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SHORT NOTE [NOTA CORTA]

HISTOPATHOLOGIC EFFECT OF *Brucella abortus* ON CHICKEN EMBRYOS INOCULATED WITH MILK FROM SEROPOSITIVE COWS

[EFECTO HISTOPATOLÓGICO DE *Brucella abortus* EN EMBRIONES DE POLLO INOCULADOS CON LECHE DE VACAS SEROPOSITIVAS]

Evelyn Pulido-Camarillo[1], David I. Martínez-Herrera[2]*, Violeta T. Pardío-Sedas[2], Ahidé López-Merino[3], José Francisco Morales-Álvarez[1], Humberto Ramírez-Mendoza[4], Francisco Rivera-Benítez[4], Olivia Padrón-Tello[2], Álvaro Enrique de J. Peniche-Cardeña[2] and Ricardo Flores-Castro[1]

[1]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias. Centro Nacional de Investigación Disciplinaria en Microbiología Animal. Km. 15.5 Carretera México-Toluca, Col. Palo Alto, C.P.05110, Cuajimalpa, Distrito Federal, México. evlyn_18@yahoo.com.mx,morales.francisco@inifap.gob.mx, flores.ricardo@inifap.gob.mx

[2]Universidad Veracruzana, Facultad de Medicina Veterinaria y Zootecnia. Circunvalación Esq. Yáñez s/n, Col. Unidad Veracruzana, C.P. 91710, Veracruz, Veracruz, Méx. vpardio@uv.mx, apeniche@uv.mx, Oliviapadron@hotmail.com.

[3]Instituto Politécnico Nacional, Escuela Nacional de Ciencias Biológicas, Campus Santo Tomas. Prolongación de Carpio y Plan de Ayala s/n, Col. Santo Tomas, C.P. 11340, Delegación Miguel Hidalgo, México D.F. ahide99@hotmail.com

[4]Universidad Nacional Autónoma de México, Facultad de Medicina Veterinaria y Zootecnia. Circuito Exterior, Ciudad Universitaria, C.P. 04510, Del. Coyoacán, México, D.F. betosram@yahoo.es, expide@yahoo.com

**Corresponding author: dmartinez@uv.mx*

SUMMARY

The histopathologic effect caused by *B. abortus* on chicken embryos (CE) inoculated with milk from seropositive cows was determined. One hundred 7- to 9-days-old CE were used and divided into three groups: I) 80 CE divided into eight subgroups of 10 CE each, that were inoculated with milk cream from eigth cows seropositive to *B. abortus*; Group II) 10 CE inoculated with a field strain of *B. abortus* (positive control group); and Group III) 10 CE inoculated with ultra-pasteurized milk (negative control group). Bacteria with characteristics resembling *Brucella* spp. were isolated in at least 1 of each 10 CE in Group I, and in all the CE in Group II. The CE worked as a model to achieve infection and induction of damages into the cytoarchitecture of the liver and chorioallantoic membrane, this determined by the observation of inflammation and necrosis in their tissues, both in Group I and Group II. In conclusion, *B. abortus* caused morphological changes in the liver and chorioallantoic membrane of CE.

Keywords: *Brucella abortus*; inoculation; histopathology; zoonosis; milk.

RESUMEN

Se determinó el efecto histopatológico que produce *B. abortus* en embriones de pollo (CE) inoculados con leche de vacas seropositivas. Se usaron 100 CE de siete a nueve días de edad divididos en tres grupos: Grupo I) 80 CE divididos en ocho subgrupos de 10 CE cada uno que se inocularon con crema de leche de ocho vacas seropositivas a *B. abortus*; Grupo II) 10 CE inoculados con una cepa de campo de *B. abortus* (testigo positivo); y Grupo III) 10 CE inoculados con leche ultrapasteurizada (testigo negativo). Bacterias con características compatibles con *Brucella* spp. fueron aisladas en al menos 1 de cada 10 CE en el Grupo I, y en todos los CE del Grupo II. El CE sirvió como modelo para lograr infección e inducción de daños en la citoarquitectura del hígado y membrana corioalantoidea, determinado esto al observar inflamación y necrosis en sus tejidos, tanto en el Grupo I como en el Grupo II. En conclusión, *B. abortus* provocó alteraciones morfológicas en hígado y membrana corioalantoidea del CE.

Palabras clave: *Brucella abortus*, inoculación, histopatología, zoonosis, leche.

INTRODUCTION

Worldwide, economic losses due to brucellosis are of main importance, not only on animal production, but on human health (Seleem *et al.*, 2010). Bovine brucellosis produced by *Brucella abortus* occurs all around the world, except in those countries where the disease has been eradicated. Like in other countries, in Mexico the economic impact of brucellosis is high, because it affects the health and decreases milk production of cows. In Mexico, 9000 to 10000 million liters of cow's milk are produced anually, of which 35 % is consumed as unpasteurized cheese and milk; therefore, the risk of acquiring food-borne diseases, including brucellosis, is high (Luna and Mejía, 2002). Brucellosis is found throughout the country and during the national campaign for its control and eradication positive herds with different prevalence levels have been identified. In Mexico, the states with the highest prevalence of bovine brucellosis include Chihuahua, Hidalgo and Guanajuato (Luna and Mejía, 2002).

The sub-acute and chronic forms of brucellosis pose a risk for human health, so it is important to have different tools for the diagnosis of this disease. The alternative infection models, such as the chicken embryo (CE), are fundamental because they increase the possibilities to isolate viable bacteria for strain characterization and epidemiological studies. Histopathology is a fundamental tool for the identification of morphological alterations caused by *B. abortus*, and it also indicates the viability and replication of this bacteria by the changes it causes in the cytoarchitecture of the organs it has affinity with (Meador *et al.*, 1986).

The CE is a resource used for the isolation of intracellular parasites, particularly viruses (Detilleux *et al*., 1988; Nix *et al.*, 2006). However, since brucellas are facultative intracellular parasites, the CE can also be used to achieve their isolation in samples from infected individuals, on the condition that the samples are from tissues or fluids not highly contaminated (Buddingh and Womack, 1941). This infection model has many advantages, such as the easy accessibility, easy handling at the laboratory, its sterile condition, the general absence of internal contaminants and the various routes of inoculation. The substance inoculated does not activate the antibody production and has a great variety of tissues for the replication of the infectious agent (Nix *et al.*, 2006).

The CE, when inoculated directly with *B. abortus* or with contaminated dairy products, can be an alternative for the recovery of field strains in cow's milk from herds that are considered as infected. This procedure would serve to increase the number of colony-forming units (CFU) and to demonstrate the active infection within the CE tissues through histopathology. For the above mentioned, the objective of the present study was to determine, using routine histological methods, the presence and the histopathological effect of *B. abortus* in CE inoculated with milk from cows seropositive to this bacteria.

MATERIALS AND METHODS

Milk sample collection

Milk samples were collected from eight dual-purpose cows that were positive to brucellosis according to the official serological card and rivanol tests, as established by the NORMA Oficial Mexicana NOM-041-ZOO-1995, National Campaign Against Brucellosis in Animals. The milk samples were aseptically obtained in sterile 50 ml-tubes.

Bacteriological diagnosis

The milk samples were centrifuged at 1000 x g for 10 min to separate the cream. The cream was cultivated by duplicate in agar plates with Farrell's selective medium; half of the plates were incubated in aerobiosis and the other half in microaerobiosis at 37 °C and 5 to 10 % CO_2 for 20 days. Likewise, the inocula for the CE were prepared using 0.5 ml of milk cream of each cow and 4.5 ml of ultra-pasteurized milk added with the antibiotics present in the Farrell's medium at a ratio of 1:10. Each inoculum was allowed to settle with the growth inhibitors for al least 12 h at 4 °C, prior to be used in the CE.

Chicken embryo inoculation

One hundred 7- to 9-days-old CE were used, which were allowed to settle for 12 h in an incubator at 37 to 37.5 °C and 50 to 60 % relative humidity (RH) at their arrival at the laboratory (Hitchner *et al*., 1980). Three experimental groups were formed. Group I included 80 CE, that were divided into eight subgroups of 10 CE each, which were inoculated with milk cream from the eight cows seropositive to *B. abortus*. Group II included 10 CE inoculated with the *B. abortus* field strain 2308 and it was the positive control group. Group III included 10 CE inoculated with ultra-pasteurized milk and it was the negative control group. The milk cream in Group I and the strain 2308 were re-suspended with ultra-pasteurized milk added with the supplement of the Farrell's medium, which contains 5 mg nalidixic acid, 25 IU bacitracin, 10 mg

cycloheximide, 5,000 IU polymyxin, 100,000 IU nystatin and 2 mg vancomycin. The ultra-pasteurized milk that was inoculated into Group III was also added with the antibiotics of the Farrell's medium. Using an ovascope, the air chamber in each egg was located and the shell area disinfected with 70 % isopropyl alcohol and perforated at the center of the air chamber; the CE was located inside of the yolk sac and 0.5 ml of the inoculum were introduced in it (Hitchner *et al*., 1980). The shell perforation was sealed with white wax and the CE were placed in the incubator at 37 °C, with 50 to 60 % RH. All the CE were checked every 24 h for three consecutive days, to observe their viability until completing 72 h of incubation; then, the CE were sacrificed by refrigeration at 4 °C for 3 h.

Histopathology of the liver and the chorioallantoic membrane

In all the CE a cross section was performed with the aim to carry out the physical inspection of the egg membranes to observe the morphological changes and macroscopic alterations of their organs. Then, the liver and the chorioallantoic membrane were removed, and one half of each of these organs were used for the bacteriological culture, which was made by continuous streak of the agar plate with Farrell's medium; this was done by duplicate. Half of the plates inoculated with each organ were incubated in aerobiosis, and the other half were incubated at 37 °C and 5 to 10 % CO_2 for eight days; all the plates were checked daily until completing the incubation period. Additionally, the same portion of these organs were used to make smears that were fixed in methanol and stained by the modified Ziehl-Neelsen technique (Díaz *et al*., 2001). According to their morphological characteristics, the colonies with morphology resembling *Brucella* spp. were identified using routine biochemical techniques (Alton *et al*., 1988).

The other half of each organ of the CE were fixed in buffered formalin at 10 % for 24 h; then, the tissues were dehydrated and included into paraffin in a tissue processor. Of each tissue, 4 µm sections were made, which were placed on slides covered with silane at 2 % and poly-L-lysine, to be stained using the hematoxylin and eosin method (H-E; Prophet *et al*., 1995).

Statistical analysis

The results were analyzed using the Kruskal-Wallis test, available in the statistical package STATISTICA 6.0.

RESULTS AND DISCUSSION

Bacteriological diagnosis

The milk samples that were obtained from seropositive cows and cultured in specific media for *Brucella* spp. before being inoculated into the CE, showed no development of colonies resembling this bacteria after 20 days of incubation, which might indicate that the initial concentration of bacteria in the milk was less than 100 CFU, that is the minimum concentration required for the bacteria development to occur in an artificial culture medium; however, the absence of development does not mean that the sample has no potential to be infectious (Buddingh and Womack, 1941).

The isolation of bacteria resembling *Brucella* spp. from the CE and in the different groups, is shown in Table 1. As it can be observed in Group I, in seven out of the eight subgroups the isolation of bacteria resembling *Brucella* spp. was possible in at least 1 of the 10 CE inoculated with milk cream from seropositive cows. In Group II, which included CE inoculated with the *B. abortus* field strain 2308, the isolation was possible in all the CE. In Group II no bacteria resembling *Brucella* spp. were isolated.

Table 1. Number of isolates of bacteria resembling *Brucella* spp. from chicken embryos inoculated either with milk cream from cows seropositive to *Brucella abortus* (Group I), *Brucella abortus* field strain 2308 (Group II), or ultra-pasteurized milk (Group III).

	Isolated / inoculated*
Group I	
Subgroups	
1	7/10
2	6/10
3	7/10
4	4/10
5	8/10
6	0/10
7	6/10
8	7/10
Group II (positive control)	10/10
Group III (negative control)	0/10

*Chicken embryos in which *Brucella abortus* was isolated / embryos inoculated

The isolates characteristics to which reference is made include the observation of Gram-negative short bacilli (coccobacilli) from liver smears and fixed smears obtained from the cultures, as well as the observation of partially acid-fast bacteria stained by the modified Ziehl-Neelsen technique. These findings are considered important, since they mean that the CE was an excellent alternative to increase the initial concentration of the bacteria from the milk cream. This finding confirms the advantage the CE has over the direct culture of clinical samples, since in the direct culture the lack of a concentration of bacteria sufficient to be recovered by that means is evident. Some authors such as Detilleux *et al.* (1988) and Samartino and Enright (1993), have demonstrated that to increase the concentration of *Brucella* spp. in tissue samples from infected individuals, the bacteria can be inoculated in 3- to 7-days-old CE, and after incubation for 72 h the bacteria can be recovered in a sufficient amount in artificial culture media.

Seven isolates of Group I and one isolate of Group II were selected and subjected to polymerase chain reaction and immunohistochemical and immunofluorescent techniques for bacteria identification, and all were positive to *B. abortus*.

Histopathology of the liver

In the livers of the CE inoculated with milk cream of seropositive cows and in which *B. abortus* was isolated, as well as in the CE inoculated with the strain 2308, it could be observed the loss of the cytoarchitecture of the hepatocytes, which were partially separated, with alteration in the conformation of the cords; the cytoplasm showed vacuolization (vacuolar degeneration). The lesions observed are evidence that the bacteria concentrations in the samples processed may not be sufficient for their isolation, but the amount is sufficient to cause infection and tissue damage (Yeager *et al.*, 1967). An addition, there was also loss of structure around the blood vessels of the liver tissue, and it was frequently substituted by fibrous tissue.

These results can be compared to those described by Bruce in 1886, who isolated *B. melitensis* from the liver and spleen of patients who died as a consequence of complications caused by brucellosis (Seleem *et al.*, 2010), and in the liver abundant cells of the mononuclear phagocyte system present could be observed (Tizard, 2009). Also, the lesions identified in this infection model can be compared to those described by Molello *et al.* (1963), that indicate fibrosis and loss of the cytoarchitecture caused by *B. abortus* in goats and sheep used as infection models for the isolation of this microorganism. In the liver of CE used as the negative control group, no apparent histopathological changes were observed.

Histopathology of the chorioallantoic membrane

The incubation period used once the CE were inoculated was long enough for the bacteria to infect and reproduce in the immature cells of the chorioallantoic membrane. In these cells, when stained with H-E and observed in the microscope, the degenerative changes produced were evident, similar to what happened in the chorioallantoic membrane of the CE from the positive control group inoculated with the *B. abortus* reference strain 2308, where loss of the cytoarchitecture and presence of an eosinophilic amorphous hyaline material at the center of the membranes were observed. In the negative control group, the chorioallantoic membrane maintained a tissue structure with no apparent pathological changes.

This situation allowed to compare the lesions observed in the chorioallantoic membrane with the morphological alterations described by Buddingh and Womack (1941) when infecting CE with *B. abortus*, who indicated that the microscopic degenerative changes such as the presence of an eosinophilic amorphous hyaline material in the tissues were identified between 48 and 72 h post-inoculation, although they did not identify destruction of the ectodermal epithelium, which occurs until the cells are filled with the bacteria. These authors also indicated that the macroscopic characteristics of the lesions produced by *B. abortus* were indistinguishable, even if the virulence was low, from those produced by *B. suis*, and that when the CE are infected, they do not survive longer that 96 to 120 h, which differs to what was found in the present study, where changes in the structure of the chorioallantoic membrane were observed at 72 h post-inoculation.

The components of the *Brucella* spp. external membrane, when interacting with the different cell and humoral systems, unchain a cascade of responses that participate in the pathogenic mechanisms of the disease, which explains once again that the fact that the CE does not belong to a mammal does not prevent its immature cells to be infected with *B. abortus* (Orduña *et al.*, 2001). This study was of use to demonstrate that the infection, determined by the proliferation of inflammatory cells, damage induction in the cytoarchitecture and necrosis, causes in the CE the characteristic damages observed in the aborted fetuses from cows infected with the *Brucella* spp. field

and vaccine strains, as has been described by Xavier *et al.* (2009), who indicated the evidence of granuloma necrosis and perivascular infiltration in cows infected with the vaccine strains, as well as fetuses with pleurisy and fibrinogen peritonitis. Thus, the main characteristic of the *Brucella* genus is its ability to survive inside of phagocytic and non-phagocytic cells.

Using the Kruskal Wallis test, the results obtained from the histopathological analysis of the liver and chorioallantoic membrane of the CE from the negative control group were compared with those of the groups of CE infected with milk from cows seropositive to *B. abortus*, observing significant differences among groups ($P < 0.05$). The rank sum of the tissues of the negative control group was 49, whereas for the tissues of the groups inoculated with milk cream and with the field strain it was 147 and 210, respectively.

It is convenient to highlight that in this study the use of CE as infection model for *B. abortus* was not intended to be used to diagnose the disease, but to have an alternative model to assure obtaining viable microorganisms that can be used in characterization studies and with epidemiological purposes. Likewise, histopathology was used as a tool to be used as an indicator of proliferation and pathogenicity of bacteria.

CONCLUSION

The infection model used in this study, even though did not belong to a mammal, was of use to achieve infection and induction of damages in the cytoarchitecture of the liver and chorioallantoic membrane after inoculation of milk from cows seropositive to *B. abortus*. This is indicative of proliferation and pathogenicity of this bacteria in CE, which increases the probability of isolating *B. abortus* after being inoculated into this infection model.

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