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# Diagnosis of Caprine Brucellosis by Serology and Multiple PCR

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***Abstract:*** *Our aim was to perform the diagnosis of caprine brucellosis by serology and multiple Polymerase Chain Reactions (PCR). The field work was conducted on the Ejidos la Victoria MunicipioTlahualilo Public Lands of San José de Bellavista y Bermejillo, Mapimí Municipality, state of Durango, Mexico. Meetings were held with the producers to explain to them the objectives and benefits to be obtained with the carrying out of this investigation. Samples were taken from 114 native breed animals crossed with Saanen and Alpine breed animals from the jugular vein, obtaining 114 blood samples in tubes without anticoagulant for the obtention of serum in order to process the Rose Bengal Plate Test (RBPT) and 114 whole blood samples for processing multiple PCR. We carried out DNA extraction of control strains of Brucella abortus RB51 and Brucella melitensis RM1 employing the phenol:chloroform:isoamyl alcohol method with the oligonucleotide sequence of Brucella genus, Brucella abortus, Brucella melitensis, Brucella suis, and IS711. General seroprevalence was 26.31% and seroprevalence by Tlahualilo Municipality was 41.86%, while this in Mapimí was 18.18%. In the multiple PCR sample analysis, we found that 30 samples corresponded to B. melitensis, obtaining 100% sensitivity and specificity. The PCR technique described in this study presented 100% sensitivity and specificity with the RBPT, allowing for the simultaneous identification, between and genus and species, the implementation of the multiple-PCR variant capable of identifying different species of the Brucella genus, the latter leading to a better diagnosis of the disease.*

***Keywords:*** *Brucella melitensis, goats, sensitivity, specificity.*

## I. Introduction

Brucellosis is cataloged as a bacterial zooonosis found worldwide whose genus is *Brucella* and that consists of multiple species [1]. Its incidence ranges from between 1.3 and 70.0 cases per 100,000 inhabitants, differences due to the characteristics of each nation. Mexico is one of the countries with the greatest incidence of human brucellosis in Latin America, causing economic loses generated in domestic cattle-raising and its impact on public health [2]. The highest brucellosis incidence rates were found in bovines, followed by caprines and ovines. The genus *Brucella* includes three important species for human pathology: *Brucella melitensis*, which preferentially affects goats, but that can affect bovines and pigs. In Mexico, the highest incidence of bovine brucellosis is observed in stabled livestock and in high-animal-density areas, such as the central, southeastern, and coastal zones. Caprine brucellosis possesses a wider distribution with greatest frequency registered in entities with high goat concentrations [3].

At present, the Mexican Ministry of Health carries out educative health activities, informing the population of the public health problem represented by the consumption of non-pasteurized lactic products and contact with the meat of animals suffering from brucellosis. Preventive measures for professional risk include the following: consume pasteurized milk, subproducts, and derivatives of these, rejecting those of doubtful origin; limit close cohabitation with animals; wash hands with soap and water before eating and after contact with animal or subproducts and waste; cleanliness, disinfection, and separation by means of fences in places for the raising/breeding of livestock (caprine, bovine, and porcine); identification and elimination of sick animals and vaccination prior to 3 months of age; timely stimulation of medical care and treatment termination; motivation of the medical and paramedical area to conduct patient follow-up at 30, 60, 90, and 120 days, and maintain surveillance of blood donors being brucellosis-negative [4].

Maximal focus cases have been reported of up to 18.3% of brucellosis in humans, caused by the ingestion of caprine- and ovine-origin lactic products in which *B. abortus* and *B. melitensis* were involved.

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Mexico places worldwide as one of the regions with the greatest prevalence of bovine brucellosis, with 3.42% seropositivity in humans. In the Mexican state of Zacatecas, the campaign against brucellosis is found in the control phase. Brucellosis prevalence at the domestic level in herds/flocks was 2.94% and 0.61% in heads of cattle; thus, it is referred that caprine brucellosis was determined as 10.35%, and for ovine brucellosis, this was 0.61% for the same year [5].

Reinforcing zoosanitary status has repercussions on public health, in that it mitigates the risk of contagion in humans due to the consumption of unpasteurized lactic products[6]. The Comarca Lagunera is a brucellosis-endemic zone, and this disease tends toward chronicity due to ignorance in terms of its diagnosis, treatment, and implications for public health, in that it causes arthritis, endocarditis, meningitis, and osteomyelitis, while it also leads great economic losses on being a zoonosis.

In that brucellosis is a zoonosis, it is economically important for humans and livestock, and its worldwide distribution is caused by Gram-negative bacteria, facultative intracellular, that belongs to the genus *Brucella*. The identification and differentiation of *Brucella* species is based on the culture, a long and laborious process, in addition to there being a risk of infection. Although the serodiagnosis of brucellosis is performed by means of the detection of specific antibodies, it is necessary to confirm the result with molecular methods. Therefore, the study of novel techniques that improve the diagnosis of this disease is fundamental.

In addition, there is scarce information on the relative knowledge of the blood profile in goats with brucellosis; thus, it is pertinent to know the changes among the biochemical and hematological metabolites that can reflect the effects of brucellosis on animal health. Because with a zoonosis, it is essential to know the hematological and blood biochemical values, in that they are indispensable indicators for carrying out diagnostic health evaluations at the individual as well as at the population level. Having access to reference data supplied by healthy persons contributes to the early detection of individual diseases and/or organic dysfunctions. Therefore, the objective of the present investigation was to perform a comparison between the serological and molecular method in the diagnosis of brucellosis with multiple PCR multiple in healthy bovine cattle and in those with brucellosis.

## II. Materialsand Methods

**Type, place of study, and ethical aspects**

The present analytical study was approved by the Research Ethics Committee of the FCQ of the University of the State of Durango (UJED) N°123301538X0201 COFEPRIS. The field work was carried out in the Ejidos la Victoria MunicipioTlahualilo Public Lands, San José de Bellavista y Bermejillo, Mapimí Municipality, state of Durango, Mexico. Meetings were held with the producers to explain the objectives and benefits to be obtained with the performance of this investigation. The sampling was consecutive in gestating goats 12 months of age with and without brucellosis. We collected approximately 10 mL of blood per goat; sampling was conducted in tubes[2,3], and immediately afterward, the tubes were transported in coolers for their processing. The serum was separated from the blood by centrifugation[4] at 3,000 rpm during 10 min.

Each sample was labeled utilizing codes that described each animal. One hundred fourteen native goats crossed with Saanen and Alpine breeds were sampled by the jugular vein, obtaining blood samples in a tube with anticoagulant to obtain the serum for processing by means of the RBPT[5] at the Regional University Unit of Arid Zones (URUZA) of the Autonomous University of Chapingo (UACh) and 114 samples of whole blood for multiple PCR processing at the FCQ, Durango Unit, UJED. We carried out DNA extraction of the control strains of *Brucella abortus* RB51 and *Brucella melitensis* RM1 employing the phenol:chloroform:isoamyl alcohol method with two wrinkled *B. abortus* RB51 and *B. melitensis* RM1 control strains, with the oligonucleotide sequence of *Brucella genus* (BG), *Brucella abortus* (BA), *Brucella melitensis* (BM), *Brucella suis* (BS), and IS711 (IS). Meetings were held for the producers to explain the objectives and benefits to be obtained with the performance of this investigation. We performed multiple PCR applying this to each of the samples: Identification of *Brucella* spp. was based on the amplification of the genomic region of the *bcsp31*[6] gene. The DNA sequence constituted by the animals' feeders [7] is shown in table 1.

**DNA extraction of control strains**

To carry out the genomic extraction of the control strains of *Brucella*, this began from a 5-day growth in Trypticase Soy Agar (TSA) broth. We placed 1.5 mL of culture in an Eppendorf tube and centrifuged this for 2 min at 13,000 rpm. The supernatant was eliminated and the cellular pellet was resuspended in 1 mL of lysis

[2]BD Vacutainer® Suero/SERUM 13 × 100 mm.
[3]BD Vacutainer® K2E/K2 EDTA 13 × 75 mm.
[4]SOLBAT® Modelo C-600, México.
[5]ABA test card at 3% *Brucella Abortus* Antigen (BAA) , PRONABIVE® México.
[6]GenBank access #M20404.

buffer (EDTA 50 mM, NaCl 100 mM, pH 7.5), was mixed in a vortex until resuspension, and was centrifuged for 2 min at 13,000 rpm to recover the bacterial pellet again and the supernatant was eliminated. To recover this, we resuspended this again in 300 µl of lysis buffer. After this, we added 100 µl of lysozyme (10 mg/mL) drop-by-drop and shook this with a vortex; the mixture was incubated for 15 min at 37°C. We added, to the cellular extract, 30 µL of Sodium DiSulfate (SDS) at 20% and incubated this at 37°C during 5 min. We extracted the lysate with a similar volume of phenol-chloroform-isoamyl alcohol (24:25:1) u/u; this was mixed and centrifuged at 13,000 rpm for 5 min (206.4:215:8.67 µL) [8]. The aqueous phase was transferred, we added 30 µL of sodium acetate 3M, and filled the tube with cold ethanol at 95%. We inverted the tube, observing that the chromosomic DNA formed a pellet. The DNA was left to dry and later was resuspended in nuclease-free water. Finally, we proved the presence and integrity of the DNA in agarose gel 1% stained with Ethidium Bromide (EtBr) 0.5 mg/mL, after having submitted it to electrophoresis (120 V/208 mA) in a Tris-Acetate-EDTA (TAE) 1X buffer. DNA concentration and purity were calculated in NanoDrop™[7] by means of a 260/280 ratio.

**Extraction of genomic DNA in whole blood**

Extraction of genomic DNA in whole blood consisted of placing 500 µl of whole blood in an Eppendorf tube, to which we added 1 mL of red-blood lysis solution, 0.01 M Tris-HCl pH 7.6, 320 mM sucrose, 5 mM $MgCl_2$, and 1% Triton X 100[8]. This was centrifuged at 7,000 rpm for 2 min, the supernatant was discarded, and this step was repeated two or three times until we observed no excess of hemoglobin. We added 400 µl of nucleic-acid lysis solution (0.01 M Tris-HC1, 11.4 mM sodium citrate, 1 mM EDTA, and 1% of SDS). We then added 100 µL of NaCl (5 M) and 600 µL of $CHCl_3$; this was centrifuged at 7,000 rpm for 2 min and we transferred 400 µL of the supernatant into a new tube. We added to this 800 µL of cold absolute ethanol (–20°C), vortexed it, and afterward, centrifuged it for 1 min at 12,000 rpm. Finally, we added 50 µL of TE to conserve the DNA at 4°C until its use. The DNA integrity was observed in agarose gel at 1% stained with EtBr 0.5 mg/mL after having been submitted to electrophoresis (120 V/208 mA) in a TAE 1X buffer. DNA concentration and purity was calculated in NanoDrop™ by means of a 260/280 ratio.

**Conditions of multiple PCR**

Multiple PCR was carried out in the whole blood samples as well as in the genomic DNA of the strain. This was performed in a total volume of 25 µL of reaction that contained the following: 5 µL of PCR buffer reaction; 0.2 mM of each deoxyNucleotideTriPhosphate (dNTP), and 1.5 mM of $MgCl_2$. The feeders were mixed at concentration of 800 nM BG-F and -R, 600 nM of IS, 350 nM of *Brucellaabortus* (BA), 450 nM of *BrucellaSuis*(BS), and 200 nM of *Brucellamelitensis*(BM). We added, for each reaction, 2 µL (~500 pg) of DNA template and 1 U of Taq polymerase [7].

Amplification consisted of an initial denaturalization at 95°C during 1 min, 30 amplification cycles with denaturalization at 95°C during 5 sec, hybridation at 64°C during 10 sec, extension at 72°C during 30 sec, and a final extension at 72°C during 1 min in the thermocycler[9]. The presence and integrity of the genomic DNA of the PCR products were proven in agarose gel 1%[10], stained with EtBr 0.5 mg/mL after having been submitted to electrophoresis (120 V/208 mA) in a TAE 1X buffer.

## III. Results

General seroprevalence was 26.32%, and for Tlahualilo Municipality this was 41.86%, while in Mapimí it was 18.18%. We observed the band pattern of the strains of each species. We carried out the determination of DNA concentration and purity obtained employing NanoDrop™ equipment, the results of which were as follows: for the *B. melitensis*RM1 strain, there was a concentration of 149 ng/µL and 1.7 purity in the 260/280 ratio, while for the *B. abortus* RB51 strain, there was a concentration of 120 ng/µL and 1.7 purity.

**ExtractionofGenomic DNA of Whole-blood Samples**

The application of multiple PCR in this study required having an adequate DNA-extraction method of blood samples, due to that *Brucella* is an intracellular-growth microorganism it is fundamental for the bacterial DNA (if it were to exist in the sample) to be extracted from the interior of the macrophage which is where it inhabits, in the most integral form possible and at concentrations that are adequate for performing its later amplification. We observed an average concentration of 230 ng/µL with a Standard Deviation (SD) of 15.7 and 1.63 purity with an SD of 1.5 in the 260/280 ratio, which permitted us to perform amplification of the samples.

[7]ThermoScientificNanoDrop™ 2000®, USA.
[8]Sigma-Aldrich®, USA.
[9]MyCycler,Bio-Rad®, USA.
[10]Agarose, LE, Analyticalgrade, Cat. # V3125 Promega®, Spain.

**Multiple PCR**

We carried out DNA amplification of the control strains to determine their band pattern. We observed that the band that defined the genus *Brucella* determined by feeders of the *bcsp31* gene genomic region presents 208 pb, while bands of the species *B. abortus*and *B. melitensis* that are determined by the feeders that are directed to the specific integration of element IS711 in the genome of the respective species evidenced the *B. melitensis* species in the 731-pb band and the *B. abortus* species, in the 498-pb band (figure 1).

On analysis of the samples that were RBPT-positive, on processing the multiple PCR, we found that these samples corresponded to *B. melitensis* with a 731-pb band (figure 2).

**Sensitivity and Specificity of the Multiple PCR Technique for Diagnosis of Brucellosis**

To determine the sensitivity and specificity of the multiple PCR technique, we employed the Odds Ratio (OR) statistical method, utilizing BR as the standard diagnostic test. On applying the 2 × 2 table, we obtained the sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) described in tables 2 and 3.

## IV. Discussion

At present, there are specific diagnostic methods of brucellosis, including serological, molecular, or microbiological methods, and RB continues to be utilized as a diagnostic test [9]. Molecular tests permit us to know the species of the genus *Brucella*. Diverse authors have reported techniques for the molecular diagnosis through PCR and have cited its high sensitivity and specificity employing PCR assays in real time. Nagalingam and collaborators in 2012 reported that by means of serological tests, it is not possible to differentiate positive brucellosis in infected and in vaccinated animals; however, through the multiple PCR technique, it is possible to differentiate between vaccine strains S19, RB51, and Rev1 and infecting strains of the genus *Brucella* of livestock and humans [10]. In this study, multiple PCR was carried out in whole blood samples, such as the genomic DNA of the strain, conditions that have already been considered in the identification of the genus and species of *Brucella*, on which multiple PCR was performed directly from clinical samples, affording certainty to the diagnosis. In this respect, it has been determined that the identification of *Brucella* spp. by conventional tests involve time, risk, and requires interpretation by an expert, while the PCR technique is rapid, sure, and easy to interpret. In the present investigation, 30 serum samples were confirmed as positive for RBPT, and from the same sampling with 30 samples of whole blood, the isolation was confirmed of *B. melitensis* with multiple PCR. In the same manner, Irajian et al. analyzed 68 isolations by PCR from humans as well as from animals, among which *B. melitensis* predominated in 36 isolates, two of *B. abortus* and one of *B. suis* of the animal specimen, and 24 isolates of *B. melitensis* and six of *B. abortus* of the human specimen [8]. In this regard, it was documented that *B. melitensis* presents very severe clinical conditions in humans [2].

In the present work, in the results of the amplification of the RBPT-negative samples, bands were observed, with which we obtained 100% sensitivity and specificity; thus, PPV and NPV were 100%. Cevallos and coworkers (2010) also performed a conventional PCR technique, obtaining a sensitivity value of 75% and one of 92% for specificity, in comparison with the RBPT technique [11]. On developing the PCR technique utilizing whole blood, it was observed that the technique is fast, that its manipulation is facilitated, and that it diminishes the risk of infection; in addition, it allowed for simultaneous identification of the genus and the species due to the use of the multiple-PCR variant, and it provides overwhelming epidemiological reports due to that it identifies the most prevalent species in a determined population. The latter renders the technique feasible for it to be applied in the diagnostic laboratory.

In addition to the consumption of unpasteurized lactic products, due to which it is necessary to provide technical advice to small producers on the knowledge of brucellosis, of how to prevent the infection in order to not only control the latter, but also to eradicate it from brucellosis-endemic zones. The results of this demonstrate that PCR is an effective method for identifying brucellosis in humans from peripheral-blood samples. Also, PCR could be employed as a rapid and confirmatory instrument for samples for which a doubt arises in terms of RBPT. For future investigations, it is recommendable to utilize multiple PCR for the diagnosis of the different species of the genus *Brucella* because it amplifies different DIANA sequences, as well as the simultaneous identification of diverse genes. It is recommended to process multiple PCR such as Western blot that detects specific proteins, in that there are cases in which genes are detected that encode for toxins or other virulence factors.

## V. Conclusion

The multiple PCR technique described in this study can be utilized for the diagnosis of human brucellosis employing whole blood as sample analysis. Also, it possesses a high grade of sensitivity and specificity as a diagnostic test: Its rapidity is noteworthy in addition to that its economy of resources renders it feasible for its application in the diagnostic laboratory. The results demonstrate that RBPT continues to be an

inexpensive and totally reliable test for performing a good diagnosis of human brucellosis in the absence of PCR for a population with a high level of antibodies in endemic areas.

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**Table 1.** Primers for detection of *Brucella* using specific Single Nucleotide Polymorphism (SNP)

| | Sequence 5'- 3' |
|---|---|
| *Brucella* genus | F: CAATCTCGGAACTGGCCATCTCGAACGGTAT<br>R: ATGTTATAGATGAGGTCGTCCGGCTGCTTGG |
| *B. abortus* | GACGAACGGAATTTTTCCAATCCC |
| *B. melitensis* | AAATCGCGTCCTTGCTGGTCTGA |
| *B. suis* | GCGCGGTTTTCTGAAGGTTCAGG |
| IS711 | TGCCGATCACTTAAGGGCCTTCA |

**Table 2.**Use of the 2 × 2 table for the evaluation of the diagnostic test.

| **Result of multiple PCR** | **Result of RBPT** | | **Total** |
|---|---|---|---|
| | **Positive** | **Negative** | |
| Positive test | 30 | 0 | 30 |
| Negative test | 0 | 84 | 84 |
| Total | 30 | 84 | 114 |

**Table 3.**Values of sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV).

| | Value (%) |
|---|---|
| Sensitivity | 100 |
| Specificity | 100 |
| PPV | 100 |
| NPV | 100 |

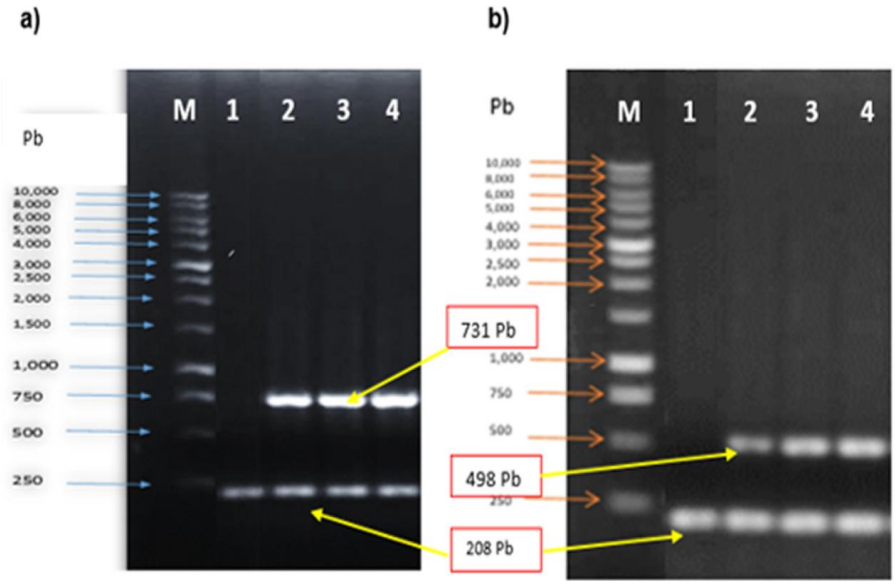


**Figure 1.**MultiplePolymerase Chain Reaction (PCR) of the control strains. Electrophoresis in agarose gel at 1%, stained with EtBr, showing in M the molecular weight marker of Kb.

**a) Products of the multiple PCR of the control strain of *Brucella melitensis* RM1**. **Lane 1**: Amplification utilizing solely the genus feeders (with a 208-pb band corresponding to the genus *Brucella*); Amplification **Lanes 2–4** utilizing these genus and species feeders (a >731-pb band corresponding to the species *Brucellamelitensis).*

**b) The multiple PCR products of the control strain of *B. abortus* RB51. Lane 1:** Amplification only utilizing the feeders of the genus (with a 208-pb band corresponding to the genus *Brucella*); **Lanes 2–4**, amplification employing feeders of the genus and species (a >498-pbband corresponding to the species *B.abortus*).

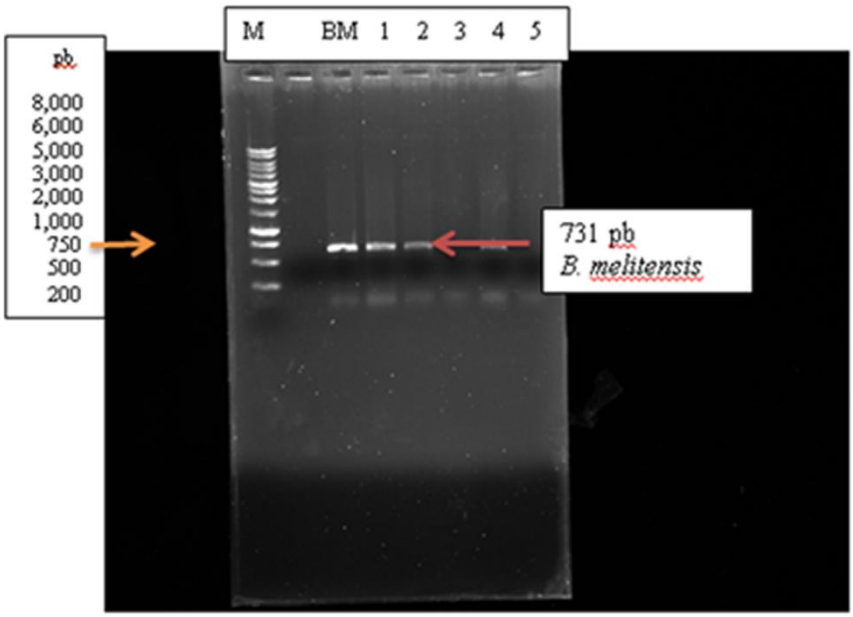


**Figure 2.** Multiple PCR of Rose Bengal (RB)-positive samples.