

VETERINARIA RIVISTA DI SANITÀ PUBBLICA VETERINARIA **ITALIANA**

Special Issue Brucellosis



Pathogenesis of *Brucella suis* biovar 1 in the armadillo (*ChaetophRACTUS villosus*)

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Veterinaria Italiana, Special Issue Brucellosis DOI: 10.12834/VetIt.3007.25510.2

Abstract

Brucella suis biovar 1 has the broadest animal host spectrum. Affects domestic animals and wildlife species. The aim of our study was to investigate the pathogenesis of *B. suis* biovar 1 infection in the armadillo (*ChaetophRACTUS villosus*) under experimental conditions. One gravid female and three adult males were inoculated with a suspension containing 1×10^6 CFU/mL (colony-forming units) of *B. suis* biovar 1 by oral route. In addition, the gravid female and one male received the same suspension by the conjunctival route. A young male and two females not inoculated were kept in contact with the animals inoculated. The animals that tested seropositive were euthanized. All inoculated armadillos showed positive antibody titres 2 weeks post-inoculation. Of the three uninoculated animals, one female was seropositive for *Brucella* infection. *Brucella* was isolated from the spleen, liver, mesenteric lymph nodes, uterus, testes, and urine. Characteristic histologic lesions were found in the epididymis. These results suggest that armadillos can act as a reservoir for the spread of *B. suis* infection, and the persistence of *Brucella* in armadillo tissues constitutes a risk for humans, because of the cultural practice of armadillo meat consumption in rural communities.

Keywords

armadillo, *Brucella suis* biovar 1, *ChaetophRACTUS villosus*, Experimental infection, Pathogenesis

Introduction

Brucellosis is one of the most important diseases in livestock and a major zoonotic disease worldwide. It is caused by various species of the facultative intracellular gram-negative bacteria *Brucella* (Godfroid *et al.* 2010). The causative species are classified largely based on pathogenicity; host preference; growth characteristics; and serological, bacteriological, and/or molecular methods (Whatmore *et al.* 2006). Thus far, several *Brucella* species have been recognized, of which *B. melitensis*, *B. suis*, and *B. abortus* are the most pathogenic organisms isolated from different animal species (Godfroid *et al.* 2010). The main symptoms of *Brucella* infection in most animal species are abortion and stillbirth.

B. suis biovar 1 infection is an increasingly emerging veterinary and public health problem, as the organism has been isolated from pigs (Godfroid *et al.* 2011) and humans in different Latin American countries (Samartino *et al.* 2002, Godfroid *et al.* 2005, Lucero *et al.* 2008), including Argentina.

B. suis biovar 1 can naturally infect cattle (Ewalt *et al.* 1997). It has been isolated from cattle in the Americas and Australia, thus indicating localization of this organism in the mammary tissue; however, there was no evidence of abortion or infertility (Ewalt *et al.* 1997, Cook and Noble 1984).

It has been isolated from wildlife animals as wild pig (*Sus scrofa*, Corn *et al.* 1986), peccary (*Tayassu tajacu*, Lord and Lord 1991), European hare (*Lepus europaeus*, Szyfres *et al.* 1968, Fort *et al.* 2012), armadillo (*ChaetophRACTUS villosus* Kin *et al.* 2014), as well as hunting dogs (Ramamoorthy *et al.* 2011). In La Pampa, Argentina, wildlife surveys revealed seroprevalences of 6.6%, 17.1%, and 16.0 % in European hare, grey fox (*Lycalopex gymnocercus*), and armadillo, respectively (Baldone *et al.* 2007, Fuchs *et al.* 2009, Kin *et al.* 2014).

The knowledge of cultural rituals and practices related to the consumption and handling of wild animals in rural communities as well as information regarding other risk factors is essential for guiding the development of effective control strategies for *Brucella* infection.

Because armadillo meat is consumed in rural communities in Argentina, the present study was conducted to investigate the pathogenesis of *B. suis* biovar 1 in armadillos under experimental conditions.

Materials and methods

Experimental infection

Seven armadillos, which were captured in the province of La Pampa, Argentina, were used in this study. They were kept in enclosures with food and water ad libitum. In group A, two females were kept in one enclosure (A1 and A2), and in group B, three adult males (B1, B2, and B3), a young male (B4), and an adult female (B5) were kept in another enclosure. The inoculated animals included one gravid female (A1) and three adult males (B1, B2, and B3). The strain used was *B. suis* biovar 1 isolated from armadillo (Kin *et al.* 2014).

The animals were orally inoculated with a suspension containing 1×10^6 CFU/mL (colony-forming units) at a dose of 0.1 ml per animal. This dose was chosen because it was found to be effective with other animals (Fort *et al.* 2012). In addition, female (A1) and male (B1) received the same suspension by the conjunctival route. Although the intraconjunctival route is the most used in experimental infections with brucella, we had no reference on its effectiveness in armadillos. It was for this reason that oral and conjunctival infection was carried out on two armadillos. Since there was a limited number of animals to carry out the test.

Blood samples were collected from all the animals at the 80, 49 and 32 days before inoculation, at the beginning of the experiment (day 0), and at 7, 10, 17, 25, 38, and 55 days after inoculation. The serum samples were analysed using the Buffered Plate Antigen test (BPA, Biotandil Lab, Argentina) and Fluorescence Polarization Assay (FPA, Biotandil Lab). For the detection of *Brucella* antibodies, a cut-off score of 82 mP was kept for the latter test (Kin *et al.* 2018).

After 55 days, the animals that tested positive for *Brucella* (5 animals) were euthanized under anaesthesia (tiletamine and zolazepam, 5.0 mg/kg/I.M).

Tissue samples from different organs (uterus, vagina, bulbourethral glands, epididymis head, epididymis tail, liver, spleen, lungs, mesenteric lymph nodes, axillary lymph nodes, and kidneys) were taken. Urine samples were also collected. Bacteriological culture was made in blood agar, *Brucella* agar, MacConkey agar, and Salmonella-Shigella agar, and the culture media were incubated in 10% CO₂ atmosphere at 37 °C and examined daily for 7 days. The presence of colonies showing the characteristics of *Brucella* were analysed by biochemical tests, namely oxidase, catalase, urease, CO₂ requirement and H₂S production, as well as by growth on media containing basic fuchsin (20 mg/ml) and thionin (20 mg/ml) (Quinn *et al.* 2002).

For histopathological analyses, tissue samples collected were immersed in 10% buffered formaldehyde solution. After fixation, tissues were trimmed, dehydrated and embedded in paraffin. Sections of 3µm thickness were obtained with a microtome and rehydrated by immersion in xylene and decreasing alcohol solutions and stained with haematoxylin and eosin following the standard procedures.

The results were analysed statistically by using the chi-square test for significance.

Ethics

Experiments with armadillos were performed in compliance with the regulations of the Institutional Animal Care and Use Committee (CICUAE) of INTA (66/2014). It should be noted that armadillo is not an endangered species (Ojeda *et al.* 2012).

Results

All serological tests (BPA and FPA) were negative at 80, 49 and 32 days before inoculation, at the beginning of the experiment (day 0), and up to 10 days post- inoculation.

At day 17 post-inoculation, three inoculated animals tested positive (A1-B1-B3) and one noninoculated (B5) tested positive for the BPA test. The only exception was inoculated male (B2), which tested positive at day 25. The uninoculated armadillos, female (A2) and the young male (B4), did not develop infection and tested always negative.

The female that was pregnant at the time of the challenge (A1) had miscarriage 3 days after the inoculation. It was not possible to recover the foetuses to perform the corresponding bacteriological cultures. For FPA test inoculated males (B1) and (B3) and female (A1) showed positive titres at day 17. The other inoculated male (B2) showed positive values at day 25. With regard to the animals that were not inoculated, only the female (B5) showed positive result in FPA test at day 17.

In all animals that tested positive, the antibody titres steadily increased until the end of the trial. The animals inoculated by both routes conjunctival and oral (B1 and A1) showed the highest titres in the FPA test in the shortest period of time (Figure 1).

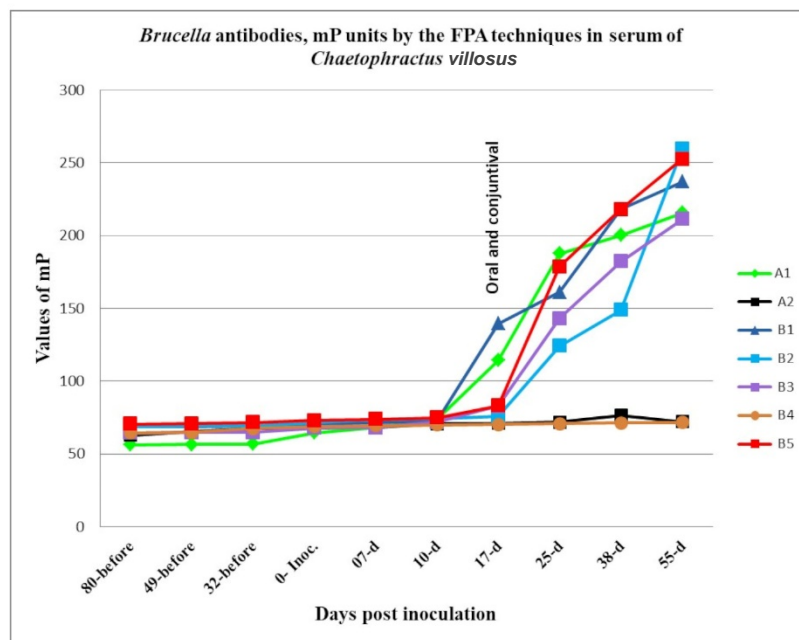


Figure 1. Brucella antibody titres in serum of armadillos (mP-FPA values), uninoculated and inoculated with the strain of *B. suis* biovar 1, according to sex (A2 and B5: adult females uninoculated, A1: inoculated gravid female, B1, B2, B3: adult male inoculated, B4: juvenile male uninoculated).

The three males and two females that showed positive result in the serological brucellosis test were euthanized at day 55 post-inoculation. Macroscopic lesions were not observed in any of the necropsied animals. *Brucella* was isolated from the spleen, liver, and mesenteric lymph nodes of all the four inoculated animals as well as from the in-contact female (B5). The organism was also isolated from the uterus of both females that tested positive and from the vagina of the female who had miscarriage. *Brucella* was also isolated from three urine samples that were collected from armadillos (B5, A1 and B3). In only three animals (B2, B3 and B5), *Brucella* was isolated from the lungs. In addition, *Brucella* was isolated from the two samples of axillary lymph nodes (A1 and B5). With regard to the reproductive organs of the three males, *Brucella* was isolated from the epididymis tail of two males and from the bulbourethral glands of one male (Table I).

Animal Number	B5	A1	B1	B2	B3
Infection	by in contact	challenged ^a	challenged	challenged	challenged
Sex	Female	Female	Male	Male	Male
Lungs	pos	neg	neg	pos	pos
Spleen	pos	pos	pos	pos	pos
Liver	pos	pos	pos	pos	pos
Kidneys	neg	neg	neg	neg	pos
Mesenteric lymph nodes	pos	pos	pos	pos	pos
Axillary lymph nodes	pos	pos	nd	nd	nd
Parenchyma of the testis	--	--	neg	neg	neg
Epididymis tail	--	--	pos	neg	pos
Bulbourethral glands	--	--	pos	neg	neg
Urine	pos	pos	nd	nd	pos
Uterus	pos	pos	--	--	--
Vagina	neg	pos	--	--	--

pos: positive; neg: negative; nd: not done; ^amiscarriage

Table 1. Isolation of *B. suis* biovar 1 from different organs of seropositive armadillos.

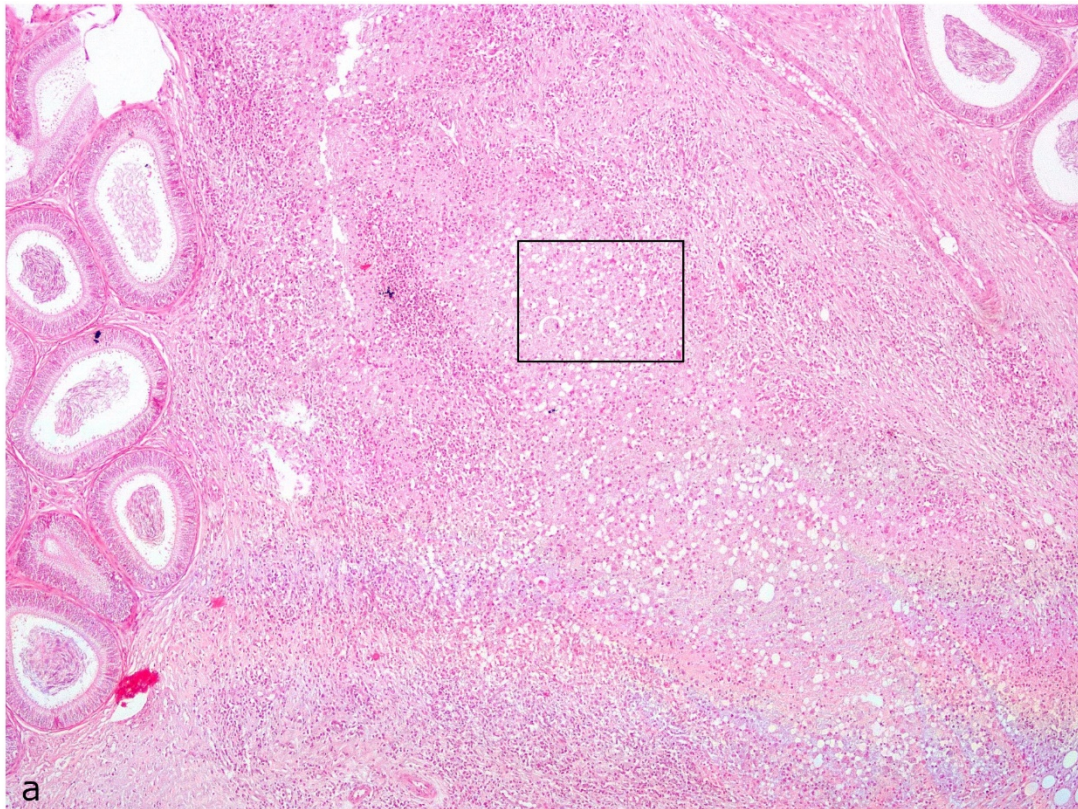


Figure 2a. Testicle: Interstitial infiltration of eosinophilic material with macrophages, lymphocytes and few neutrophils composing granulomatous orchitis. Hematoxylin/eosin, 40x magnification.

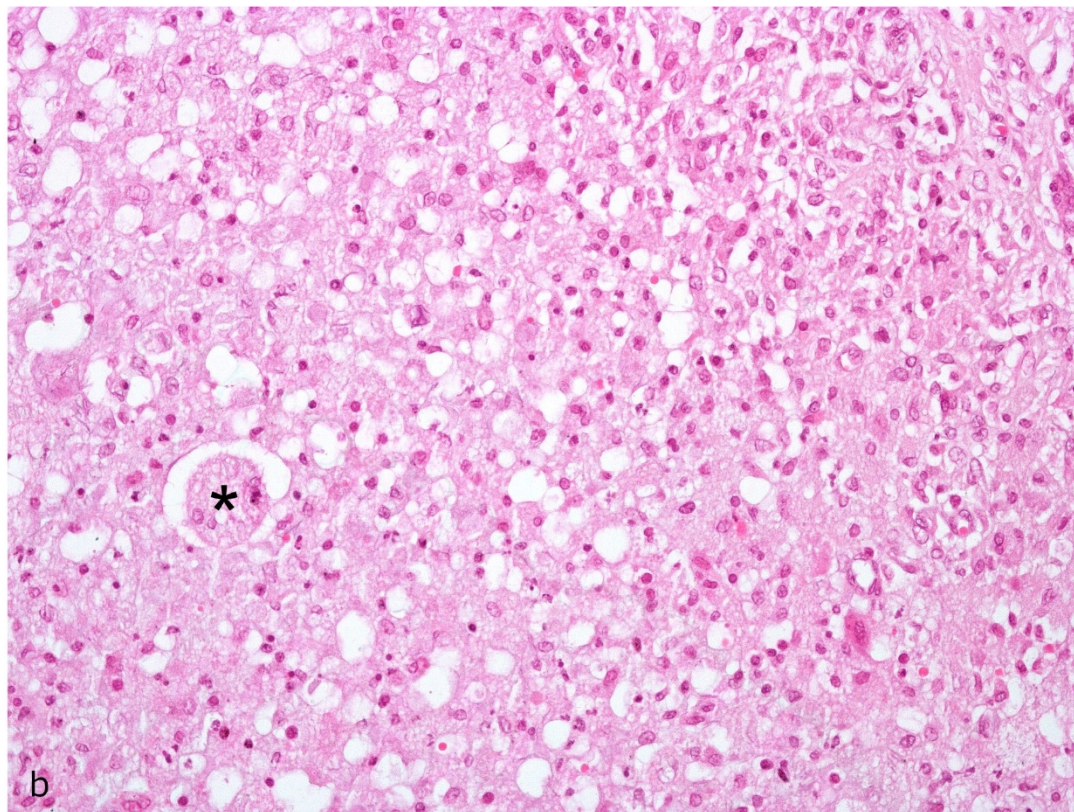


Figure 2b. Testicle : Detail of boxed area in A showing giant cell (*) and granulomatous inflammatory infiltrate. Hematoxylin/eosin, 200X magnification.

Discussion

The sources and routes of *B. suis* infection in armadillos are unknown. Armadillo shares its natural habitat with domestic animals such as pigs, horses, and cattle as well as with other wildlife animals. Thus, armadillos could become infected through contact with these domestic or wild animals, including those in whom the presence of *Brucella* is still unknown. Given the high prevalence of *Brucella* found in the natural habitat of armadillos (16%, 24/150, Kin *et al.* 2014), it is possible that the infection was acquired through intraspecies transmission.

In the present study, the possibility of infecting armadillos with *Brucella* through both oral and conjunctival routes was confirmed. The transmission of the infection was also observed in an uninoculated female that shared the enclosure with three inoculated males. In addition, the study demonstrates that a pregnant female inoculated with *Brucella* through oral and conjunctival routes had miscarriage 3 days later; this finding suggests a relationship between abortion and *Brucella* infection. The pregnant female was in the last third of pregnancy (approximately between days 50 and 65). With regard to this early abortion, it is important to highlight that a hyperimmune reaction due to the high inoculation dose could be responsible for the abortion, rather than a typical inflammatory process elicited by the bacterium. It is also important to note that *Brucella* was found 55 days after inoculation in the uterus and vagina of this female.

The infection of the uninoculated adult female, which was kept in captivity with the three males that were experimentally infected, suggests possible contagion through the venereal route. Considering the presence of the organism in the uterus of female (B5) and in the epididymis tail of the inoculated males, we can speculate that sexual transmission of this organism it could be possible. In pigs transmission during copulation also occurs frequently, and *B. suis* excretion in semen has implications for those practising artificial insemination.

In pigs, after the initial bacteraemia, *B. suis* colonises cells of the reproductive tract of either sex. In females, placentas and fetuses are invaded, while in males, invasion occurs in one or more of the following: testes, prostate, epididymides, seminal vesicles or bulbo-urethral glands (WOAH 2021).

The significant difference ($p \leq 0.0003$) observed at 17 days post inoculation in antibody titres between animals that were inoculated by both routes (oral and conjunctival) compared to those that received a single oral dose, is due to inoculum size. This indicates that the eye is also permeable to the passage of *Brucella*, allowing the bacteria to reach

the bloodstream faster than through the oral route.

Contrary to the results observed in rabbits (Fort *et al.* 2012) infected with *B. suis* biovar 1, the inoculated armadillos did not show macroscopic lesions. In the histopathological studies, lesions were observed only in the epididymis tail of armadillo (B1); this was identical to the lesions observed in domestic species (Foster, 2007). The presence of *Brucella* in the epididymis and the lesions found in one of the infected animals could be associated with infertility problems in affected armadillos. No histopathological changes were observed in organs from armadillos (A1, B2, B3, and B5) or changes were too minor (e.g., slight infiltration of neutrophils or lymphocytes) to be associated with the infection. However, isolation of *B. suis* from most organs corroborated the presence of infection in these animals. The discrepancy in the lesions between species could be related to a variation in the pathogenesis of the infection. Future studies are needed to determine the pathology associated with long-term infection of *B. suis* in armadillos.

The positive urine culture of both male and females indicates that armadillos might not be occasional hosts of *B. suis* biovar 1, but rather suggests the possibility of horizontal transmission between them and to other species (Forbes and Tessaro 1993, Meng *et al.* 2009).

There is no available evidence indicating that armadillos can transmit brucellosis to domestic animals, humans, or other wildlife animals, nevertheless the high incidence of *Brucella* in armadillos (Kin *et al.* 2014) and the cultural practice of armadillo meat consumption indicate that this species could be a risk to humans as well as for domestic and wildlife animals in contact with them. Therefore, there is a need for epidemiological investigations at the wildlife/livestock/human interface to guide the development of effective control strategies for *Brucella* infection.

Conclusion

This study demonstrated that the armadillo acquires *B. suis* biovar 1 infection through both oral and conjunctival routes. Further, most of the armadillo organs studied were colonized by the bacteria for at least 55 days. While it was not possible to verify the occurrence of *Brucella* in aborted fetuses, its presence in the epididymis and uteri indicated its affinity to sexual organs, which could therefore cause reproductive problems in the infected animals. The shedding of *Brucella* through urine is another important factor that should be considered as a route of transmission not only to armadillos but also to other animal species, and especially to humans.

Acknowledgments

The authors would like to acknowledge the National Institute of Agricultural Technology (INTA) Anguil for providing the fund as a part of PAMSL – 1282102 project and Research project Universidad Nacional de La Pampa (UNLPam) PB 208 Res CS 161/09.

The authors would like to thank Virginia Bermejo for her support in developing the histopathological tests.

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