypes. The IgM is produced in the first week post-vaccination, followed soon thereafter by IgG1, which are detectable in the BAPA test [18].

This fact can be explained by the functional similarity between IgY and IgG, besides that the antibody response of hens is usually similar to that of mammals [21]. After a single inoculation of a protein antigen, there is a peak in IgM between the 4th and 8th day, which declines rapidly and is accompanied by the production of IgY antibodies [21].

It is noteworthy that the reactivity of Y immunoglobulins is due to the high specificity for binding with foreign substances, such as antigens, which are able to invade the body [12].

Upon analyzing the results of the BAPA and 2-ME tests on IgY samples extracted from the egg yolks of hens, it was observed that Group 2 remained reactive to the two tests.

A previous study showed that the immunization of a hen resulted in the transfer of specific serum antibodies to the egg yolk [13]. The transovarian passage of IgY takes approximately 3 to 6 days [19]. The amount of IgY transferred to the egg yolk has been reported to be proportional to the concentration of IgY in maternal serum [11].

The data obtained in the BAPA test of Group 2 in this study is not in agreement with others authors, who discovered that the antibody response of the IgY extracted from the pool of egg yolks of this group was delayed in relation to previously reported data, because it was reactive two weeks after the serum IgY presented a reaction in the test [19].

In the 2-ME test, it was found that Group 2 presented three inconclusive reactions in weeks 5, 6 and 7, showing a titer of 200 in slow agglutination test in tubes and NR (no reagent) in 2-Mercaptoethanol test. This indicates that the concentration of IgY in these samples may have been below the limit of detectability of the 2-ME test, since in the weeks after week 13, the hens were reactive to the test, presenting a titer of up to 200:200.

The ELISA results of Group 2 performed with hen serum were similar to others researches which reported that hens begin to produce antibodies after the first inoculation, and that within a few days, these antibodies can be found in their egg yolks [10]. The antigen used to immunize the hens was able to perform antibody activity, as shown in Figure 2.

B19 antigen is comprised of proteins with molecular weights greater than 1000 Da, thus activating the immune system of hens and production of antibodies. Substances with molecular weights of less than 1000 Da are not able to elicit the production of antibodies. Essentials factors for the production of antibodies are macromolecules such as proteins and polysaccharides highly immunogenic and the ability of the immunized animal to recognize the antigen and produce proteins with high affinity and specificity [22].

With respect to the indirect ELISA performed on the IgY samples extracted from the egg yolks of Group 2, the observed increase in the absorbance values coincides with the response obtained in the BAPA test of the IgY extracted from the pooled egg yolks. Once again, this indicates that the transfer of serum antibodies to the egg was delayed in comparison to that reported in other researches [17], because this study indicated that the detection of antibodies in the yolk occurred about fifteen days later than in serum.

This study demonstrated that hens immunized with strain B19 produced IgY antibodies reactive to this antigen, which were detectable by indirect ELISA and by official tests for the diagnosis of bovine brucellosis, BAPA and 2-ME, representing a good choice for the production the polyclonal antibodies, like showed in study with the production of high titers of specific polyclonal IgY antibodies to Leptospira [24].

CONCLUSION

IgY antibodies produced by stimulation of B19 vaccine antigen can be considered useful tools in diagnostic tests aimed at detecting the antigen, given their satisfactory response to the tests evaluated in this study, and the fact that they ensure the animal welfare.

MANUFACTURERS

[1]Sigma-Aldrich Co. St. Louis, MO, USA.
[2]MilliporeSigma Life Science Center. Burlington, MA, USA.
[3]Thermo Scientific Scientific. Waltham, MA, USA.
[4]Amresco Inc. Solon, OH, USA.
[5]Shenzhen Poly Colorte Printing Co. Ltd. Shenzhen, China.

Ethical approval. This experiment was approved under the guidelines of Ethics Committee for Animal Use of Federal University of Uberlândia, under Registration No. 071/12.

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Declaration of interest. The authors declares that there is no conflict of interest.

REFERENCES

1 **Akita E.M. & Nakai S. 1993.** Comparison of four purification methods for the production of immunoglogulins from eggs laid by hens immunized with a enterotoxigenic *Escherichia coli* strain. *Journal of Immunological Methods*. 2(160): 207-214.

2 **Akita E.M., Li-Chan E.C. & Nakai S. 1998.** Neutralization of enterotoxigenic Escherichia coli heat-labile toxin by chicken egg yolk immunoglobulin Y and its antigen-binding fragments. *Food and Agricultural Immunology.*10(2): 161-172.

3 **Araújo A.S. 2007.** Produção de antiveneno botrópico em ovos de galinha. 57f. Belo Horizonte, MG. Dissertação (Mestrado em Ciência Animal) - Escola de Veterinária da Universidade Federal de Minas Gerais.

4 **Barbas III C.F., Burton D.R., Scott J.K. & Silverman G.J. 2001.** *Phage Display: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press, 736 p.

5 **Bernardo A.R. 2009.** Tecnologia IgY: produção de anticorpos aviários para *Leishmainia* (*Leishmainia*) *amazonensis* com o uso ético dos animais de experimentação. 49f. Rio de Janeiro, RJ. Dissertação (Mestrado em Ciências Veterinárias) - Instituto de Veterinária, Universidade Federal do Rio de Janeiro.

6 **Brasil, Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Departamento de Defesa Animal. 2006.** *Manual Técnico do Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose - PNCEBT: legislação.* Brasília: MAPA/SDA/DSA, 188p.

7 **Carlander D. 2002.** Avian IgY Antibody. *In vitro* and *in vivo.* 53p. Uppsala, SWE. Dissertation of Department of Medical Science, Clinical Chemistry, University Hospital, Uppsala, SWE.

8 **Chacana P.A., Terzolo H.R., Calzado E.J.G. & Schade R. 2004.** Tecnologia IgY o aplicaciones de lós anticuerpos de yema de huevo de gallina. *Revista Medicina Veterinaria.* 85(5): 179-189.

9 **Devi C.M., Bai M.V., Lal A.V., Umashankar P.R. & Krishnan L.K. 2002.** An improved method for isolation of anti-viper venom antibodies from chicken egg yolk. *Journal of Biochemical and Biophysical Methods*. 51(1): 129-138.

10 **Guimarães M.C.C., Correia V.G. & Gama Filho R.V. 2008.** Produção de anticorpos em galinhas. *Perspectivas on line*. 2(7): 122-129.

11 **Hamal K.R., Burgess S.C., Pevzner I.Y. & Erf G.F. 2006.** Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks inmeat lines of chickens. *Poultry science*. 85(8): 1364-1372.

12 **Karlsson M., Kollberg H. & Larsson A. 2004.** Chicken IgY: utilizing the evolutionary advantage. *World's Poultry Science*. 60(3): 341-348.

13 **Klemperer F. 1893.** Ueber natürliche lmmunität und ihre Verwerthung für die Immunisirungstherapie. *Archiv für Experimentelle Pathologie und Pharmakologie*. 31: 356-382 apud **Narat M. 2003.** Production of antibodies in chickens. *Food Technology and Biotechnology*. 41(3): 259-267.

14 **Kumar S., Kulshrestha R.C., Bhatia K.C. & Kaushik R.K. 1984.** Brucellosis in poultry – an experimental study. *Internacional Journal of Zoonoses*. 11(2): 133-138.

15 **Leslie G.A. & Clem L.W. 1969.** Phylogeny of immunoglobulin structure and function. Immunoglobulins of the chicken. *The Journal of Experimental Medicine*. 1130 (6): 1337-1352.

16 **Michael A., Meenatchisundaram S., Parameswari G., Subbraj T., Selvakumaran R. & Ramalingam S. 2010.** Chicken egg yolk antibodies (IgY) as an alternative to mammalian antibodies. *Indian Journal of Science and Technology*. 3(4): 468-474.

17 **Narat M. 2003.** Production of Antibodies in Chickens. *Food Technology and Biotechnology*. 41(3): 259-267.

18 **Nielsen K.H., Gall D., Kelly W., Vigliocco A., Henning D. & Garcia M. 1996.** Immunoassay Development: Application to Enzyme Immunoassay for the Diagnosis of Brucellosis. Nepean, Ontario: Animal Disease Research Institute. *O.I.E. Reference Laboratory of Brucellosis. Agriculture and Agri-Food Canada.* Austria: IAEA,16p.

19 **Patterson R., Younger J.S., Weigle W.O. & Dixon F.J. 1962.** Antibody production and transfer to egg yolk in chickens. *Journal of lmmunology.* 89(2): 272-278.

20 **Svendsen L., Croeley A., Ostergaard L.H., Stodulski G. & Hau J. 1995.** Development and comparison of purification strategies for chicken antibodies from egg yolk. *Laboratory Animal Science*. 45(1): 89-93.

21 **Schade R., Calzado E.G., Sarmiento R., Chacana P.A., Porankiewicz-Asplund J., Terzolo H.R. 2005.** Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and human and veterinary medicine. *Alternative to Laboratory Animals: ATLA*. 2(33): 129-154.

22 **Sousa C. M. 2008.** Produção de Anticorpor IgY de galinhas e IgG de coelhos para análise de auxina e citocininas. 61f. Rio de Janeiro. Tese (Doutorado em Fitotecnia). Instituto de Agronomia - Universidade Federal Rural do Rio de Janeiro.

23 **Tan S.H., Mohamedali A., Kapur A., Lukjanenko L. & Baker M.S. 2012**. A novel, cost-effective and efficient chicken egg IgY purification procedure. *Journal of Immunological Methods*. 380(1-2): 73-76.

24 **Tavares T.C.F., Soares P.M., Naves J.H.F.F., Soares M.M., Ferreira Junior A., Souza D.L.N., Ávila V.M.R. & Lima-Ribeiro A.M.C. 2013.** Produção e purificação de imunoglobulinas Y policlonais anti-*Leptospira* spp. *Pesquisa Veterinária Brasileira*. 33(9): 1097-1102.

25 **Vasconcelos G.A.L.B.M. 2010.** Produção de anticorpos IgY específicos para o vírus da hepatite A purificados de gema de ovo de frangas imunizadas e sua possível aplicação em diagnóstico do vírus no fígado. 121f. Rio de Janeiro. Dissertação (Pós-graduação em Biologia Parasitária) - Instituto Oswaldo Cruz.

26 **Warr G.W., Magor K.E. & Higgins D.A. 1995.** IgY: clues to the origins of modern antibodies. *Immunology Today*. 16(8): 392-398.