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Enzyme-linked immunosorbent assay (ELISA) for detection of IgY Anti-*Borrelia anserina* antibodies in *Gallus gallus domesticus**

Ensaio de imunoadsorção enzimático (ELISA) indireto para detecção de anticorpos IgY Anti-*Borrelia anserina* em *Gallus gallus domesticus*

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Abstract

This study aimed to promote the standardization of an indirect, enzyme-linked immunosorbent assay (ELISA) for the serological detection of *B. anserina* in *Gallus gallus domesticus*. An aliquoted sera from vaccinated chicken with *B. anserina* antigen (GI), experimental infected chickens with *B. anserina* (GII) and rustic poultry rearing of *G. gallus* (GIII) were tested with in-house ELISA developed to detect serum antibodies against B. anserina in *G. gallus domesticus*. On average, the experimentally infected chickens became positive at 9 DPI a mean ± standard deviation (SD) ODI value of 163.11 ± 70.65. The highest observed Optical Density Index (ODI) was 372.54 ± 132.39, at 26 DPI, and the highest overall ODI value was 626.51. The vaccinated chickens became positive between 8 and 10 DPV, with an ODI of 245.59 at 10 DPV, with an overall maximum ODI of 543.13. A total of 108 blood samples were collected from poultry raised on rustic farms. Of the total samples collected, 58.33% (63/108) were considered positive for *B. anserina.* The maximum ODI found among these rustic chickens was 283.24. This stardardization provided a sensitivity and specificity of 100%.

Keywords: poultry, borrelioses, argasidae.

Resumo

Este estudo teve como objetivo promover a padronização de um ensaio imunoenzimático indireto (ELISA) para a detecção sorológica de *Borrelia anserina* em *Gallus gallus domesticus.* Um frango vacinado com antígeno de *B. anserina* (GI), frangos infectados experimentalmente com *B. anserina* (GII) e frangos criados de forma rústica (GIII) foram testados com ELISA indireto *in house* desenvolvido para a detecção sorológica contra *B. anserina* em *G. gallus domesticus*. Em média, os frangos infectados experimentalmente tornaram-se positivos aos 9º dia pós-inoculação (DPI), um valor do índice de densidade óptica (ODI) médio ± desvio padrão (SD) de 163,11 ± 70,65. O maior ODI observado foi 372,54 ± 132,39, em 26ºDPI, e o maior valor geral de ODI foi 626,51. Os frangos vacinados tornaram-se positivos entre 8º e 10° DPV, com um ODI de 245,59 a 10 DPV, com um ODI máximo geral de 543,13. Um total de 108 amostras de sangue foram coletadas de aves criadas em fazendas rústicas. Do total de amostras coletadas, 58,33% (63/108) foram consideradas positivas para *B. anserina.* O ODI máximo encontrado entre essas galinhas rústicas foi 283,24. Essa padronização proporcionou sensibilidade e especificidade de 100%.

Palavras-chave: aves domésticas, borrelioses, argasidae.

Introduction

avian borreliosis is an acute and septicemic disease caused by the spirochete *Borrelia anserina* and affects different avian species worldwide. This disease was described in a geese farm in Russia (Sakharoff, 1891). *Argas miniatus* make the transmission of this bacterium, a species of a tick of the family Argasidae that parasitizes mainly poultry of the species *Gallus gallus.* These ticks live in cracks of chicken coops, making birds of rustic breeding with more significant risks of infestations (Dickie and Barrera, 1964; Cepeda et al., 2016; Ouchene et al., 2020)

The main clinical manifestations of avian spirochetes are hyperthermia, polydipsia, drowsiness, anorexia, inappetence, greenish diarrhea, and mucous pallor (Boero, 1967; Lisboa et al., 2009). As a prophylactic measure, combating the biological vector is essential and, on the other hand, the use of antibiotics from the group of Penicillins, Streptomycins, and Terramycin is helpful in the treatment of sick birds (Hutyra et al., 1947; Boero, 1967).

Few studies are referring to this agent's epidemiology, since with the development of industrial avian breeding, this disease is no longer a significant problem of economic loss. However, at the same time, the consumption of organic poultry products has been increasing considerably, mainly in communities of small and medium-sized rural owners, in the peri-urban areas of the metropolitan regions of large cities and, in this way, avian

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borreliosis can again cause problems since we have the vector (Lima et al., 2020).

This study aims to promote the standardization of the indirect enzyme-linked immunosorbent assay (ELISA) for serological detection in *B. anserina* in *G. gallus domesticus*.

Material and methods

This study was conducted following ethical principles of animal experimentation, approved by the Ethics Committee of the Universidade Federal Rural do Rio de Janeiro (CEUA-IV UFRRJ) for the use of animals under protocol number 123/2014.

Borrelia anserina strain PL obtained from naturally infected *G. gallus* isolated in Barbour Stoenner Kelly (BSK) culture medium (Ataliba et al., 2007) was used for this study. An aliquot was thawed from liquid nitrogen and inoculated into a 15mL flask containing BSK medium. When the culture reached the peak of spirochetes concentration (approximately 14th day post-infection - DPI), the culture flask was centrifuged at 9000 xg for 20 minutes at 4º Celsius, and the formed pellet was washed three times in phosphate saline buffer (PBS), pH 7.4. Then, the pellet was suspended with 2 mL of the same solution and subjected to sonication for 30 cycles of 30 seconds with an interval of 30 seconds each cycle. This solution was subsequently filtered at 0.45µm and aliquoted. The protein concentration of the total antigen extract was measured according to the Lowry et al. (1951).

To produce hyperimmune serum, a 30-day-old chicken was inoculated (0.4 mg/application) with *B. anserina* antigen (Group I - GI). Three subcutaneous applications were made at 15-day intervals (0, 15- and 30-days post-vaccination - DPV). This animal's blood was collected with an interval of three days in the first 30 days and, after that period, periodically up to 90º DPV.

Ten 36-day-old chickens (commercial strain "Redbro Plumé" belonging to the same batch and immune to Marek's disease and avian bouba) housed in individual suspended cages (Group II - GII) were infested with four third-stage nymphs of the *A. miniatus* species previously infected with *B. anserina* (Cepeda et al., 2016). The infected nymphs were exposed to feed until their engorgement was completed. In this group, blood samples were collected on days 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 17, 21, 24, 31 and 44 post-infestation (DPI).

From January to June 2014, residences were visited in the municipality of Seropédica-RJ, which have rustic poultry rearing by *G. gallus* (Group III - GIII). Inspection for ticks' presence was made both on the body of the animals studied and in the environment. The ticks found were placed in isopropyl alcohol and identified in the laboratory using a dichotomous key, according to Barros-Battesti et al. (2006).

The animals were contained manually, and the blood samples were obtained through the pulsation of the alar vein, using disposable syringes of 3 mL, transferring them immediately to sterile bottles without anticoagulant sequentially, kept refrigerated. Subsequently, the blood was centrifuged at 3400 rpm, and the aliquoted serum was kept at negative 20º Celsius until serological analysis.

In-house ELISA was adapted from the methodologies of Voller et al. (1976) for avian sera. For validation, microwells (NUNC® Surface Maxisorp) medium binding plates were coated with 100 µl/well of solubilized *B. anserina* antigen at three concentrations of 5, 10 and 15 µg/ml solubilized in 0.05M sodium carbonate/bicarbonate. buffer (pH 9.6) and incubated at 4 °C overnight. Following incubation, plates were washed three times with PBS (pH 7.4) containing 0.05% Tween-20 (PBST). Plates were blocked for 90 min at 37 °C with 200 µl/well of 6% skim powdered milk solution in PBST (Milk/PBST) and washed three times in PBST. Negative and positive sera were diluted in duplicate at 1:100, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700 e 1:800 in 5% milk/PBST and 100 µl was added to the appropriate microwells.

Plates were incubated for 90 min at 37 °C and washed three times in PBST. Then, 100 µl Anti-Chicken IgY (whole molecule) Alkaline Phosphatase (KPL®) diluted 1:4000 in 5% Milk/PBST was added to each well and plates were incubated for 90 min at 37 °C. Plates were washed three times in PBST, and 100 µl (1mg/ml) substrate p-nitrophenyl phosphate (pNPP - SIGMA®) was solubilized in diethanolamine buffer (pH 9.8) was added to each well and incubated for approximately 25 min at room temperature. The optical density (OD) of each plate was measured at 405 nm using a microplate reader. We used negative samples from young chickens raised in cages without contact with ticks and a positive sample of a vaccinated chicken (60º DPV) as described above.

The results in OD obtained after reading on a spectrophotometer were crossed to verify which combination of dilution of the antigen (5, 10 or 15 µg/ml) and the serum (1: 100 to 1: 800) provided the most significant difference between the averages of optical densities of positive and negative sera.

In-house ELISA tested the aliquoted sera (GI, GII, and GIII) following the protocol described above and using antigen and serum concentrations defined by the best average found. The cut-off point was calculated using the mean of the negative controls (12 samples) by Frey et al. (1998). To correct the effect of the variation in OD obtained with the reading of each tested plate, each plate's cut-off value was equal to 100 (DO test serum x 100/cutoff), and the results of each test serum expressed as an Optical Density Index (ODI).

For the statistical analysis, we used the Student–Newman–Keuls (SNK) test with a significance level of 5% to compare means.

Results and discussion

The results of the standardization of the test are shown in Figure 1. The most significant difference between positive and negative occurred in combining the sensitization concentration of 5 µg/ml of antigen and a dilution of 1:800.

The serological test results for the samples of the vaccinated chicken, the experimentally infected chickens, and the hens of rustic farms in the municipality of Seropédica-RJ are displayed in a mixed graph as ODI in Figure 2.

The experimentally infected chickens on average were positive at the 9th DPI with an ODI of 163.11 ± 70.65 (mean ± SD) (Figure 2). The mean of the highest observed ODI was 372.54 ± 132.39 occurred at the 26th DPI, and the highest ODI found was 626.51. The vaccinated chicken was positive between 8th and 10th DPI with an ODI of 245.59 at the 10th DPI and reached a maximum ODI of 543.13.

Figura 1: Optical density (OD) of the combination of antigen concentration (5, 10, or 15 µg/ml) and serum dilution (1:100 to 1:800) according to the differences between the average OD values of positive and negative sera.

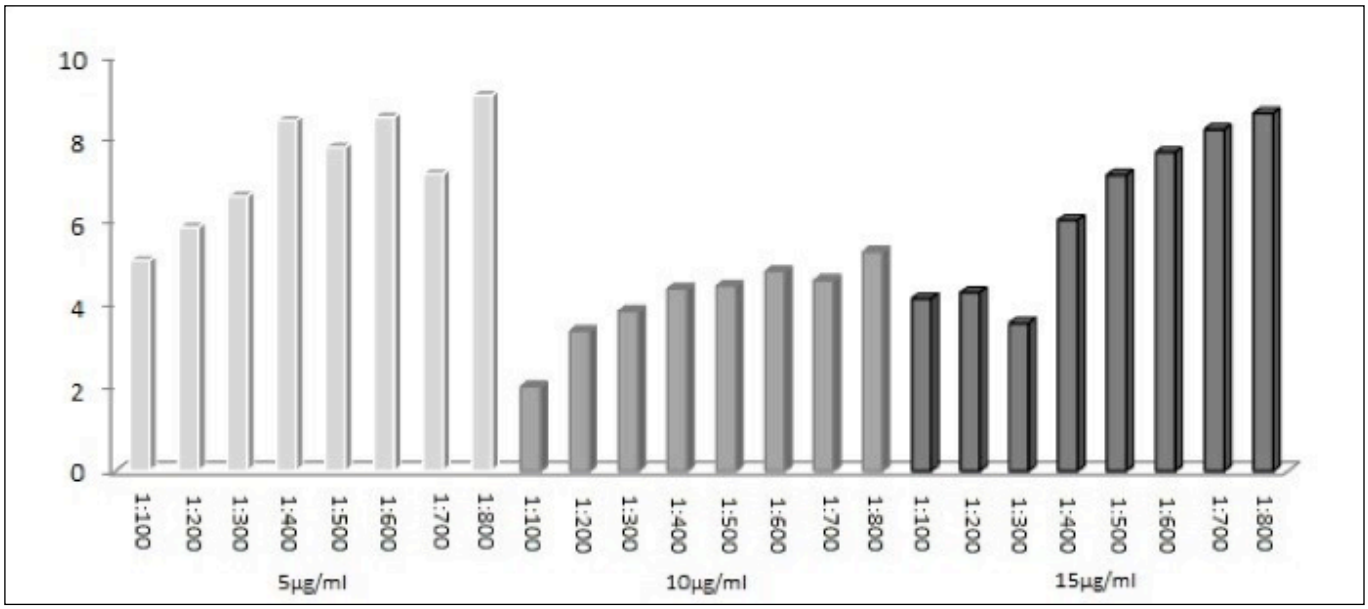
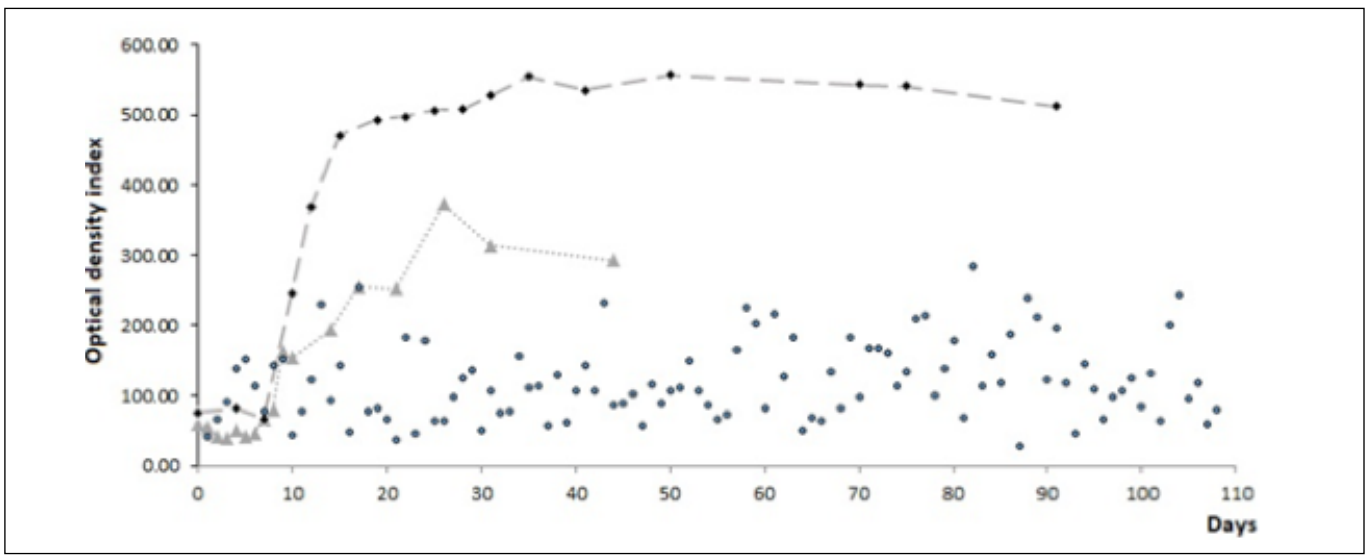


Figura 2: Distribution of optical densities of animals in relation to the *cutoff* (DOx100/*cutoff*) obtained from the indirect ELISA test for *Borrelia anserina* in chickens sera from the municipality of Seropédica – RJ, Brazil (scattered data), vaccinated chicken (dashed line) and experimental infected chickens (dotted line).



In all, 108 blood samples were collected of poultry from rustic farms of the 14 properties in the municipality of Seropédica, state of Rio de Janeiro. In none of the properties where the collections were made ticks of the species *A miniatus* were found, but in four chickens of three properties, eight ticks of the species *Amblyomma sculptum* were found.

Of the total fowls collected, 58.33% (63/108) were considered positive (Figure 2). The maximum ODI found in these rustic chickens was 283.24.

Avian borreliosis is an acute septicemic disease, after clinical cure, it is difficult to find these spirochetes in the blood (Ataliba et al., 2007). This study proposes a new diagnostic method for *B. anserina* in *G. gallus*, a serological detection.

The diagnosis of Avian Borreliosis can be made by observing the characteristic clinical signs, correlated with ticks in fowls. When in the acute phase of borreliosis, the diagnosis can be made through peripheral blood smears or histopathological examinations of affected organs stained with hematoxylin-eosin and silver impregnation (Cepeda et al., 2016; Cepeda et al. 2021). Complementary tests such as hematological (LISBÔA, et al 2006) and biochemical (Cepeda et al., 2016) tests, although unusual because they are a production animal, provide rapid results.

The In-house ELISA method is widely used in diagnosing diseases caused by agents of the genus *Borrelia*, such as Lyme Borreliosis, mainly in epidemiological surveys, representing as a primary immunological tool, due to its high sensitivity and specificity (Magnarelli et al., 1987). The ELISA standardization developed in our study was prioritized to differentiate positive from negative. This same methodology was used by Machado et al. (1997) when standardizing an In-house ELISA for *Babesia bovis* clearly defining the difference between negative and positive sera, calibrating the ELISA absorbance as a score over a wide range of antibody levels.

Our results showed a difference between the tested samples. The GI showed a high ODI at the peak of antibody production (543.13), visually higher than the infected animals' average through feeding by positive *A. miniatus* (GII). However, in a more detailed analysis, it is visible that the ODI varied widely among the infected animals; thus, one of the animals obtained a higher ODI than the vaccinated fowl (626.51). It is worth mentioning that the GI animal was vaccinated with a high concentration of *B. anserina* crude antigen mixed with Freund's adjuvant. The mean ODI value of the anti-*B. anserina* infected group (352.53±132.39) was 3.53 times greater than that obtained with non-infected sera ($p = 0.0002$), clearly discriminating between the mean absorbances of the positive and negative reference sera. This data gave a sensitivity and specificity of 100%.

The ODI values observed in GIII are below the maximum values observed in GI and GII. Although the occurrence of *Argas* ticks has not been reported, there was a seroconversion of many animals to *B. anserina*. In 100% of the properties visited, the hens lived free in an uncemented environment, but in general, surrounded, most hens had contact with different species of animals such as horses, cattle, pigeons and dogs. Thus, the chickens studied were parasitized, possibly by other tick species. However, we only found the species *A. sculptum*.

Species of ticks of the genus *Argas* are heteroxenous, nesting, preferentially feed, at night, which makes it difficult to find specimens. Most fowl farmers are unaware of its existence. The *Argas* genus presents in its biological cycle evolutionary phases of the larvae (which remain on the host for 3 to 7 days to complete its meal), 4 or 5 nymph phases (undergoing changes or ecdysis at each phase change) and adult phase, feeding on 10 to 45 minutes and showing little sexual dimorphism (Santos et al., 2008; Santos et al., 2010). These characteristics are especially important for the transmission of Borreliosis. After the spirochetes disappeared from the chicken' bloodstream, immediately after the acute phase of infection, they can only be detected through serological tests. Thus, antibody research can allow for a detailed study of outbreaks and help establish a disease prevention program.

Thus, we understand that the results found in these domestic chickens can be given as a possible cross-reaction with other species of the *Borrelia* genus. And this fact can be seen as an important result for public health, as they raise suspicions that chickens may be sentinels and/or reservoirs of many species of the *Borrelia* genus pathogenic to humans. Nevertheless, the indirect ELISA as a diagnostic tool for *B. anserina* suggested in our study proved to be quite efficient as an important means of screening in properties with suspected avian Borreliosis. However, it is necessary to associate with other direct diagnostic methods, such as blood smears, or indirect, such as Indirect Immunofluorescence Assay (IFA).

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References

ATALIBA, A. C. et al. Isolation and molecular characterization of a Brazilian strain of *Borrelia anserina*, the agent of fowl spirochaetosis. *Research Veterinary Science***,** v. 83, n. 2, p. 145-9, Oct 2007.

BARROS-BATTESTI, D. M.; ARZUA, M.; BECHARA, G. H. *Carrapatos de importância médico-veterinária da região neotropical: um guia ilustrado para identificação de espécies*. 1ª ed. São Paulo: Vox/ICTTD-3/Butantan, 2006.

BOERO, J. J. *Parasitosis animales*. Buenos Aires, Argentina: Universidade de Buenos Aires, 1967.

CEPEDA, M. B. et al. Alterações bioquímicas, anatômicas e histopatológicas em fígado de *Gallus gallus* Linnaeus, 1758 experimentalmente infectados por *Borrelia anserina* Sakharoff, 1891. *Pesquisa Veterinária Brasileira***,** v. 36, n. 8, p. 687-693, 2016.

CEPEDA, M. B.; CORDEIRO, M. D.; BAETA, B. A.; FONSECA, A. H. Experimental infection by *Borrelia anserina* strain PL in *Gallus gallus. Semina: Ciências Agrárias* v. 42, n. 4, p. 2429-2438, 2021.

DICKIE, C. W.; BARRERA, J. A Study of the Carrier State of Avian Spirochetosis in the Chicken. *Avian Diseases***,** v. 8, n. 2, p. 191-195, 1964.

FREY, A.; DI CANZIO, J.; ZURAKOWSKI, D. A statistically defined endpoint titer determination method for immunoassays. *Journal of Immunological Methods***,** v. 221, n. 1, p. 35-41, 1998. ISSN 0022-1759.

HUTYRA, F.; MAREK, J.; MANNINGER, R. Espiroquetosis de las Aves de corral: Spirochaetosis avium. *Patología y Terapéutica Especiales de los Animales Domésticos,* v. 1, p. 716-722, 1947.

LIMA, S. K.; GALIZA, M.; VALADARES, A.; ALVES, F. *Produção e consumo de produtos orgânicos no mundo e no Brasil*. Instituto de Pesquisa Econômica Aplicada. - Brasília: Rio de Janeiro: Ipea, 2020.

LISBOA, R. S. et al. Avian spirochetosis in chickens following experimental transmission of *Borrelia anserina* by *Argas (Persicargas) miniatus*. *Avian Diseaseas,* v. 53, n. 2, p. 166-8, 2009.

LOWRY, O. H. et al. Protein measurement with the Folin phenol reagent. *Journal of biological chemistry***,** v. 193, p. 265-275, 1951.

MACHADO, R. et al. An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against Babesia bovis in cattle. *Veterinary Parasitology***,** v. 71, n. 1, p. 17-26, 1997.

MAGNARELLI, L. A. et al. Clinical and serologic studies of canine borreliosis. *Journal of the American Veterinary Medical Association***,** v. 191, n. 9, p. 1089-1094, 1987.

OUCHENE, N.; NEBBAK, A.; OUCHENE-KHELIFI, N. A.; DAHMANI, A.; ZEROUAL, F.; KHELEF, D.; BITAM, I.; BENAKHLA, A.; PAROLA, P. Molecular detection of avian spirochete *Borrelia anserina* in *Argas persicus* ticks in Algeria, *Comparative Immunology, Microbiology and Infectious Diseases*, v.68, 2020. https://doi.org/10.1016/j.cimid.2019.101408

SAKHAROFF, M. Spirochaeta anserina et la septicémie des oies. *Annales de l'Institut Pasteur***,** v. 5, p. 564-566, 1891.

SANTOS, H. A. et al. The influence of the fasting period on the number of nymphal instars and on the sex ratio of Argas (Persicargas) miniatus (Acari: Argasidae). *Revista Brasileira de Parasitologia Veterinária***,** v. 19, n. 3, p. 164-168, 2010.

SANTOS, H. A. et al. Biological aspects of *Argas* (*Persicargas*) *miniatus* Kock, 1844, (Ixodoidea; Argasidae) larvae in Gallus gallus. *Revista Brasileira de Parasitologia Veterinária***,** v. 17, p. 45-49, 2008.

VOLLER, A.; BIDWELL, D.; BARTLETT, A. Enzyme immunoassays in diagnostic medicine: Theory and practice. *Bull Word Health Organ***,** v. 53, n. 1, p. 55, 1976.