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ORIGINAL ARTICLE

Comparison of the tests polymerase chain reaction, serology, and blood culture with respect to sensitivity and specificity for detection of *Brucella* spp in human samples

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Abstract

Objective*: The aim of this study was to evaluate the sensitivity and specificity of polymerase chain reaction for detection of* Brucella *spp in human blood samples compared with the serological tests and blood culture.* **Material and Methods:** *In 2005, a total of 92 people were sampled from the towns of Anáhuac and Sabinas Hidalgo, Nuevo León, where an outbreak of human cases had taken place in the same year as this study. The sera collected were analyzed by serological tests according to the NOM 022-SS2-1994. DNA was obtained using CTAB extraction method and it was used to amplify a fragment of 223 bp of the coding sequence for a protein of 31 kDa present in all* Brucella *species.* **Results:** *The polymerase chain reaction test detected 23 positive samples. The sensitivity and specificity compared with RB was 44.68 and 95.56%, respectively. Compared with mouse antibody production, it was 51.61 and 88.52%, and 2-mercaptoethanol was 53.57 and 87.50%. When isolation (positives cultures) was compared with polymerase chain reaction, we obtained 100.0% sensitivity and 80.23% specificity, taking into account people with positive and negative serology.* **Conclusions:** *The polymerase chain reaction test can be an alternative tool to bacterial culture in human brucellosis diagnosis.* (Gac Med Mex. 2015;151:579-85)

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Introduction

Brucellosis is an infectious disease produced by bacteria of the *Brucella* genus, which are characterized for being facultative intracellular pathogens. It is a classical zoonosis (anthropozoonosis) that causes health problems among individuals who ingest foods originating in infected animals and represents an occupational risk for people who work with or maintain close contact with infected cattle. The disease is distributed throughout the world and it is endemic in some countries, where it represents an important public health problem[1]. The control of the disease in animals has a huge impact on incidence reduction in humans[2].

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The incidence of brucellosis in the human population of Mexico is variable, with variations in time depending on the geographical area. The Epidemiology General Direction of the Ministry of Health reported 2,073 confirmed cases in the year 2010. The states with the highest incidence were: Sonora with 248, Guanajuato with 317, Jalisco with 179, Nuevo León (N.L.) with 154 and Michoacán with 145 cases[3]. From the year 2000 to 2009, the records of nation-wide new cases of brucellosis in humans show how the incidence rate of 1.66 reported in 2006 increased to 2.38/100,000 inhabitants in 2010[3].

In countries like ours, the risk for acquiring the infection by human beings is correlated with hygienic and alimentary habits. The mobilization of dairy products to urban zones, as part of the commercialization process, has contributed to a large extent to the dissemination of the disease, regardless of how far places are from the endemic zones[4]. Animals so far accepted as carriers often have very close contact with man, which explains the dimension of the problem posed by this zoonosis. On the other hand, brucellosis shows poorly defined symptoms in humans, which makes early detection of infected subjects difficult and favors evolution to chronicity, thus complicating treatment alternatives and definitive cure[4]. Brucellosis diverse clinical spectrum, especially in the chronic form, can make for diagnosis to be overlooked or delayed if the physician does not suspect its existence. Brucellosis definitive diagnosis is based on isolation of the bacterium[3,4]. However, the proportion of positive cultures ranges from 15 to 85%[5,6]. The Mexican Official Standard 022-SS2-1994, for the prevention and control of brucellosis in man at the primary care level, establishes the serologic diagnosis of brucellosis by means of the Rose Bengal (RB), standard plate agglutination test (PAT) and 2-mercaptoethanol (2-ME) methods, in addition to confirmatory tests such as a positive blood or bone marrow culture[7].

Molecular techniques have been shown to possess great sensitivity and specificity for the detection of *Brucella* in different biological samples (blood, bone marrow, milk, urine, etc.)[8,9]. The polymerase chain reaction (PCR) test has taken prominence for rapid and efficient diagnosis, leaving isolation for epdemiological studies[9,10]. To be routinely used in diagnostic laboratories, the technique has to be validated, i.e., the test has to be assessed, with clinical samples, for sensitivity, specificity and quality control aspects[11]. This work aimed to assess the PCR test with regard to its sensitivity and specificity for the detection of *Brucella* spp, in human blood samples, compared with serological methods and blood culture.

Material and methods

Sampling sites

The study was conducted in the Anáhuac and Sabinas Hidalgo municipalities, in the state of N.L., which were identified and selected, based on brucellosis outbreaks that ocurred there and were reported by the Ministry of Health of the state government in the year 2005.

Sample size

The sample size was determined using the following formula: $n = 3.84p\ (1\text{-}p)/T^2$; where n is the required size, p is the unknown population prevalence, and T is the quantity or upper and lower limits of P in percentage points; in other words, the degree of accuracy and in the level of confidence estimation, 100 (1-a)%[12]. In this case, a prevalence of 0.0345 for the state of N.L., reported in 1992, was considered[13]. Considering a 95% level of confidence (T = 0.05) and applying the formula, the sample size (n) was 51. Considering that the prevalence value used to calculate the sample size came from a report of 1992, the number was increased to 92 analyzed samples in this study[13].

Study population

Ninety-two individuals from different socioeconomic strata were recruited in the study, including persons with symptoms, in addition to persons without symptoms but who lived together with sick people from the rural settlements related to the human brucellosis outbreak.

Type of sample

Whole blood was used for blood cultures and PCR. Serum was used for serologic testing. The samples were taken by personnel of the Ministry of Health; 10 ml of whole blood were drawn from each patient and were divided in 2 parts: 5 ml without EDTA (for serum) and 5 ml with EDTA.

Serum analysis and isolation

The RB and PAT tests, as well as microagglutination in the presence of 2-ME were carried out according to the NOM 022-SSA-1994 (2000) standard. In addition, 3 ml of each individual's blood were inoculated in Ruiz Castañeda's modified biphasic medium, in order for isolation to be performed by means of blood culture.

Molecular methods

As positive control, DNA of the *Brucella melitensis* Rev1 vaccine strain was used in the PCR tests.

DNA extraction from blood culture isolates, control strain and samples using the CTAB method

A sample was taken from the colony with an innoculation loop and placed in 1.5-ml capacity Eppendorf® tubes, 400 µl of TE 1X pH 8.0 (10 mM Tris-HCl, 1 nM EDTA) were added and were inactivated at 95 °C for 20 minutes. Then, the extraction was carried out using the Wilson 1993 modified method[14]. For the bacterial DNA extraction from white blood cells, 400 µl of whole blood were placed separately in Eppendorf® tubes, were added 900 µl of TE 1X pH 8.0 (10 mM Tris-HCl, 1 mM EDTA), were centrifuged at 10,000 rpm/5 min in a SIGMA® microcentrifuge, the supernatant was decanted and the obtained button was used for DNA extraction.

PCR amplification conditions

Primers that amplify part of the gene that codifies for an immunogenic 31 KDa protein of the external membrane of *Brucella abortus* (BCSP31) reported by Bayle et al. in 1992 were used; the B4 (5'-TGGCTCGGTTGCCAATATCAA-3') and B5 (5'-CGCGCTTGCCTTTCAGGTCTG-3') sequences amplify a 223-base pair (bp) fragment. BCSP31 is specific of the *Brucella* genus and is preserved in *B. abortus, B. melitensis* and *B. suis*[15].

The PCR tests were carried out in volumes of 25 µl, in a PCR Express thermal cycler (Hybaid Thermo, Middlesex, United Kingdom). The PCR mix contained 25 pmol of each primer, 200 mM of each one of the 4 deoxynucleoside triphosphates (Bioline, Inc., Randolph, MA, USA), 1.0 mM $MgCl_2$, 1 X of reaction regulator (100 mM Tris-HCi, 500 mM KCl, 15 mM $MgCl_2$ pH 8.3), 2.5 U of Taq DNA polymerase (Roche® Applied Science), approximately 100 ng of template DNA and deionized water for a final volume of 25 µl.

The reaction mixture was subjected to the following thermal cycle conditions: an initial denaturalization cycle at 93 °C for 2 min, followed by 35 3-step cycles: denaturalization at 93 °C for 60 s, primer allignment at 60 °C for 30 s and an extension at 72 °C for 30 s. With a final extension at 72 °C for 10 min.

Agarose gel electrophoresis

The amplified fragments were analyzed by means of agarose gel electrophoresis (Promega, Inc.) at 15%; in a TBE buffer solution (Tris base 445 mM, boric acid 445 mM, EDTA 10 mM), stained with ethidium bromide (10 µg/ml). 8 µl of each sample (amplicon) were placed, mixed with 2 µl of loading buffer solution (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 30% [w/v] glycerol [pH 8.0]). The 100 bp DNA ladder (Bioline) was used as molecular weight marker. Migration was at 100 V for 1 hour. The amplification products were visualized with an UV transluminator (Spectroline Transluminator, model 7C-254R, Electronics Corporation, Westbury, NY, USA) under UV light. The images were captured with a Polaroyd cammera and A667 film adapted with ultraviolet light filter (Fig. 1 and 2).

Statistical analysis

To determine the usefulness of PCR in the diagnosis of brucellosis, it was compared with the blood culture, RB, PAT and 2-ME tests results as the official tests (NOM-022-SSA2-1994). The relative sensitivity and specificity of the tests was calculated using a 2-row by 2-column contingency table; this way, the PCR (variable in rows) and serology and blood culture (variable in columns) were compared. In addition, the reported formulas were used[16], according to the following concepts: relative sensitivity is the capability of the alternative method to detect the targeted organism as compared with the standard method (serology and blood culture). Relative specificity is the capability of PCR to fail to detect the targeted organism when it is not detected by the standard method.

In addition, the Kappa index test was used to assess the level of agreement between tests[17]. All statistical analyses were performed with the OpenEpi v3 program.

Results

Serologic tests

Of the 92 analyzed samples using serological methods for the diagnosis of brucellosis, positive results were obtained in 47, 31 and 28 for RB, PAT and 2-ME, respectively (Table 1).

PCR tests of DNA from blood samples and blood culture

The DNAs of 92 human blood samples from individuals related to the human brucellosis outbreak of the

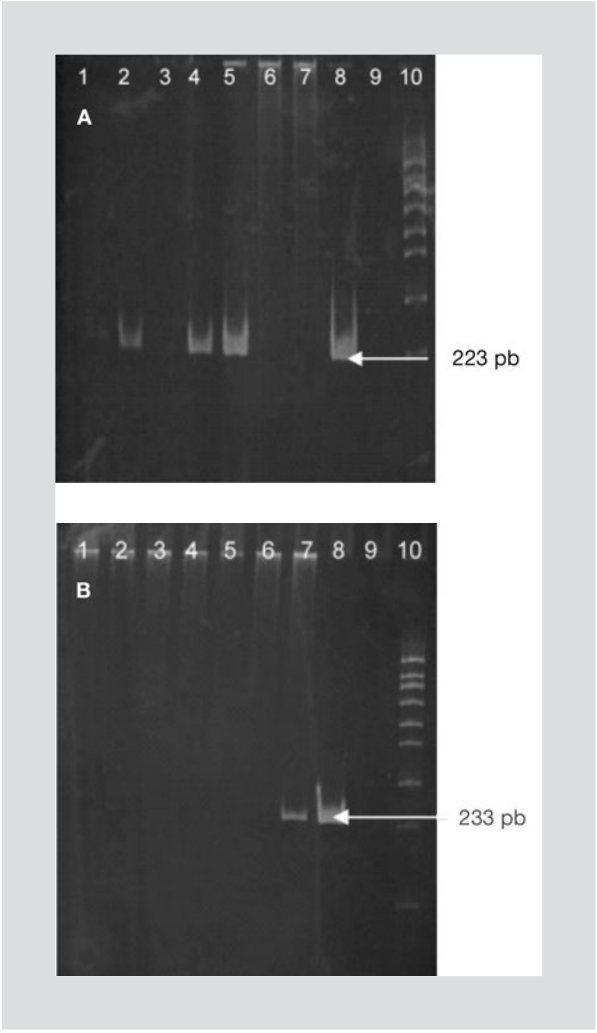

Figure 1. *Human blood samples DNA amplification electrophoresis. Panel* **A** *and* **B***: Lanes 1 to 7, blood samples; lane 8, positive control (Rev1 Strain), lane 9, negative control (water) and lane 10, molecular weight marker (Ladder 10).*

Anáhuac and Sabinas Hidalgo municipalities, N.L., were analyzed by means of PCR; 23 of the samples (Table 1) clearly showed amplification of the expected 223 bp fragment.

The PCR test, with blood samples from individuals related to the human brucellosis outbreak, allowed for 25% (23/92) of positive samples to be detected, in contrast with the RB test, which detected 51%, PAT with 33.70%, 2-ME 30.43% and blood culture 6.52% (Table 1).

Blood culture

Brucella spp was isolated in 6 blood cultures of the 92 collected blood samples. The isolates were confirmed by the Institute of Epidemiological Diagnosis

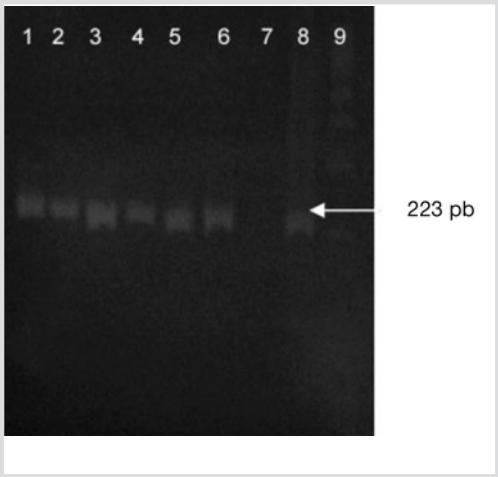

Figure 2. *Blood culture isolates amplification electrophoresis. Lanes 1 to 6, isolates; lane 7, negative control (water); lane 8, positive control (Rev1-strain DNA) and lane 9, molecular weight marker (Ladder 100).*

and Reference as *B. melitensis*. These samples were positive for PCR and for serology with all methods.

Comparison analysis of PCR agreement with serological tests and blood culture

By means of the contingence analysis performed for each serological test and blood culture versus the PCR technique, the following results were obtained: when PCR was compared with the RB test, PCR showed 44.68% sensitivity, 95.56% specificity and a slight agreement (0.398). When PCR was compared with PAT, 51.61 and 88.52% were obtained for sensitivity and specifity, respectively, in addition to moderate agreement (0.429). When PCR was compared with 2-ME, 53.57 and 87.50% sensitivity and specificity, respectively, were obtained, in addition to moderate agreement (0.432). When PCR was compared with blood culture, PCR obtained a 100.0% sensitivity, 80.23% specificity and a slight level of agreement (0.346) (Table 2).

Of the 92 analyzed samples, no data on the patients' clinical condition were obtained; in addition, whether the sample was taken when the patient was on teratment, if it was the first time the infection ocurred or if the patient was experiencing relapses was not known, since these patients were seen at their ranchs and this population does not attend the healthcare center regularly.

Table 1. Serology, blood culture and PCR tests results of the analysed samples from the Anáhuac and Sabinal Hidalgo municipalities, N.L.

	Test				
Result	**RB**	**PAT**	**2-ME**	**Blood culture**	**PCR**
Positive	47 (51%)	31 (33.70%)	28 (30.43%)	6 (6.52%)	23 (25%)
Negative	45 (49%)	61 (66.30%)	64 (69.57%)	86 (93.48%)	69 (75%)
Total sera	92	92	92	92	92

RB: Rose Bengal; PAT: standard plate agglutination test; 2-ME: 2-mercaptoethanol.

Table 2. PCR sensitivity, specificity and Kappa indices results for the study samples

Parameter	**RB**	**PAT**	**2-ME**	**Blood culture**
Sensitivity	44.68%	51.61%	53.57%	100.0%
Specificity	95.56%	88.52%	81.16%	80.23%
Kappa index	0.398	0.429	0.432	0.346

RB: Rose Bengal; PAT: standard plate agglutination test; 2-ME: 2-mercaptoethanol.

Comparative analysis of brucellosis diagnostic tests

The results of the samples from the Anáhuac and Sabinas Hidalgo municipalities, N.L., indicate that 47 positive samples were obtained by RB, 31 by PAT, 28 by 2-ME and 6 by isolation. For PCR, only 23 positive samples were obtained; each one of these results was grouped in 11 cases, which are shown in table 3.

Discussion

Some studies have demonstrated that when the infection is endemically established in an area, practically all persons have or have had contact with the pathogen, without necessarily showing symptoms, as it was the case of the 13 individuals in group 1, where 13 patients were RB-positive, with the rest of the tests being negative. The presence of antibodies, revealed by the RB test, shows that these individuals were sometime infected and have remained positive. This finding was brought to light by the national seroepidemiological survey conducted in the country in apparently healthy individuals[13].

In group 2, 2 patients were (+) for PCR and (–) for serology and blood culture; possibly the sample was taken in an early phase of the disease and/or the individual had an infection with very few circulating bacteria and, therefore, no *Brucella* was isolated and if this individual resolved the infection soon, no antibody formation was induced[4,18].

In some cases, isolation and identification of the etiologic agent has been found not always to be possible, especially in some clinical presentations of the disease, and that blood cultures can be negative when there was no apparent acute phase or when the diagnosis was not established during its course[4,7,19]. On the other hand, presumptive and confirmatory serological tests indicate being negative, since no antigen-antibody reaction occurred and, therefore, the case was not considered to be positive. The RB test can be negative in people with few days of evolution or with chronic disease. Importantly, the test may yield a positive result even after treatment and recovery of the patient, even for years; therefore, in isolation and with no clinical data available, this result is of poor value. However, in group 4, the test was positive together with PCR, which suggests performing an analysis of the clinical status and to retest the patients' blood culture and, in case of having an isolate, start a treatment to control brucellosis[20]. One patient was PCR (+), RB (+) and PAT (+), and 2-ME and blood culture-negative. The blood culture was possibly negative because the amount of blood was insufficient; the authors recommend 5 to 10 ml per container. Other investigators have observed that blood cultures are not always positive when serology is positive because the *Brucella* has to be viable and at sufficient concentration and it requires a prolonged incubation period, since it is a slow-growing bacterium[21]. On the other hand, an antigen-antibody reaction ocurred in the RB test, and it was confirmed with PAT, indicating that the patient had

Table 3. Association of serological, PCR and blood culture test-results

Nº of group	RB	PAT	2-ME	Blood culture	PCR	Number of samples
1	+	–	–	–	–	13
2	–	–	–	–	+	2
3	+	+	–	–	+	1
4	+	–	–	–	+	5
5	+	+	+	–	+	9
6	–	–	–	–	–	41
7	+	+	+	–	–	12
8	–	+	–	–	–	1
9	–	+	+	–	–	1
10	+	+	–	–	–	1
11	+	+	+	+	+	6
Total						92

RB: Rose Bengal; PAT: standard plate agglutination test; 2-ME: 2-mercaptoethanol.

agglutinating IgM antibodies. Possibly, the infection was at an initial stage, since IgM antibodies are generated early in the disease and progressively decrease over the course of 3-6 months, with or without the disease being cured[7]. When PCR (+), RB (+), PAT (–), 2-ME (–) and blood culture (–) results were obtained, probably it was because the pathogen was present in blood and was detected by PCR. Positive presumptive serology was due to an antigen-antibody reaction, indicating that in these patients there is production of specific antibodies[19].

The PCR (+), RB (+), PAT (+), 2-ME (+) and blood culture (–) results obtained in 9 patients confirmed the presence of *Brucella* in blood; however, the bacteria could not be isolated because there were low levels of the pathogen at the moment the blood sample was taken. Furthermore, when the patient presents the disease in chronic or focal stage there are few circulating bacteria, which makes isolation difficult[22].

The group of 41 patients who were negative to all tests represented the true negatives, since the samples were taken randomly, from patients with and without symptoms.

On the other hand, in areas where the disease is endemic, serological tests often yield positive results even in the abscence of symptoms; i.e, antibodies do not only appear in the serum of patients with brucellosis over the course of the disease, but also are found in apparently healthy individuals who have the infection subclinically or in an inapparent manner. Therefore, serological tests have limited value for the diagnosis of brucellosis at the outbreak point of origin, as it was the case of the group of 12 individuals in whom PCR was (–), RB (+), PAT (+), 2-ME (+) and blood culture (–), while the presumtive and confirmatory serological tests were positive, since the specific diagnostic-value titrations ocurred for each test[19,21].

On the other hand, the RB test detects the presence of agglutinating antibodies such as IgM, IgG and IgA over the first days the symptoms of the disease occur. In 2005, Elfaki concluded that antibiotic therapy limits the presence of *Brucella*-specific IgM antibodies, but fails to eliminate residual IgG antibodies in treated patients[22,23]. With regard to the results obtained with confirmatory serologies, knowing the patient's history and assessing clinical characteristics is required.

The PCR (+), RB (+), PAT (+), 2-ME (+) and blood culture (+) results, obtained in 6 patients, indicate that PCR detected the pathogen in blood in our study. PCR has been used in patients detecting other sequences such as that of ribosomal RNA (16S and 23S) and genes encoding proteins Omp25 and Omp31[24,25], and even has been used to differentiate *Brucella* species. In a study conducted by Morata et al.[26], PCR combined with ELISA was shown to reach a sensitivity up to 94.9% and specificity of 96.5% and hence it has been recommended as the diagnostic method of choice. In addition, PCR has been shown to be useful not only as a diagnostic method but also to have prognostic implications, since it can be used to assess the therapeutic response; recently, Vrioni et al.[8] were able to demonstrate, by means of real-time PCR, that *B. melitensis*

DNA persists in spite of clinical cure, which explains disease relapses and would suggest the possibility that, once acquired, brucellosis remains as a latent infection[8,27].

The final diagnosis is based on *Brucella* isolation in cultures of blood, bone marrow, liver and other tissues. The development of the microorganism in Ruiz Castañeda's biphasic medium usually occurs at 7 to 21 days, although there are cases of late growth that can reach up to 35 days[6,25]; this method is one of the most widely used, although it has the disadvantage that the bacteria grow slowly. As the disease progresses, the probability of positive blood cultures decreases, thus making isolation from lymph nodes, liver or spleen necessary[8].

Finally, we were able to detect 23 positive samples from individuals of the Anáhuac and Sabinas Hidalgo municipalities, N.L., by means of PCR; 21/23 samples coincided with one or more positive serological tests. The serological tests (RB, PAT and 2-ME) detected a higher percentage of positive results compared with PCR and the RB test was confirmed as the best brucellosis screening test.

Taking PAT and 2-ME as reference tests, according to the NOM 022-SSA2-1994 standard, in the identification of humans positive to brucellosis, these were superior by 8.7 and 7.43, respectively, compared with PCR. With regard to the RB, PAT, 2-ME and isolation methods, PCR obtained sensitivity values ranging from 44.68 to 100.0% and specificity of 80.23 to 95.56%. In addition, a moderate level of agreement was obtained between PCR and serological tests (MAP and 2-ME); however, with regard to blood culture and RB, PCR showed a slight level of agreement.

PCR showed a sensitivity of 100.0% when it was compared with blood culture, which indicated a huge value of the molecular test to be used in the detection of the pathogen in human blood. As for specificity (80.23%), it was lower than sensitivity; this might be due to the difficulty to isolate the bacterium in blood because of the low circulating levels at the moment the blood culture sample was obtained.

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