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ORIGINAL ARTICLE

Comparison of serology, culture, and PCR for detection of brucellosis in slaughtered camels in Iran

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Abstract Brucellosis is a zoonotic disease which is characterized by abortion and reduced fertility in many species. Camel brucellosis is caused by *Brucella abortus* and *Brucella melitensis*. To investigate sensitive methods in the detection of camel brucellosis, PCR was used to overcome the limitations of serology and culture methods. Three hundred ten camels were examined for brucellosis infection using serological tests (RBPT, mRB, Wright, and 2-ME). In addition, 100 serological tested cases (39 mRB positive and 61 mRB negative) were analyzed with both bacteriological (lymph node culture on *Brucella* agar supplemented with antibiotics) and PCR (nested-PCR on sera and blood samples) methods. The nested-PCR was genus-specific and amplified the 16S rDNA locus. Six out of 310 (1.94 %) of the examined camels were positive using the serological tests, whereas, no bacteria was isolated from lymph tissues. Nested-PCR was positive in six and nine individuals in sera and blood samples, respectively. The genus-specific nested-PCR assay on blood samples detected a higher number of camel brucellosis compared with serological and classical culture methods. These results have identified a sensitive PCR method which could be used as a complement test for the detection of brucellosis in live camels with the lowest risk of infection to laboratory workers.

Keywords Camel · Brucellosis · Serology · Bacteriology · PCR

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Introduction

The camel has been considered an aid to man for thousands of years, in many different respects, and has a high economic value by providing meat, milk, and wool as well as transportation and labor. The camel is the most suitable domestic mammal for use in climatic extremes due to its physiological attributes. Pathogenic diseases, poor nutrition, and traditional management systems have restricted their full utilization (Bekele 2002).

The camel is a domestic animal that may be infected by *Brucella.* Camel brucellosis is characterized with lesion in lymph nodes and joint capsules, inflammation of the uterus, abortion, and reduced fertility (Abbas and Agab 2002; Kudi et al. 1997; Wernery and Kaaden 2002). Furthermore, it seems, the problem of *Brucella* in the camel has potentially important implication for public health and the implementation of brucellosis control programs. Firstly, the camel may act as a reservoir for dissemination of contaminated secretion to other domestic animals and humans. Secondly, in many countries, no formal surveillance and eradication program for camel brucellosis has been proposed (Abbas and Agab 2002; Paling et al. 1988).

The eradication of brucellosis as a major zoonotic problem in animals is a necessary step to control the disease in man (Corbel 2006; Reviriego et al. 2000).

Currently, diagnosis of brucellosis is based on serological and microbiological tests. Serological methods are not always

sensitive or specific (Diaz-Aparicio et al. 1994; Goudswaard et al. 1976; Perry and Bundle 1990), mainly due to cross-reactivity with other antigens (Diaz-Aparicio et al. 1994; Hess and Roopke 1951; Perry and Bundle 1990).

Isolation and identification are the most reliable methods in the diagnosis of brucellosis, although not always successful, and represent a major infection risk for technicians (LòpezMerino 1991). Microbial culture may be used for some suspected cases, but is not used for surveying the disease in camel populations (Alshakh 2007). In recent years, progress has been made in applying new molecular and genetic techniques to improve the diagnosis of brucellosis (Bricker 2002). One of these techniques, the polymerase chain reaction (PCR) is a sensitive, rapid, and relatively inexpensive technique and is particularly useful in the detection of *Brucella* DNA in tissues and body fluids contaminated with non-viable or low number of *Brucella* (Fekete et al. 1990; Herman and Ridder 1992; Leal-Klevezas et al. 2000). There are few publications on using PCR in the detection of camel brucellosis (Alshakh 2007).

The objective of this study was to evaluate nested-PCR assay for the detection of *Brucella* DNA in serum and blood samples from camels and to compare its performance with bacteriological method. Additionally, the aim was to determine the agreement between PCR and serological tests.

Materials and methods

Blood and lymph node samples were collected from 310 camels slaughtered in Najaf abad's abattoir, Isfahan province, Iran. The animals were apparently healthy at the time of slaughter and none were previously immunized against *Brucella*. All samples were collected under sterile hygienic conditions. Twenty milliliters of whole blood (without anticoagulant) was aseptically taken from the jugular vein and immediately divided into 10 ml aliquots in two sterile tubes. The first tube contained EDTA (as anticoagulant) and was used for PCR; and the second blood sample (without anticoagulant) was used for serological tests. After slaughtering the animals, lymphoid tissues were sampled from the subscapular lymph nodes and immediately placed in a different sterile container. All samples were kept on ice and transported to the laboratory. Blood, sera, and lymph tissue samples were kept frozen (−20 °C) until analysis.

Serological examination

Seroprevalence of brucellosis was investigated using different serological tests including Rose Bengal plate test (RBPT) (Morgan and McDiarmid 1960), modified Rose Bengal (mRB) (Ferreira et al. 2003), Wright, and 2-mercaptoethanol tests (2-ME) (Alton et al. 1988). The agglutination titer 1/40 in Wright and 1/20 in 2-ME tests was regarded as serologic positive result of active camel brucellosis. Standardized antigens were supplied by the Razi institute (Karaj, Iran).

Bacteriological examination

One hundred lymph nodes consisting of 39 positive cases by mRB and 61 negative cases (as negative control) were examined by bacteriological tests. These samples were used to prepare slide smears stained by the Gram, modified Ziehl-Neelsen, and modified Koster methods (Alton et al. 1988) and examined with a microscope for *Brucella*-like organisms.

Lymph tissue samples, of approximately 5 g, were homogenized manually with 5 ml of TSB culture. One milliliter of each lymph tissue homogenate was immediately cultured onto *Brucella* agar plates (Merck, Germany) containing 5 % (*v*/*v*) inactive horse serum (Baharafshan, Iran) supplemented with *Brucella* selective supplement (SR083A, Oxoid). The following concentrations of antibiotics were added per liter of media: Bacitracin, 12,500 IU, Nystatin, 50,000 IU, Nalidixic acid 2.5 mg, Cyclohexamide 50.0 mg, Vancomycin 10.0 mg, and Polymixin B 2,500 IU.

Lymphoid tissue cultures were incubated in a 5–10 % CO_2 incubator at 37 °C for at least 7 days. Cultures were periodically checked for the presence of colonies. Suspected colonies were identified according to the methods adopted by Alton et al. (1988).

DNA extraction from serum and blood samples

DNA from 100 serum and blood samples from 39 positive cases by mRB and 61 negative cases (as negative controls) were extracted for the isolation of *Brucella* DNA using ZistFannavari DNA Purification kit as describe by the manufacturer (with some modification for blood samples).

DNA amplification and detection of PCR products

A genus-specific nested-PCR Nest1 primers (Bruc1F: 5′ ATAGCTGGTCTGAGAGGATGATCAG 3′ and Bruc1R: 5′ TTCGGGTAAAACCAACTCCCATGG 3′) were amplified 1,126 bp and Nest 2 primers (Bruc2F: 5′ ATATTGGA CAATGGGCGCAA 3′ and Bruc2R: 5′ AGCGATT CCAACTTCATGCA 3') were amplified 959 bp of *Brucella* 16S rRNA gene (Kazemi et al. 2008).

PCR was carried out in a total volume of 25 μl, using 50 mM KCl, 10 mM Tris–HCl (pH 8), 1.5 mM $MgCl_2$, 0.2 mM each the four deoxynucleotide triphosphate and 0.05 IU of Taq polymerase, 0.4 mM of each primer, and

Table 1 Positive results of serological tests out of 310 camel sera samples

Serological test	Numbers	(%)
mRB[a]	39	12.58
RBPT[b]	27	8.71
Wright	7	2.26
2-ME[c]	6	1.94

The agglutination titer 1/40 in Wright and 1/20 in 2-ME tests regarded as serologic positive result of active camel brucellosis

[a] Modified Rose Bengal

[b] Rose Bengal plate test

[c] 2-Mercaptoethanol tests

2 μl template DNA. The amplification was performed in a DNA thermal cycler at a denaturation temperature of 95 °C for 5 min; this was followed by 25 cycles at 94 °C for 45 s, 64.9 °C for 1 min, and 72 °C for 1 min and one final extension at 72 °C for 7 min. Second PCR reaction was carried like the first except that the annealing temperature was 58.5 °C for 35 cycles.

Negative controls containing all the reagent but distilled water instead of template DNA were routinely processed exactly as described above to monitor contamination with *Brucella* DNA. Positive control with genomic DNA isolated from a suspension of *Brucella abortus S19* was kindly supplied by the Razi institute.

Electrophoresis

The PCR products were dissolved in a 1 % (*w*/*v*) agarose gel containing 1× TBE buffer (100 mM Tris–HCl (pH 8), 90 mM boric acid, and 1 mM Na_2EDTA), stained with an ethidium bromide solution (0.5 μg/ml) and visualized under UV light.

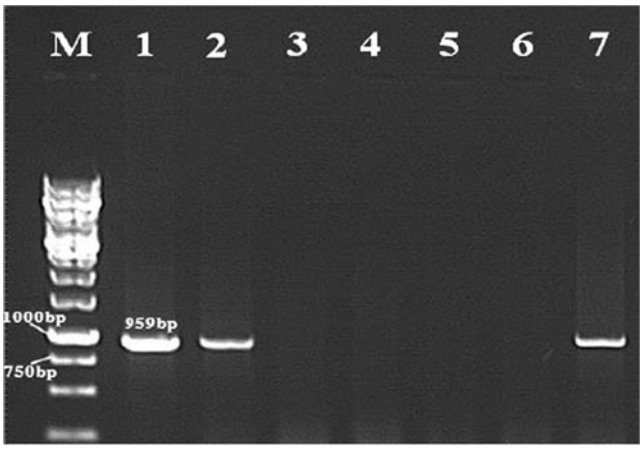

Fig. 1 Nested-PCR of blood samples: *M* GeneRuler™ 1 kb DNA ladder (Fermentas, Cat. No: SM0311). *Lane 1* control positive (*Brucella abortus S19*); *lane 2* culture negative, serology positive; *lanes 3, 4, and 7* culture negative, serology negative; *lane 5* control negative nested 1; *lane 6* control negative nested 2

Table 2 Frequency of *Brucella*-positive and *Brucella*-negative cases diagnosed based on serology and serum PCR methods

Serum PCR Serology	Positive	Negative	Total
Positive	5	1	6
Negative	1	93	94
Total	6	94	100

Results

In this study, blood and lymphoid tissue samples were collected from 310 camels.

Serology examination

Of the 310 camel sera, 39 (12.58 %) were determined to be positive by mRB, 27 (8.71 %) by RBPT, seven (2.26 %) by Wright, and six (1.94 %) by 2-ME. The results of serological tests are shown in Table 1.

Bacteriological examination

None of the lymph nodes smears showed partial acid-fast organisms. We were not able to isolate bacteria from lymphoid tissue cultures.

Serum PCR

PCR products with a molecular size of 959 bp were obtained from 6 % (6/100) of sera samples (Fig. 1). Of the six PCR positive sera samples, 83.33 % (5/6) were obtained from seropositive camels. So one serum sample was negative by the serological tests but its serum was positive by PCR (Table 2).

Table 3 Frequency of *Brucella*-positive and *Brucella*-negative cases diagnosed based on serology and blood PCR methods

Blood PCR Serology	Positive	Negative	Total
Positive	6	0	6
Negative	3	91	94
Total	9	91	100

Blood PCR

Nested-PCR was positive in 9 % (9/100) of blood samples (Fig. 1); 66.67 % (6/9) of positive PCR samples were also positive by serological tests. Three blood samples were negative by the serological tests but the blood was positive by PCR (Table 3). Serum and blood control subjects were PCR negative for all 61 cases.

Discussion

Since brucellosis is a zoonosis, the fight against this disease in humans and animals relies mainly on veterinary sanitation measures focused on the reduction or eradication of this disease in farm animals. A critical tool for the success of these measures is, without doubt, an accurate and early diagnosis of the disease.

Camels are popular and important multipurpose local animals in Iran; more than 200,000 dromedary camels live in the arid and semiarid deserts of Iran (Oryan et al. 1994; Sharifiyazdi et al. 2011), but camel brucellosis has received little research interest, despite its importance in transmission of *Brucella* to human beings. It is important because there is no global program for the control of camel brucellosis such as vaccination, test, and slaughter of reactors. The present research compared the classical methods (serological and microbiological methods) with PCR for diagnosis of camel brucellosis.

In this study, examination of serum samples by mRB, RBPT, Wright, and 2-ME led to seropositivity being detected in 12.58, 8.71, 2.26, and 1.94 % samples, respectively. Thus seroprevalence in this population is 1.94 %.

No bacteria were isolated from cultured tissue samples. There may be some reasons for this finding: In the lymphoid tissue, the bacteria may be present in very low numbers. This is consistent with the small number of detection colony forming units in this material by classical bacteriological methods. The stage of infection may influence the number and location of *Brucella* organisms in lymph tissue glands (Gupta et al. 2006; Ilhan et al 2008; Leal-Klevezas et al. 2000).

To our knowledge, although there are many reports on the detection of camel brucellosis in Iran and other parts of world, this is the first time that nested-PCR has been applied in the diagnosis of camel brucellosis and has obtained satisfactory results with good sensitivity and specificity. PCR technique could detect significantly more infected animals compared to serological methods. *Brucella* DNA detection was 6 and 9 % from serum and blood PCR, respectively. Blood and serum PCR positive results, with negative serological tests, were obtained for three and one cases, respectively. This provides an indication that PCR can be valuable for laboratory diagnosis of chronic infections or primary stage when antibodies are not routinely diagnosed (Ilhan et al. 2008; Leal-Klevezas et al. 2000). Also, another advantage of PCR may be the speed of reaction, less than a single working day can provide useful, early information to help make an appropriate decision (Alshaikh et al. 2007; Bricker 2002; Elfaki et al. 2005; Fekete et al. 1990; Herman and Ridder 1992). Hence, an appropriate PCR method could be used as a supplement and complement test for identification and differentiation of *Brucella* in camel with the lowest risk of infection to laboratory workers.

Several studies have documented the presence of circulating pathogen DNA in serum samples (Elfaki et al. 2005; Kawamura et al. 1999). Zerva et al. (2001) also found sufficient DNA in the serum fraction, rather than whole blood, to detect acute human infection by *Brucella* using PCR methods. Based on our findings, whole blood PCR assay for *Brucella* may be preferred over its reaction on sera samples. This correlates with the findings of Takele et al. (2009) but is not consistent with previous studies by Zerva et al. (2001). In the present study, in three cases PCR detected *Brucella* DNA in whole blood, but the serum PCR was negative. This could be due to the amount of circulating DNA in serum at later/chronic stage of infection is presumably lower as a result of it being an intracellular pathogen but is still detected in whole blood (Leal-Klevezas et al. 2000; Queipo-Ortuno et al. 1997; Takele et al. 2009).

Although PCR inhibitors are often detected in whole blood specimens (Morata et al. 1998), in this study we manage these problems by using red blood cell lysis, washing repeatedly by centrifugation and adjusting the concentration of isolated DNA to a maximum dilution. In addition, nested PCR technique reduced the effect of PCR inhibitors by sample dilution. Therefore, nested PCR evaluated in this study resulted in a more sensitive analysis than the other assays, due to the double round of PCR amplification and also by potentially eliminating PCR inhibitors (Gohari et al. 2010).

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