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Short communication

Duplex PCR for the diagnosis of *Brucella melitensis* and its differentiation from the REV-1 vaccine strain

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ABSTRACT

Caprine brucellosis is a chronic infectious disease caused by *Brucella melitensis*, whose main symptoms are abortion in females and orchitis in males. Vaccination by mistake of pregnant goats with the REV-1 vaccine may cause abortions. The aim of this study was to develop a duplex PCR that could identify and differentiate the DNA of *B. melitensis* field strains from that of the REV-1 vaccine strain. The results showed that the duplex PCR performed on DNA isolated from infected tissues is very convenient as it significantly shortens the time of detection of the etiological agent, yields a reliable result and has an acceptable cost.

1. Introduction

Caprine brucellosis is a chronic infectious disease caused by *Brucella melitensis*, whose main symptoms are abortion in females and orchitis in males (Alton, 1990). Furthermore, it is one of the most important zoonoses worldwide (Pappas et al., 2005). In Argentina, the disease is present in the provinces of Salta, Formosa, Chaco, Catamarca, Tucumán, La Rioja, Mendoza and San Luis, with areas with more than 40% of the herd infected (Robles et al., 2014). The disease is usually diagnosed by a blood test to detect antibodies to the causative agent or by microbiological tests for the isolation of *B. melitensis* in cases of abortions (Crespo-León, 1994).

Vaccination of goats with the REV-1 vaccine, an attenuated *B. melitensis* strain, minimizes the appearance of the disease. However, vaccination by mistake of pregnant goats with this vaccine may lead in many cases to abortions. In these circumstances, the identification of the bacterium is imperative to define weather the abortion was caused by the field strain or the vaccine strain. Achieving an accurate diagnosis by microbiological methods is not always possible, because samples sometimes arrive at the laboratory in bad conditions. Moreover, in the cases in which the bacterium is isolated, the culture and identification of the isolates demand at least two working weeks to confirm the diagnosis.

In the last two decades, differentiation of *B. melitensis* field strains from the REV-1 vaccine strain has been achieved by using molecular techniques. The techniques used include the PCR-RFLP (Cloeckaert et al., 2002; Bardenstein et al., 2002) and multiplex PCR for all species of *Brucella spp* (Garcia-Yoldi et al., 2006; Mayer-Scholl et al., 2010). However, these methods are laborious and expensive to apply in diagnostic cases.

The aim of this work was to develop a simple and affordable duplex PCR that can differentiate the DNA of *B. melitensis* field strains from that of the REV-1 vaccine strain.

2. Methods

2.1. Bacterial strains and growth conditions

The bacteria used in this study were the commercial vaccine strain REV-1 (OCUREV, batch 07002, CZV laboratory, Spain), three *B. melitensis* field isolates from goats, three *B. ovis* field isolates from sheep, *B. abortus* strain 2308, and commercial vaccine strains RB51 and S19. The strains were plated on Columbia agar (Britania laboratories, Argentina) supplemented with 7% of sheep blood and then incubated at 37 °C with 20% CO_2 (*B. ovis*), 10% CO_2 (*B. abortus* and RB51) or under aerobiosis (S19, *B. melitensis* and REV-1).

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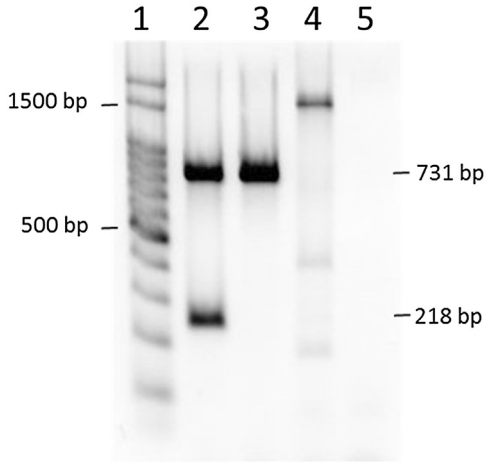

Fig. 1. Duplex PCR on DNA isolated from bacterial culture. Lanes: 1, Molecular weight maker; 2, REV-1 vaccine strain; 3, *B. melitensis* field strain; 4, *B. ovis* field strain; 5, negative control.

2.2. Tissue culture from aborted fetuses

After necropsy, samples of spleen, liver and lung from four aborted goat fetuses were homogenized with sterile saline solution using a Stomacher (AES Laboratoire, France). Then, 200 μl of the solution or abomasum content was plated on Columbia agar (Britania laboratories) supplemented with 7% of sheep blood and then incubated at 37 °C for seven days. The colonies were identified using biochemical tests (Alton, 1990).

2.3. DNA isolation

DNA from pure strains was extracted using the Accuprep Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions. DNA from a fraction of tissue homogenates was isolated by three methods: the phenol/chloroform (F/C) method (Matrone et al., 2009), the Chelex method (Bio-Rad) (CH) and the Chelex plus proteinase K method (CHK) (Silva et al., 2014). In the F/C method, DNA was extracted from 500 μl of macerated thawed tissue samples, as previously described (Matrone et al., 2009). In the CH method: a 20-μl aliquot of tissue homogenate was added to 200 μl 5% Chelex-100 (Bio-Rad). The mixture was vortexed, incubated at 65 °C for 20 min, and boiled in a water bath for 8 min. After vigorous vortexing for 15 s, the sample was centrifuged at 16000 x *g* for 3 min, and the supernatant was collected and stored in a new tube at −20 °C until use. In the CHK method, DNA was extracted using the CH method as described by Silva et al. (2014) with small modifications. A 20-μl aliquot of tissue sample was added to 200 μl 5% Chelex-100 (Bio-Rad), with the subsequent addition of 5 μl of 20 mg/ml proteinase K (Sigma). The mixture was vortexed, incubated at 37 °C for 1 h, and boiled in a water bath for 15 min to inactivate proteinase K. After vigorous vortexing for 15 s, the sample was centrifuged at 16000 x *g* for 3 min, and the supernatant was collected and stored in a new tube at −20 °C until use.

2.4. Amplification by PCR

PCR was performed using specific primers Bmel Fw:−5′ TGC CGA TCA CTT AAG GGC CTT CAT-3′ and Bmel Rv: 5′-AAA TCG CGT CCT TGC TGG TCT GA-3′, which amplify a 731-bp fragment (Bricker and Halling, 1994) in REV-1 and *B. melitensis* field strains, and primers REV Fw: 5′-CAG GCA AAC CCT CAG AAG C-3′ and REV Rv: 5′-GAT GTG GTA ACG CAC ACC AA-3′, which amplify a 218-bp fragment (Garcia-Yoldi et al., 2006) in REV-1. Primers were analyzed using OligoAnalyzer 3.1 (Integrated DNA Technologies). DNA from *B. melitensis* and REV-1 strains was used as positive control. DNA from *B. ovis*, *B. abortus* 2308, S19 and RB51, *Histophilus somni, Actinobacillus seminis* and water were used as negative controls. The PCR reaction mixture consisted of Buffer 1× (PB-L, Argentina), 200 μM of each dNTP (PB-L), 0.5 μM of primers Bmel, 0.01 μM of primers REV (Life Technologies, USA), 2 mM of $MgCl^2$, 0.05 U of Taq DNA polymerase (PB-L) and 100–500 ng of DNA in a final volume of 25 μl. The amplification parameters were: 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 64 °C for 1 min, and 72 °C for 1 min, with a final step of extension at 72 °C for 5 min. PCR products were resolved by 1.5% agarose gel electrophoresis stained with GelRed (Biotium, USA). Amplified bands were viewed under a UV transilluminator and the image of the gel was captured using the FOTO/Analyst® 107 Investigator/Eclipse System (Fotodyne, USA).

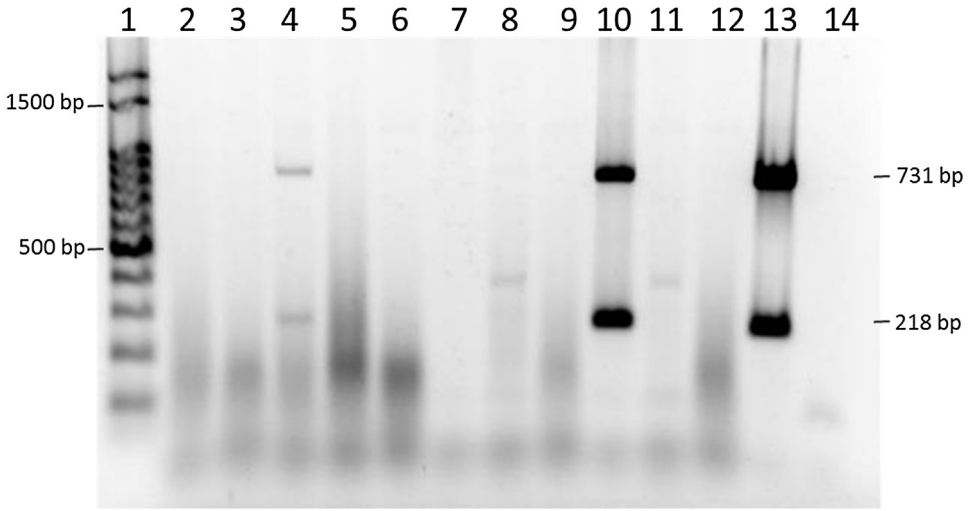

Fig. 2. Duplex PCR on DNA isolated from fetuses tissues by CHK method. Molecular weight marker, lane 1; animal 1, lanes 2–4 (spleen, liver, lung); animal 2, lanes 5–8 (spleen, liver, abomasum content, lung); animal 3, lanes 9–10 (liver, abomasum content); animal 4, lanes 11–12 (abomasum content, spleen animal); positive control, lane 13; negative control, lane 14.

3. Results

3.1. PCR on pure strains

We first chose PCR primers to differentiate *B. melitensis* field strains from REV-1. Thus, we selected a primer pair described by Bricker and Halling (1994) that amplifies a 731-bp fragment in all *B. melitensis* biovars and in REV-1 and a primer pair described by Garcia-Yoldi et al. (2006) that amplifies a 218-bp fragment only in REV-1. These primers detect a specific mutation in the *rpsL* gene of the vaccine strain REV-1 that differentiates it from the *B. melitensis* reference strain (Bricker and Halling, 1994).

We adjusted the PCR conditions modifying different parameters such as primer concentration and melting temperature. The analytical sensitivity of PCR was determined by serial dilutions of DNA as 0.1 ng for both REV-1 and *B. melitensis* (data not shown), being higher than other diagnostic PCRs published before (Moustacas et al., 2013; Qasem et al., 2015).

Moreover, the analytical specificity was determined by performing PCR on DNA of *B. ovis*, *B. abortus* and vaccine strains S19 and RB51 and other pathogenic bacteria of small ruminants such as *A. seminis* and *H. somni*. A 1500-bp band and two smaller faint bands were obtained for *B. ovis*, *B. abortus* and vaccine strains S19 and RB51. No bands were obtained for *A. seminis* or *H. somni*. The results showed that the PCR was specific and differential for *B. melitensis* and REV-1 (Fig. 1).

3.2. PCR on tissue samples

To determine whether the PCR was able to detect the presence of DNA of *B. melitensis* or REV-1 directly on tissues, we performed the PCR on DNA extracted from tissues from aborted goat fetuses suspected of being infected with *B. melitensis* or REV-1. Microbiological diagnosis was carried out in parallel.

Microbiological culture and biochemical tests showed positive results for REV-1 in the four animals analyzed. However, animals 2 and 4 showed a large number of contaminants or a low number of colonies of REV-1.

The PCR was able to detect REV-1 DNA in tissues of two out of the four animals analyzed (lungs from animal 1, lungs and abomasum content from animal 3). In animals 2 and 4, REV-1 was not detected by PCR (Fig. 2). Dilution of the samples to remove PCR inhibitors did not alter the results.

The sharpest bands were obtained by the F/C DNA extraction method. However, this method is laborious and expensive. Of the Chelex extraction methods, which are faster and cheaper than the F/C method, the protocol that includes proteinase K (CHK) was the most advantageous (data not shown).

4. Discussion

Several authors have attempted to differentiate *B. melitensis* field strains from the REV-1 vaccine strain (Samadi et al., 2010; Banai, 2002; Benkirane et al., 2014). The techniques used include PCR-RFLP (Cloeckaert et al., 2002), which is more expensive and time-consuming than conventional PCR, and multiplex PCR for all *Brucella* spp. (Garcia-Yoldi et al., 2006; Mayer-Scholl et al., 2010), which requires the use of many primer pairs, making it expensive and laborious.

The duplex PCR developed in this work was able to differentiate *B. melitensis* from the vaccine strain REV-1, with only two primer pairs in one step. It also allowed differentiating *B. ovis* from *B. melitensis*, which could be useful in regions where sheep and goats are bred.

Moreover, the CHK DNA extraction method, coupled with PCR, considerably shortened the working time compared to culture and biochemical identification. This PCR could be implemented to identify *B. melitensis* or REV-1 in colonies from the microbiological culture, as well as to perform direct molecular diagnosis from tissue samples (preferably abomasum content and lung), considerably shortening the traditional diagnosis time. Although analysis of a greater number of samples is needed to validate this PCR, results presented here demonstrate that this duplex PCR is a promising tool to diagnose brucellosis in goats.

5. Conclusion

The PCR developed in this work is very convenient because it allows the differentiation of *B. melitensis* field strains from REV-1, significantly shortens the detection time of the etiological agent, yields a reliable result and has an acceptable cost.

Conflict of interest statement

The authors declare no conflict of interest.

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