VETERINÁRIA NOTÍCIAS

ARTICLE

Received: July 2022. Accepted: September 2022

ISOLATION AND AMPLIFICATION OF *Anaplasma marginale* IN CHICKEN EMBRYO FIBROBLAST CELL CULTURE

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ABSTRACT

Anaplasma marginale (A. marginale) is a worldwide pathogen that infects a variety of ruminants, but mostly cattle. The present study aimed to describe an isolation technique for *A. marginale*, using chicken embryo fibroblast (CEF) cell culture. Blood and tick samples were collected from 5 calves from 2 to 3 months old, which were considered to be infected with *A.marginale* due to anemia, jaundiced mucous membranes, and prostration. DNA extraction and PCR were performed for diagnosis using blood and tick samples. All tick and blood samples tested positive in PCR. Additionally, ticks were crushed with the aid of a blender for inoculation in CEF cell culture. After inoculation, the cultures were kept at 37ºC and 5% CO2 for 15 days. The cell supernatant of cell cultures was again analyzed using PCR and Wright stain method to confirm *A. marginale* isolation. Cell cultures tested positive in PCR, and the presence of the agent was demonstrated by Wright stain. Therefore, by using CEF cell culture it was possible to isolate and amplify the *A. marginale* in a concentration of 1.3 x 107.2 bodies per mL. The CEF cells are undemanding and easy to preserve; they are an option for isolation and production of *A. marginale* under laboratory conditions.

KEYWORDS: in vitro cultivation of *Anaplasma marginale,* bovine parasitic sadness, anaplasmosis, *Anaplasma* sp.

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1. INTRODUCTION

Anaplasma marginale (A. marginale) is a worldwide pathogen that infects a variety of ruminants, but mostly cattle. It is an obligate intraerythrocytic rickettsia of ruminants, transmitted biologically by the tick *Rhipicephalus microplus* (SILAGHI et al., 2017). These vectors are favoured by climatic conditions found in most regions of Brazil, which enable the long survival of both ticks and bacteria in herds across the country. In addition to transmission by ticks, *A. marginale* can be transmitted by blood-sucking insects and fomites (GUEDES JÚNIOR et al., 2008). Vaccines for anaplasmosis do exist but they only moderate symptoms, i.e., they do not prevent that the animal become a carrier and spread the pathogen. As of today, no more effective solution has been developed, as shown by Curtis et al. (2020).

Anaplasmosis is a disease that leads to severe, progressive anemia, high fever (above 40°C), weight loss, abortion, pallor of mucous membranes, jaundice, dehydration, loss of appetite, reduced performance and, in several cases, death. Surviving animals keep on carrying the agent and become an important source of concern, as they may lead to further disease outbreaks (HAIRGROOVE et al., 2015; CURTIS; COETZEE, 2021). During necropsy, the most frequently observed macroscopic lesions are clotted blood, anemic or jaundiced mucous and serous hepatosplenomegaly, dark, enlarged kidneys, gall bladder with lumps and dense content, and cerebral congestion (BRITO et al., 2019).

Besides its solo effect, *A. marginale* is also one of the causative agents of bovine parasitic sadness (BPS), a complex of diseases comprising anaplasmosis and babesiosis, which is caused by the protozoa *Babesia bigemina* and *Babesia bovis* (GUEDES JÚNIOR et al., 2008). BPS is responsible for major economic losses such as herd mortality, reduced milk production, and decreased weight gain, in addition to expenses with control and prophylaxis (GRISI et al., 2002; KOCAN et al., 2010; ZABEL; AGUSTO, 2018). There are two types of commercially available vaccines to prevent BPS in Brazil, both composed of the three attenuated microorganisms (*A. centrale*, which is used against *A. marignale*, *B. bovis* and *B. bigemina*). However, despite satisfactory results in immunization, there are cases of severe reactions to the vaccine and development of symptoms (DANTAS-TORRES; OTRANTO, 2017).

Even though the isolation and propagation of this agent under laboratory conditions is an important means to study its biology, it represents a great challenge. Considering the growing concern regarding animal welfare, especially when it comes to animal experimentation, it is advised that this kind of endeavour only use long-lasting cell lines (SILAGHI et al., 2017). Therefore, the present study aimed to demonstrate the isolation, maintenance, and amplification of *Anaplasma marginale* in chicken embryo fibroblast (CEF) culture.

2 - MATERIAL AND METHODS

2.1 - SAMPLE COLLECTION

The study was carried out in an animal health diagnosis laboratory located in the City of Uberlândia, State of Minas Gerais, Brazil. This study was previously approved by the Animal Ethics Committee of Laudo Laboratório (Approval Number 01.0420.2016). The samples were obtained from a property located in a rural area in Uberlândia. Blood and tick samples were collected from five calves aged 2-3 months that had apathy, jaundiced mucous membranes, and fever. After collection, the samples were sent to the laboratory for diagnosis. Tick samples were stored together as a sample pool, while the blood samples, which were collected individually from each calf, remained separated. Blood samples were collected by jugular puncture, using a 10mL ETDA syringe and 25x0.8mm needles. Ticks were collected from different regions of the animals' body surface. After collection, the samples were kept in an isothermal box containing ice for transport to the laboratory.

The PCR technique was performed for diagnosis. For DNA extraction from the ticks, approximately 15 grams of ticks were macerated using a shredder, and saline (PBS) was added in a proportion of 1:10 (w / v) (15g of tick, and 150 of PBS). Then, this mixture was centrifuged at 10,000 rpm for 5 min, and 200 µL of the supernatant was used for DNA purification. The samples were PCR tested using the nested PCR extraction method, with the membrane protein MSP4 being used for detecting *A. marginale* (LASMAR et al., 2012). Not only did the pool of tick samples tested positive for *A.marginale*, but 3 out of the 5 blood samples tested positive too. Positive samples in the PCR were prepared for inoculation in a CEF cell culture at Laudo Laboratório Avícola LTDA/Inata Produtos Biológicos.

Cell cultures were prepared using 20 embryonic eggs from SPF (Specific Pathogen Free) chickens, with 10 days of incubation. After embryo euthanasia, the chorioallantoic membrane was removed and left in agitation, with a 10% trypsin-based solution for 20 minutes, after which the supernatant was collected and resuspended in culture medium 199 (gibco). The fibroblasts were quantified. CEF cells were maintained in the laboratory in medium 199 (gibco) with 10% fetal bovine serum (FBS). Cell cultures were prepared 24 hours in advance in cell culture plates (1.000.000cells/mL). 1mL of the supernatant from the tick mash was used as an inoculum. Blood samples were isolated by using 1mL of sample previously diluted in saline with antibiotics (Penicillin, Gentamicin and Fungison). After inoculation, the samples were kept in a greenhouse at 37ºC in an anaerobic atmosphere for 1 hour.

The inoculum was removed, and the cultures were washed three times with saline containing antibiotics. After the last wash, the cells were kept in culture medium 199 containing 10% FBS at 37ºC with an atmosphere of 5% CO2 for 15 days. On post-inoculation (pi) days 5, 7, 10, 12 and 15, the plates were observed under a microscope to assess bacterial growth in cell culture. On post-inoculation day 15, the samples were

submitted to the Wright stain method and evaluated under a microscope. Staining was carried out as follows: all the culture medium on the plate was removed while trying to keep the cell layer as intact as possible; then, 1mL of Wright stain was inoculated and left in contact with the cell layer for 40 seconds; finally, 0.5mL of water was added to the dye, and the substance was homogenized again and left in contact with the cell layer for another minute . After this process, the dye was removed, and the cell layer was gently rinsed with water. After drying, the cell layer was observed under a microscope.

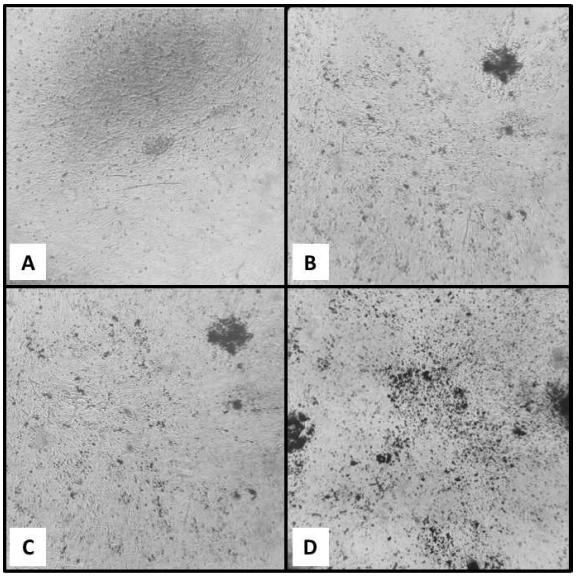
At pi day 15, the cultures and the supernatant were collected and centrifuged at 9000g at 4ºC for 15 min. The pellet formed was homogenized and incubated with 5mL of trypsin at a concentration of 10% at 37ºC for 20 minutes. After this step, 5mL of sterile PBS was added, and the cells were mechanically dismembered and centrifuged at 2300g at 4 ºC for 5 minutes. The supernatant was collected and quantified.

3- RESULTS AND DISCUSSION

3.1 - CULTIVATED SAMPLE RESULTS

The crops were monitored to assess changes that could be consistent with the growth of *A. marginale*. After post-inoculation (pi) day 7, the cell culture formed small structures, like vacuoles, circulating in the cell medium (Figure 1a). On pi day 10, vacuoles had increased in size and number, and smaller structures like corpuscles were floating in the cell cytoplasm (Figure 1b). On pi day 12, the vacuoles were larger, measuring approximately 20mm (Figure 1c). Those were different from the ones observed upon 15 days of inoculation, when vacuoles regressed and the number of circulating corpuscles increased in the cytoplasm (Figure 1d).

Figure. 1 Cultivation of chicken embryo fibroblasts (CEF). a - 7-day post-inoculation (pi) of positive material in the PCR for *Anaplasma marginale*. The initial formation of "corpuscles inside vacuoles". b - Increased number of "corpuscles inside vacuoles" and floating structures (free corpuscles). c - Increased corpuscles measuring about 20 mm. d - Breakage of the corpuscles and increased floating vacuoles



The samples were submitted to the Wright stain method and evaluated under an optic microscope. Corpuscles could be found in both cultures, from the tick and blood samples (Figure 2), and *A. marginale* was fixed on a microscope slide after exclusion of cell remainders by centrifugation (Figure 3). After this procedure, the samples underwent PCR analysis again to confirm agent isolation; they did tested positive for *A. marginale*.

Figure. 2 Cultivation of chicken embryo fibroblast (CEF). a- vacuoles with the presence of *A. marginale* corpuscles upon 15-day cultivation. b - free corpuscles in the cell cytoplasm.

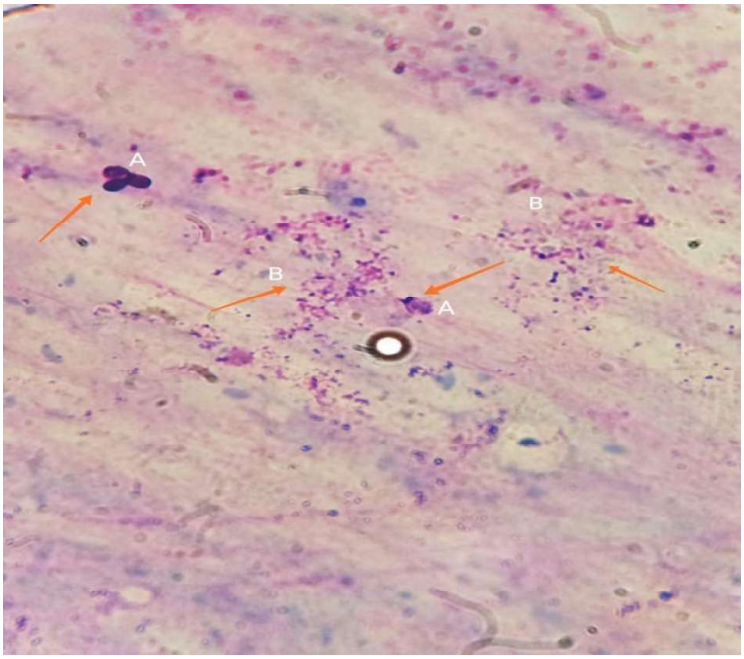
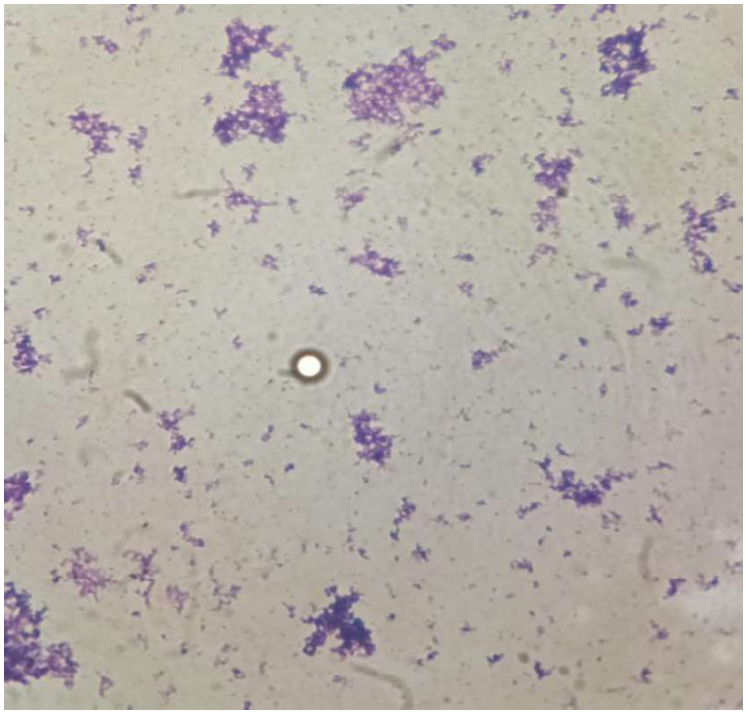


Figure. 3 *Anaplasma marginale* fixed on a microscope slide, after a centrifugation procedure to remove cell remainders and use of the Wright stain method.



Then a pool of the isolated samples was used to quantify the agent in a hematimetric chamber; the resulting isolate contained approximately 1.3 x 107.2 initial bodies of *A. marginale.*

Several researchers have sought to find ways to culture *A. marginale.* Dennis et al. (1970) used this in the culture of bovine erythrocytes by inoculating an attenuated strain of *A. marginale* in live cattle to produce specific antibodies against this agent. However, this presented some limitations, such as the production of a vaccine, which requires the use of pathogen-free animals, and the separation of agent from infected erythrocytes for antigen purification. These limiting factors make this technique unfeasible for culturing *A. marginale* and producing a vaccine.

According to Passos (2012), in vitro cultivation of *A. marginale* is a useful tool to better understand the interaction between the pathogen and its host cell. Thus, much research has used cell cultures for growth and development of these rickettsiae -- e.g., cultures of bovine lymph node cells (HIDALGO, 1975), rabbit bone marrow cells (MARBLE; HANKS, 1972) and *Aedes albopictus* mosquito cells (MAZZOLA; AMERAULT; ROBY, 1979) -- but no study has reported conclusive findings for agent growth and propagation.

Munderloh et al. (1996) cultivated *A. marginale* in an IDE8 cell derived from embryos of the tick *Ixodess capularis* and observed its growth and propagation. Several studies have followed suit and used this type of cell culture. According to Lasmar et al. (2012), even with the good agent growth in IDE8 cell culture, the inactivated vaccine for *A. marginale* showed seroconversion in vaccinated calves, but all animals showed symptoms of the disease when they were challenged with the agent.

The use of CEF cells has the benefit of easy maintenance and the possibility of large-scale agent production. In addition, they do not have other types of bovine pathogens in their composition, thus providing a purer culture that can lead to a better, more specific immune response.

4 - CONCLUSION

Based on the present findings, the test performed for the isolation of *Anaplasma marginale* was satisfactory, as the agent developed broadly and grew considerably in the chicken embryo fibroblast cell (CEF). This points to new avenues for vaccine production and consequent sanitary control of bovine anaplasmosis.

5- ACKNOWLEDGMENTS

The authors thank Laudo Laboratório/ Inata Produtos Biológicos for funding the study and for making the laboratory available for testing and diagnosis.

STATEMENTS AND DECLARATIONS

Funding

This study was supported by Laudo Laboratório Avícola LTDA/Inata Produtos Biológicos, who provided funding and the laboratory where the experiment was carried out.

Competing interests

Authors Mathias Martins and Fabiana Oliveira Notário declare they have no financial interests. Author Vinícius Borges de Faria has received research funding from Laudo Laboratório.

Author Contributions

VBF and MM planned the experiment. VBF, MM and FON analyzed and interpreted data; they also wrote the manuscript. Vinícius Borges de Farias carried out laboratory analysis.

Ethics Approval

This study was approved by Credenciamento Institucional para Atividades com Animais em Ensino ou Pesquisa (CIAEP: 02.0420.2021).

Consent to participate

Not applicable

Consent to publish

Not applicable

Data availability

Not applicable

RESUMO

ISOLAMENTO E AMPLIFICAÇÃO DE *Anaplasma marginale* EM CULTIVOS CELULARES DE FIBROBLASTOS DE EMBRIÕES DE GALINHAS

RESUMO

Anaplasma marginale (A. marginale) é um patógeno mundial que infecta uma variedade de ruminantes, mas principalmente bovinos. O presente estudo teve como objetivo descrever uma técnica de isolamento para *A. marginale*, utilizando cultivo celular de fibroblastos de embriões (CFE) de galinhas. Para isso, foram coletadas amostras de sangue e de carrapatos de 5 bezerros, entre 2 e 3 meses de idade, os quais, devido a anemia, icterícia de mucosas e prostração, foram considerados supostamente infectados com *A. marginale*. Para o diagnóstico, realizaram-se extração de DNA e posterior PCR a partir das amostras de sangue e de carrapatos coletados. Todos os carrapatos e amostras de sangue foram positivas para o teste de PCR. Além disso, os carrapatos foram triturados com o auxílio de um liquidificador para inoculação em CFE. Após a inoculação, as culturas foram mantidas a 37ºC e a 5% de CO2 durante 15 dias. O sobrenadante celular das culturas foi novamente analisado por PCR e pela técnica de coloração de Wright para confirmar o isolamento de *Anaplasma marginale*. As culturas celulares foram po-

sitivas por PCR, e a presença do agente foi comprovada por meio da coloração de Wright. Portanto, utilizando CFE, foi possível isolar e amplificar o *A. marginale* em uma concentração de 1,3x107,2 bactérias por ml. As células da CEF são pouco exigentes, de fácil manutenção e uma boa opção para isolamento e produção de *A. marginale* em condição laboratorial.

Palavras-chave: cultivo *in vitro* de *Anaplasma marginale*, tristeza parasitária bovina, anaplasmoses, *Anaplasma* sp.

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