Detection of *Brucella spp.* by PCR from milk samples isolated from the state of Guanajuato

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Abstract

In the present study, milk samples collected from Cuerámaro, Guanajuato were analyzed by PCR assays in order to show the presence of the pathogenic Bacteria *Brucella spp* . Assays with attenuated vaccines containing *Brucella spp.,* showed amplicons of ~ 200 bp amplified, corresponding to the expected size of the omp-2 gene of *Brucella spp.* Nonthless, assays with milk samples showed no amplicons, indicating that there was no presence of *Brucella spp.,* making the milk suitable for consumption. This report shows the potential of PCR assays for the detection of the pathogenic bacteria Brucella in milk and also shows that the JPR and JPF primers are effective in the amplification of the omp-2 gene of Brucella spp.

Introduction

Brucellosis is a widespread zoonotic disease of public health importance caused by members of genus brucella. Brucella species are gram-negative, facultative intracellular bacteria, which lack capsules, flagellae, endospores or native plasmids. Currently, the genus Brucella consists of ten species: *B. abortus*, *B. suis, B. ovis, B. melitensis, B. canis, B. neotomae, B. pinnipedialis, B. ceti, B. microti and B. inopinata;* How ever only four species of the genus Brucella are pathogenic for humans, *B. melitensis, B. abortus, B. suis, and B. Canis ; B. Melitensis* being the most pathogenic specie. (5)

Man is an accidental host and contracts the disease directly from infected animals, and occurs mainly through direct contact with infected animals, ingestion of raw dairy products of animal origin, or consumption of infected meat from domestic livestock (5). Human brucellosis may lead to a variety of clinical presentations, such as fever, sweating, chills, headache, malaise, myalgia and even arthralgia of the large joints.

The presentations and phases of the disease may be acute, subacute, chronic, relapsed, active or inactive.

Antibiotic treatment of human brucellosis often results in high treatment failure and relapse rates. Because the clinical presentation is non-specific, laboratory testing is required for confirmation (1).

At present, there are various assays for diagnosis of human brucellosis such as standard microbiological tests for the isolation of Brucella spp. from blood, tissue specimens, body fluids and bone marrow, serological tests for the detection of anti-Brucella spp. antibodies and molecular methods for the detection of Brucella spp. DNA (5). On the other hand, diagnosis of brucellosis in live dairy cattle involve either the isolation of Brucella from milk samples or the detection of anti-Brucella antibodies in serum or milk. However, these methods are not very effective and Bacteriological isolation is time and resource intensive; it requires level 3 biocontainment facilities and highly skilled technical personnel to handle samples and live bacteria for eventual identification and biotyping.

Handling all live Brucella involves risk of laboratory infection and very strict biosafety rules must be observed. (7).

Serological methods are not conclusive, because not all infected animals produce significant levels of antibodies and because cross-reactions with other bacteria can give false-negative results (3).

In order to avoid these disadvantages, methods based on the polymerase chain reaction (PCR) are becoming very useful and considerable progress has been made recently to improve their sensitivity, specificity, and technical ease and to lower costs. (7). Some previous studies have demonstrated that PCR can be used to detect Brucella DNA in milk samples and they have shown potential to be fast, accurate, and efficient methods for detecting Brucella (3).

In the state of Guanajuato there were 195 cases of brucellosis reported in 2014 (8) and for this reason the present study involves the detection of brucellosis, focusing on the diagnosis on dairy cattle (in this case goats). Considering the advantages of the polymerase chain reaction, assays involving this method were used in order detect *B.melitensis,* which is considered the most pathogenic specie of brucella spp.

Materials and Methods

Biological samples.

Goat Milk Sampling

The goat milk samples were obtained from a farm located in Cuerámaro, Guanajuato.

Positive samples. Reference Brucella Strains were obtained from an attenuated *brucella melitensis* strain ver-1 vaccine (*Productora nacional de biologicos bacterianos*) kindly given by Dr. Abner Gutierrez. The lyophilized vaccine was rehydrated with 10 ml of destilated water .

DNA extraction from milk samples.

The extraction method used was the proposed protocol by Romero *et al. (1999)*, with slight modifications. Frozen milk was thawed at room temperature, and 500 ml of sample was mixed with 500 ml of sterile destilated water. Subsequently 100 µl of NET buffer (50 mM NaCl, 125 mM EDTA, 50 mM Tris-HCl [pH 7.6]) was added to the mixture. The mixture was cooled on ice after incubation at 80 °C for 10 min. 20 µg/ml of Proteinase K was added at 50°C for 90 minutes. DNA was extracted by collecting the aqueous phase after adding phenol:chloroform:isoamyl alcohol (25:24:1) to the mixture, and was precipitated with 1 volume of isopropanol. Alternately the mixture was centrifuged at 13,000 (rpm) for 5 min, washed with 70% ethanol, and dried under vacuum. The DNA pellet was dissolved in 25 µl of sterile distilled water and stored at -20°C until further use.

Synthetic oligonucleotide design. The nucleotide sequence of the gene coding for the outer membrane protein *omp-2* reported for *B. abortus* (1), was obtained from the GenBank database located at the National Center for Biotechnology Information of the National Library of Medicine (Bethesda, Md.). Oligonucleotides were designed and were synthesized by Sigma Aldrich, Inc. (St. Louis, MO, USA). The sequences of both primers are as shown in Table 1.

Primers	Sequences	Amplicon Sizes (bp)	Primer Pair target	References
	Forward: 5'GCGCTCAGGCTGCCGACGCAA 3' Reverse: is 5'-AC CAGCCATTGCGGTCGGTA-3'	198	External membrane protein (omp-2)	Leal-Klevezas et al. (1995).

Table 1. Oligonucleotides used to Identify Brucella spp.

PCRs. PCR was performed in a 50 µl reaction mixture with 1.5 µl of DNA obtained directly from the commercial vaccine, 1.5 µl of each primer, 10 µl of Buffer 10X + MgCl2(KCl, Tris-HCl) , 1.5 µl of the four nucleoside triphosphates (dNTPs), and 1 µl of *Taq* polymerase (New England.). The reaction was performed in a DNA thermal cycler (Bio Rad C 1000 Touch) at a denaturation temperature of 94 °C for 1 min; this was followed by 35 cycles at 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s and one final extension at 72°C for 3 min.

Dna Analysis

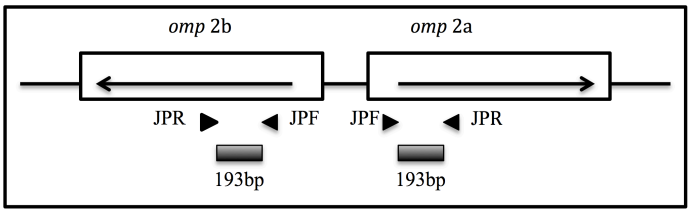
Eight microliters of the amplification reaction mixture was taken and fractionated in a 1% agarose gel containing 1X TAE (Tris Acetate EDTA), stained with an ethidium bromide solution (0.5 µg/ml), and visualized under UV light (4). Finally the gel was analysed with Image Lab™ Software by Biorad.

Results and Discussions

From the DNA sequence reported by Leal-Klevezas et al. (1995), we chose the gene that encodes for an external membrane protein (omp-2) reported for *B. abortus*. This gene has a duplication (in a head-to-head array) of the open reading frame, which is 85% homologous for this species (1).

We chose two primers designed by Leal-Klevezas et al. (1995), shown in Table 1, which targeted two homologous regions of the omp-2 gene present in both copies. (Fig. 1A).

(A)



(B)

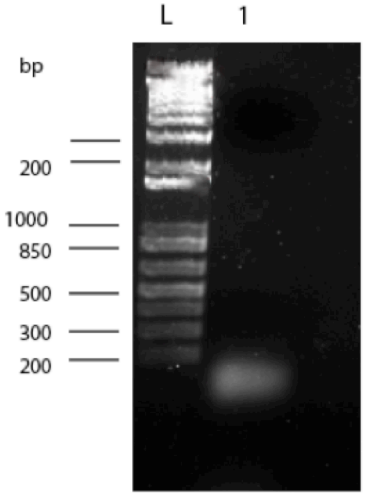


Fig. 1. Amplification map of the omp-2 gene with primers JPF, JPR, and Brucella DNA. (A) Schematic representation of the two copies of the omp-2 gene reported for B. abortus. The arrows indicate the orientations of both copies (omp-2a and omp-2b). Triangles represent the positions of primers JPF and JPR. Filled boxes represent the amplified fragments of 193 bp. **(B)** PCR amplification product from positive control; L, 1 Kb (kilobase) DNA ladder (New England Biolabs); Lane 1, B. melitensis biovar 1.

As predicted, primers JPF and JPR allowed the amplification of an aprox. 200 bp fragment from the positive Brucella control (Fig. 1B) which match the result obtained by Leal-Klevezas *et al*. (1995). Nonetheless the amplification of the DNA obtained from milk samples at the same PCR conditions were always negative (data not shown), which indicate that the milk used in the present studies was not contaminated by *Brucella spp.* making it apt for consumption.

Conclusions

Ultimately, our DNA extraction from the milk samples collected from Cuerámaro, Guanajuato showed an absence in the presence of Brucella spp. Indicating that the milk was suitable for consumption. Nonetheless the PCR assay showed the effectiveness of using the primers for JPR and JPF for the identification of Brucella in the attenuated vaccine that contained this pathogen (*B. melitensis biovar 1*). These results confirm the potential of PCR based assays and show that this technique could allow rapid and more sensitive identification of Brucella genus at the species and at the biovar level, compared with traditional techniques.

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