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Short communication

Experimental infection of rabbits (*Oryctolagus cuniculus*) with *Brucella suis* biovar 1 isolated from wild hares (*Lepus europaeus*)

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ABSTRACT

Brucella suis biovar 1 is the causative agent of brucellosis in several domestic and wild animals and it is a common agent of human brucellosis. European hares (*Lepus europaeus*) have been shown to be infected by *B. suis* biovar 1 and the transmission to other animals has been suggested. In this work, experimental rabbits (*Cuniculus orictolagus*) were infected with *B. suis* biovar 1 isolated from wild hares. Infected rabbits showed high serological response in 2 weeks after discharge and typical granulomatous lesions (2 mm diameter) were found in liver, spleen and kidneys after 50 days. *B. suis* biovar 1 was cultured from the lesion of the organs mentioned above as well as from urine, placenta and fetuses. These data suggest that hares are a potential source for horizontal transmission of *B. suis* biovar 1 to other mammalians.

1. Introduction

Brucella suis is the causative agent of swine brucellosis (Meyer and Cameron, 1963). *B. suis* has been found in domestic and wild animals and five biovars can be recognized on the basis of host specificity and laboratory tests (Godfroid et al., 2005). Biovars 1 and 3 are highly pathogenic and cause severe disease in human beings while biovars 2 and 4 are rarely pathogenic to humans and biovar 5 has never been isolated from this source. Biovar 1 has probably the broader host spectrum since it has been isolated from feral pigs, collared peccaries, cattle and humans (Corn et al., 1986; Lord and Lord, 1991; Godfroid et al., 2005; Lucero et al., 2008). The sources of human infection remain unknown in most of the cases and there are currently no requirements for monitoring and surveillance of *B. suis* in domestic pigs or in wild life.

Hares have been shown to be infected by *B. suis* biovars 1 or 2 and a role in transmission to other animals or humans was suggested (García-Yoldi et al., 2007; Szyfres et al., 1968). We undertook this study to determine if *B. suis* biovar 1 isolated from wild hares could be used to experimentally infect domestic rabbits and reproduce pathologic signs and symptoms in this species.

2. Materials and methods

2.1. Isolation of *B. suis* biovar 1 from wild hares

Hares included in the study were obtained from hunted animal stockers located at the province of La Pampa Argentina (localities sampled were: Santa Rosa, Anguil, Uriburu, Ataliva Roca, Lonquimay and Catriló). Serum samples were obtained via cardiac puncture, the blood was centrifuged (15 min – 2500 × *g*) and the supernatant stored at −20 °C. Serological detection of anti-*Brucella* antibodies was performed by standard plate agglutination (SAP) as described elsewhere (Stemshorn et al., 1985). Organs (spleen, kidneys, liver, stomach, intestine, lungs

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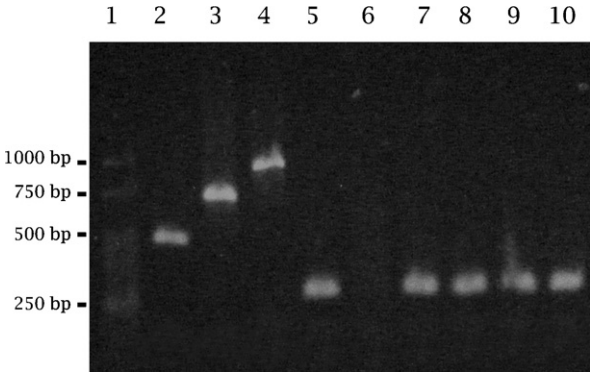

Fig. 1. AMOS-PCR performed with *Brucella* isolates from infected hares. 1: 50 bp DNA ladder, 2: *B. abortus* 2308, 3: *B. melitensis* 16 M, 4: *B. ovis* (natural isolate), 5: *B. suis* 1330, 6: blank (no DNA) 7–10: Hare isolates 68, 88, 98 and 106.

and heart) were examined for the presence of granulomatous lesions and immediately transported (at 4 °C) to the laboratory. Histopathology was carried out using the tissues conserved in 10% neutral buffered formalin. Granulomatous lesions were opened with a scalpel and streaked in blood agar and *Brucella* medium base using cotton swabs. The plates were incubated 1 week in the presence of 10% CO_2 at 37 °C. *Brucella*-like colonies were tested for motility, oxidase, urease, CO_2 requirement, H_2S production, growth on thionin (20 μg/mL) and basic fuchsine (20 μg/mL) and agglutination in the presence of A and M antisera (Alton et al., 1975). The number of viable bacteria was estimated by counting colony forming units in trypticase soy agar plates (TSA) incubated 2–3 days at 37 °C. Bacteria isolates were maintained on skimmed milk at −80 °C. For DNA extraction one loopful of bacteria was suspended in 50 μl 0.1 M NaOH, boiled for 10 min, cooled on ice and neutralized with 18 μL of 0.5 M Tris–HCl, pH 8.0. The volume was brought to 400 μL, centrifuged and 2 μL of supernatant were used as template for PCR assays. DNA amplification for *Brucella abortus, Brucella melitensis, Brucella ovis* and *B. suis* (AMOS-PCR) was performed according to Bricker and Halling, 1994.

2.2. Experimental infection in rabbits

To ascertain the pathogenesis of *B. suis* biovar 1 isolated from wild hares, one of these strains (strain 98; see Fig. 1), was inoculated in five New Zealand white rabbits. *B. suis* strain 98 was incubated for 2–3 days onto trypticase soy agar (TSA) plates at 37 °C. Three separate colonies were cultured in 50 mL trypticase soy broth in a rotary shaking (150 rpm) for 24 h at 37 °C, centrifuged 15 min at 8500 × *g*, and the pellet suspended in 0.9%,w/v NaCl (approximately $2–4 \times 10^6$ CFU/mL). Rabbits (adults of both sexes weighting 2.5–3.0 kg) were inoculated with the bacterial suspension through both conjunctiva (0.1 mL) and oral (0.2 mL) routes. Four rabbits were female two of them were pregnant at the time of infection. The rabbits, preserved with food and water ad libitum, were clinically examined and general appearance was recorded twice daily from days −15 to +50 (being day 0 the day of discharges). Blood samples were taken at days −15, 0, +25 and +50 from exposure. The animals were killed at day 50 post-infection by injecting 45 mg/kg sodium pentobarbital according to the guidelines for animal experimentation. Blood samples and organs were processed as described above and urine samples were taken by puncture of the urinary bladder during necropsy.

3. Results

The incidence of the zoonotic affection caused by *Brucella* in hares was assessed in 106 specimens collected from six stockers at the province of La Pampa, Argentina. The survey showed six individuals with lesions apparently caused by *Brucella sp.* The spleen and liver of every animal had multiple granulomatous lesions with diameters varying from one millimeter to about two centimeters. The kidneys also showed similar injuries in just two animals. Four adult hares weighting 3.8 to 4.0 kg, two males and two females, gave positive cultures for *Brucella sp.* and serum titers were detected in three out of the four animals showing values of 1/168 (one male) and 1/336 (two females). Microbiological assessment showed four isolates with the same phenotype, small rod shape, Gram (−), non-motile, producers of H_2S, CO_2-independent, not growing in presence of basic fuchsine and showed urease positive reaction within 30 min. Serotyping test showed A-dominant phenotype suggesting that the isolates were *B. suis* biovar 1. Furthermore, AMOS-PCR confirmed these results whereas every isolate showed a single band of about 285 bp typical of *B. suis* biovar 1 (Fig. 1).

Infected rabbits with *B. suis* biovar 1 (isolate number 98, Fig. 1), showed malaise and anorexia during the week after inoculation. Two animals showed conjunctivitis during three weeks and one female was affected until the time of necropsy. Serological test was positive at day 25 post challenge in all the animals assayed. Agglutination titers were 1/1024 in four cases (including the male and both pregnant females) and 1/512 in the other rabbit. Consistently with the observations in wild hares, granulomatous

Table 1
Results obtained after infection of rabbits with *Brucella suis* biovar 1 isolated from hares.

Rabbit	Sex	Lessions[a]		Culture				Pregnancy	Culture	
		Liver	Spleen	Liver	Spleen	Kidney	Urine		Fetus	Uterine
1	F	+	+	+	+	–	+	+	–	nd
2	F	+	+	–	+	–	–	–	na	na
3	M	+	+	–	+	–	–	na	na	na
4	F	+	+	+	+	+	nd	+	+	+
5	F	+	+	–	–	–	–	–	na	na

[a] White granulomatous lesions of >2 mm diameter.
(+): positive; (−) negative; nd: not done; na: not applicable.

lesions were observed in the liver and spleen in all infected rabbits (Table 1). Granuloma with dry and caseous appearance of about 2 mm in diameter, were numerous and evenly distributed over the surface of the organs. Histological examination showed nodules with necrotic centers, surrounded by fibroblastic and epithelioid cells and a fibrous outer capsule containing abundant lymphoid and polymorphonuclear cells. Although we were not able to isolate *Brucella sp.* from one of the infected rabbit that presented high serological titer, *B. suis* biovar 1 was isolated from lesions of spleen and liver of the other four infected rabbits. The bacteria was also isolated from affected kidneys of one pregnant rabbit and urine, amniotic fluid and the stomach content of the unborn rabbits of the other pregnant female (Table 1).

4. Discussion

There is no available evidence indicating hares effectively transmit brucellosis to domestic animals, humans or other wild animals. Otherwise, rabbits are not frequently used as experimental models for *Brucella* infection because they would show only partial susceptibility to *B. abortus* and *B. suis* (Silva et al., 2011). In this work, was demonstrated that hare infection can be established in rabbits reproducing most of the aspects of the disease found in the wild animal. Both male and females were infected and lesions induced in rabbit organs were highly similar to the lesions observed in hares. The granulomas of rabbits were smaller in size but this fact can be explained by the elapsed time of the infection that most probably was longer for the wild hares.

After rabbit infection, vertical transmission to the offspring in one out of two pregnant females tested was revealed. This fact suggests that lagomorphs would not be just occasional hosts of *B. suis* biovar 1. A positive culture from urine in one out of five rabbits suggests in turn another way of horizontal transmission particularly to herbivores (Meng et al., 2009) (Forbes and Tessaro, 1993). On the other hand, lateral transmission from hares to other animals as carnivores or scavenging animals would likely occur after contact with infected carcasses or abortions (Szyfres et al., 1966).

There is one previous report indicating the isolation of *B. suis* biovar 1 from hares in Argentina (Szyfres et al., 1968) and four new cases are reported here out of 106 animals examined. Although wild animals can be victims of infection spill-over from domestic animals and human activities (Godfroid et al., 2005; Reusken et al., 2011), the region sampled here does not have important pig farms or abattoirs leading to suspect human activities as the source of hares infection. Since stockers locate at >20 km far apart from each other and hare home range is about 20–25 ha, the studied specimens would came from different populations and from different infection foci (Kunst et al., 2001). These data support the idea that European hares can effectively be a source of *B. suis* biovar 1.

The occurrence of brucellosis in human beings is only sporadic but it is known that fewer than 10% of human cases may be clinically recognized or reported (Mantur et al., 2007). In Argentina, about 40% of the human isolates are *B. suis* biovar 1 but the source of infection is mostly unknown (Lucero et al., 2008). Hares would be infected with the pathogenic strain *B. suis* biovar 1 and we experimentally demonstrated it can be transmitted to other animals. Although further studies are needed in order to determine hares as true reservoir of *Brucella spp.* hunters and persons working in slaughterhouses must be educated in order to avoid unnecessary risks (Godfroid et al., 2005; Al Dahouk et al., 2007).

Conflict of interest

The authors declare that they have no competing interest.

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