

Seroepidemiology and molecular detection of *Brucella* infection in Iranian horses: A provincial study

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Key words:

horse, *Brucella* infection, PCR

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Received: 22 October 2012

Accepted: 26 December 2012

Abstract:

BACKGROUND: Brucellosis is a febrile zoonotic infection and has worldwide distribution among humans as well as animals. Although the seroprevalence of brucellosis in various animals has been described in Iran, there is only one report on equine brucellosis in the region. **OBJECTIVES:** This study was carried out to determine the seroprevalence of brucellosis in racing clubs and private horse owners in the south of Iran and risk factors associated with the disease in horses. **METHODS:** 312 randomly selected equine serum samples were investigated for the presence of antibodies against *Brucella* genus, using slide agglutination by Rose Bengal plate test (RBPT), serum agglutination test (SAT) and 2-mercaptoethanol (2-ME) test, using whole cell antigen. PCR assay was also used for detection of clinically suspected cases. **RESULTS:** Most seropositive horses in this study were asymptomatic. The true seroprevalence of brucellosis was found to be 9.9, 8 and 7% by RBPT, SAT and 2-mercaptoethanol tests, respectively. All horses with history of clinical signs (3.2% of all samples) had RBPT, SAT and 2-mercaptoethanol positive results. It was also revealed that age, sex and a history of contact with ruminants had no effect on acquiring the infection in positive cases. In the PCR, one of the three horses with fistula withers produced amplicon of 450 bp fragment of wbo sequences specific to *Brucella* spp. field strain. **CONCLUSIONS:** This study showed the seroprevalence of brucellosis in horses of Fars province and it was indicated that the PCR assay may be helpful in detection of clinically suspected horses.

Introduction

Brucellosis is a febrile zoonotic infection and has worldwide distribution among humans as well as animals. It is of major economic importance (due to reproductive disorders and reduced production of affected animals) in developing countries that do not have a national brucellosis eradication program (Radostits et al., 2007). The disease occurs in countries of the Mediterranean Basin, the Middle East and the Persian Gulf (Apan et al., 2007; Pappas et al., 2005). Although Iran have put into effect programs to control and eradicate brucellosis, the

disease still occurs in the country (Bokaie et al., 2009a). *Brucella* species are often categorized according to the principal farm animal they infect (Baek et al., 2011). Infection with *Brucella abortus* produces several consequences in horses. The most common disease is supraspinous bursitis (fistulous withers), which results from the apparent predilection of the organism for synovial structures (Reilly, 2009). This is marked by a painful swelling over the withers which may open and drain purulent material (Cohen et al., 1992). Supraatlantal bursitis (poll evil) may also be caused by *B. abortus* infection (Gul et al., 2009). Moreover, *B. abortus* infection is an in-

frequent cause of abortion in mares and infertility in the stallion (Carmichael, 1990). *Brucella suis*, transmitted from infected pigs, is an uncommon cause of bursitis or abortion in horses (Radostits et al., 2007). The seroprevalence of brucellosis in various animals such as cattle, sheep, goats, camels, poultry, dogs and human has been described in Iran (Bigdeli et al., 2011; Khadjeh et al., 1999; Sofian et al., 2008; Maadi et al., 2011; Behzadi and Mogheiseh, 2011). There is only one report on equine brucellosis in the northeast of Iran (Tahamtan et al., 2010). Because there was no published report on brucellosis in horses in the south of Iran, this study was undertaken to assess the prevalence of *Brucella* antibodies among equines in different districts of this region. The present study also describes the first application of PCR assay and its usefulness for detection of *Brucella* spp. in fistula withers discharges as a supplementary and complementary test in clinically suspected horses.

Materials and Methods

This study was conducted (during the period between April and September, 2009) on 312 randomly selected horses, to determine the seroprevalence of brucellosis and risk factors associated with the disease. Samples were taken from horse racing clubs and private horse owners of Fars Province, in the south of Iran. Blood samples were obtained by venipuncture and were transported in ice-boxes to the Shiraz University Large Animal Laboratory, as quickly as possible. The serum was then separated by centrifuging the blood samples at 2000g and kept at -20°C until tested. Serum samples were investigated for the presence of antibodies against *Brucella* genus using slide agglutination by Rose Bengal plate

test (RBPT), serum agglutination test (SAT) and 2-mercaptoethanol (2-ME) test, using whole cell antigen (Razi Vaccine and Serum Research Institute, Iran). Sex, age, history of contact with ruminants, geographical location in the province and use of horses were considered as risk factors. In suspected cases, history and clinical signs relevant to brucellosis were recorded and complete physical examination including visual inspection, palpation of the withers, and probing of fistulous tracts was performed.

Genomic DNA extraction and PCR assay: The

discharges (100 µL) from three horses with fistula withers were submitted to genomic DNA extraction. The DNA was extracted by digestion, ethanol precipitation and purification, using a commercially available kit (Qiagen DNAeasy), according to the manufacturer's protocol and stored at -20 °C before use.

The PCR assay contained the following primers: wbo1 5'-GCC AAC CAA CCC AAA TGC TCA CAA-3' and wbo3 5'-TTAAGCGCTGATGCCATT TCC TTC AC-3' for RB51 detection (Vemulapalli et al., 1999).

These primers were designed previously based on the wboA gene disruption by an IS711 element in the *B.abortus* RB51 (Vemulapalli et al., 1999). Two fragments of 1300 bp and 450 bp were amplified from RB51 vaccine and *Brucella* spp. field strain, respectively. All oligonucleotide primers used in this study were synthesized by Cinnagen Co. in Iran.

PCR amplifications were performed in a 25-µL volume using thermal cycler (MG 5331, Eppendorf, Hamburg). The following PCR conditions were applied to each assay; 50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 2 mM MgCl₂, 200 µM dNTPs, 10 pM of each primer, 1.25 U Taq DNA polymerase (Fermentas) and 2 µL of template DNA.

After initial denaturation of template DNA at 94°C for seven min, the PCR profile was as follows: 30 cycles of 45 s of template denaturation at 95°C; 45 s of primer annealing at 64°C and 45 s of primer extension at 72°C; with a final extension at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 7 µL of reaction product in a 1.5% agarose gel in TBE (89 mM Tris-HCl, 89 mM boric acid, 2.0 mM EDTA, pH = 8.0) electrophoresis buffer and visualized by staining with ethidium bromide (0.5 µg/mL) under UV light. Images were captured on a computer. Vaccine (RB51) and reference field strains of *B.abortus* 544 were used as positive controls. Also DNA from healthy horse and sterile water were used as the negative controls.

The data were analyzed by the SPSS software (version 11.5) and confidence level of 95% was assumed. Chi-square analysis was applied to determine the significance of differences in seroprevalence of brucellosis among various groups. Differences were considered significant at $p \leq 0.05$. The true seroprevalence was calculated according to Rogan and

Gladden (1978).

Results

Most seropositive horses in this study were asymptomatic. The true seroprevalence of brucellosis was found to be 9.9, 8 and 7% by RBPT, SAT and 2-mercaptoethanol tests, respectively (Table 1). Although the seroprevalence of brucellosis was high in the mares as compared to stallions, it was not significant (Table 3). A difference was also observed in seroprevalence of brucellosis of different age groups which was not statistically significant (Table 4). The prevalence was non-significantly higher in the 2-5 and 5-10 year age groups as compared to other groups. Although the seroprevalence in relation to the use of the animals was not significant ($p > 0.05$) in racing and non-racing horses, the prevalence was higher in non-racing as compared to racing horses (Table 5). All horses with history of clinical signs (3.2% of all samples) had RBPT, SAT and 2-mercaptoethanol positive results. It was also revealed that a history of contact with ruminants had no effect on acquiring the infection in positive cases (Table 6). The prevalence of the disease in different parts of Fars province and the result of SAT and 2-ME tests were presented in Tables 2 and 7 respectively.

In the PCR, one of the three horses with fistula withers (Figure 2) produced amplicon of 450 bp fragment of *wbo* sequences specific to *Brucella* spp. field strain (Figure 1). Specificity of the PCR used in this research was verified as the expected 450 bp band was not observed in the negative control tubes, including no-template DNA and DNA genomic from healthy horse.

Discussion

At the present time six species of *Brucella* are recognized: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotoma* (Bokaie et al., 2009a). Horses appear to be more resistant to *Brucella* infection than cattle, swine, and goats (Carmichael, 1990). Two *Brucella* species have been isolated in horses, namely *B. abortus* and *B. suis* biovars 1 (Cook and Kingston, 1988) and 3 (Cvetnic et al., 2005). In Iran, *B. abortus* and *B. melitensis* are more prevalent. (Zowghi et al., 2008). *B. suis*, *B. neotoma*, *B. ovis*

Table 1. Prevalence of *Brucella* infection in Horses of Fars province (Iran) as assessed by Rose Bengal plate test (RBPT), serum agglutination test (SAT) and 2-mercaptoethanol (2-ME) test.

Test result	Number of animals (%) by RBPT	Number of animals (%) by SAT	Number of animals (%) by 2-ME
Positive	31 (9.9%)	25 (8%)	22 (7%)
Negative	281 (90.1%)	287 (92%)	290 (93%)
Total	312 (100%)	312 (100%)	312 (100%)

Table 2. The number and percentage of horses and the rate of *Brucella* infection in Horses of different Locations in Fars province (Iran) as assessed by Rose Bengal plate test (RBPT).

Location	Samples	Positive Rose Bengal Test
Shiraz	40(12.8%)	5(16.1%)
Sepidan	33(10.6%)	2(6.4%)
Isfahan	52(16.7%)	5(16.1%)
Marvdasht	38(12.2%)	2(6.4%)
Kavar	25 (8%)	2 (6.4%)
Firoozabad	25 (8%)	1 (3.2%)
Nourabad	33 (10.6%)	3 (9.7%)
Kazeroun	32 (10.3%)	7 (12.8%)
Others	34 (10.9%)	4 (13%)
Total	312 (100%)	31 (100%)

Table 3. Percentage of male and female horses and the rate of *Brucella* infection in the corresponding sexes in Horses of Fars province (Iran) as assessed by Rose Bengal plate test (RBPT).

Sex	Samples	Positive
Male	89 (28.5%)	6 (19.4%)
Female	223 (71.5%)	25 (80.6%)
Total	312 (100%)	31 (100%)

Table 4. The number and percentage of horses and the rate of *Brucella* infection in different ages in Horses of Fars province (Iran) as assessed by Rose Bengal plate test (RBPT).

Age	Samples	Positive
2>	19 (6.1%)	3 (9.6%)
2-5	150 (48.1%)	11 (35.5%)
5-10	116 (37.2%)	12 (38.7%)
>10	27 (8.7%)	5 (16.1%)
Total	312 (100%)	31 (100%)

were not isolated in Iran (Khadjeh et al., 1999; Rezaei-Sadaghiani et al., 1996; Samar et al., 1996). Recently, there have been several reports suggesting the occurrence of canine brucellosis in Iran (Akhtardanesh et

Table 5. The number and percentage of horses and the rate of *Brucella* infection in racing and non-racing Horses of Fars province (Iran) as assessed by Rose Bengal plate test (RBPT).

Use	Samples	Positive
Racing	43 (13.8%)	7 (22.6%)
Non- Racing	269 (86.2%)	24 (77.4%)
Total	312 (100%)	31 (100%)

Table 6. The number and percentage of horses and the rate of *Brucella* infection in horses in contact with or no contact with ruminants in Fars province (Iran) as assessed by Rose Bengal plate test (RBPT).

Contact with ruminant	Samples	Positive
Yes	161 (51.6%)	17 (54.8%)
No	151 (48.4%)	14 (45.2%)
Total	312 (100%)	31 (100%)

Table 7. The result of SAT and 2-ME tests on *Brucella* positive samples (number and percentage).

<i>Brucella</i> antibody titer of the SAT test	SAT	2-ME
1/20>	1 (3.2%)	1 (3.2%)
2/20-3/40	5 (16.1%)	8 (25.8%)
3/80-4/40	11 (35.5%)	12 (38.7%)
>4/80	14 (45.2%)	10 (32.2%)
Total	31 (100%)	31 (100%)

al., 2011; Behzadi and Moghiseh, 2011; Mosallanejad et al., 2009). The true seroprevalence of brucellosis in our study was found to be 9.9, 8 and 7% by RBPT, SAT and 2-mercaptoethanol tests, respectively. The principal serological test used for brucellosis diagnosis is the RBPT, which is a screening test with high (>99%) sensitivity and low specificity in humans (Barrose et al., 2002) as well as in bovines (Hamidullah et al., 2009). Although no single test provides 100% sensitivity and specificity, SAT still remains the alternative test and is the test used for verification as it is a standard method for the diagnosis of brucellosis (Gul and Khan, 2007). The sensitivity and specificity of the SAT are 95.6 and 100%, respectively (Memish et al., 2002). 2-mercaptoethanol test has been used in cattle for the serological diagnosis of brucellosis. Based on 1051 sera from brucellosis free herds, the specificity of the

2-ME test was 99.8% (Stemshorn et al., 1985). Very few studies on the seroprevalence of *Brucella* species in horses have been reported. The reported seroprevalences vary from 8 to 16 per cent from 1976 to 1983 in the UK (Mac Millan, 1985) to 0.2 per cent in a tropical region of Mexico (Acosta-González et al., 2006) and 0 per cent in Eritrea (Omer et al., 2000). It has been reported that the prevalence rates of *Brucella* in horses, donkeys and mules in Egypt were 5.88%, 7.30% and 71.42%, respectively (Gul and Khan, 2007). It seems that the prevalence rate obtained in this study is somewhat higher than the prevalence of the infection in horses of Egypt (Gul and Khan, 2007). Musa (2004) reported that out of 346 horses and 28 donkeys examined in Darfur (Western Sudan), 17(4.9%) of the former and 1 (3.6%) of the latter were positive for brucellosis. Gul et al., (2009) reported that the prevalence of brucellosis in Faisalabad (Pakistan) was 20.06% in horses by RBPT and 17.15% by SAT. Abo-Shehada (2009) reported that true seroprevalence of *Brucella* species among horses in Jordan was 1.0 per cent and in donkeys it was 8.5 per cent. As it was shown, the prevalence of infection was highest in Korbali region which is well known for its large number and more densely located population of horses. In the current study, non-significant difference was observed in the seroprevalence of equine brucellosis in relation to sex. The facts regarding the prevalence of brucellosis in relation to sex is controversial and some of the research workers reported significantly higher prevalence in females than in males (Hussein et al., 2005). Bokaie et al., (2009b) reported that the incidence of Brucellosis was higher in males than females whereas others found no statistical difference between males and females (Ashenafi et al., 2007; Muma et al., 2006; Gul et al., 2009). Non-significant lower prevalence of *Brucella* infection in younger horses of our study has also been described by Nicoletti (2007). The non-significantly higher *Brucella* seroprevalence in non-racing as compared to racing horses seems to be due to close contact of non-racing horses with other domestic animals, which may increase the risk of acquiring *Brucella* infection. It has been reported that horses are infected by contact with infected cattle, other species or both and that transmission of infection from horses to cattle or between horses themselves is very unlikely

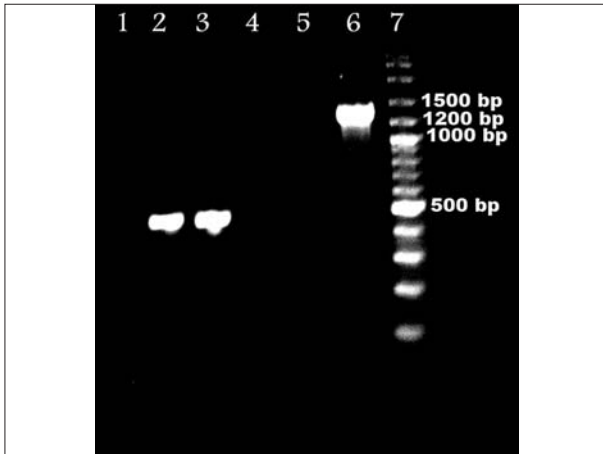


Figure 1. Electrophoretic analysis (1.5% agarose gel) of the different PCR reactions; Lane 1: negative control; Lane 2: *B. abortus* reference strain 544 (positive control); Lane 3: *Brucella* spp. field strain (positive sample); Lane 4 and 5: PCR negative samples; Lane 6: *B. abortus* vaccine strain RB51 (positive control); Lane 7: Marker 100 base pair.

(Musa, 2004). As a result, control of brucellosis in bovines and other animals in Fars Province is important for the health of horses. Most of the horses examined in this study were asymptomatic while only three showed clinical signs of the disease. The same fact was also reported by Dawson and Durrant (1975) and Mac Millan and Cockrem (1986). Although *Brucella* was not characterized in the other two horses sampled for PCR assay, its role as a potential aetiological agent for the occurrence of fistula withers could not be ruled out. *Brucella* may play a part in the pathogenesis of fistula withers in these seropositive cases, but at the time when chronic bursitis develops, no *Brucella* DNA may be detected by PCR assay. Keid et al., (2007) mentioned that a negative blood culture or PCR cannot always be relied upon to exclude a diagnosis of brucellosis, especially in chronically infected cases. Furthermore, it has been demonstrated that the stage of *Brucella* infection may influence the number and location of bacteria (O'Leary et al., 2006). Alternatively, these false negative PCR results may be explained by the presence of PCR inhibitors that are coextracted from the fistula discharges which inhibit or reduce the sensitivity of the PCR. The study indicated the seroprevalence of equine brucellosis in different districts of Fars Province and it was concluded that PCR assay may be helpful in detection of clinically suspected horses.

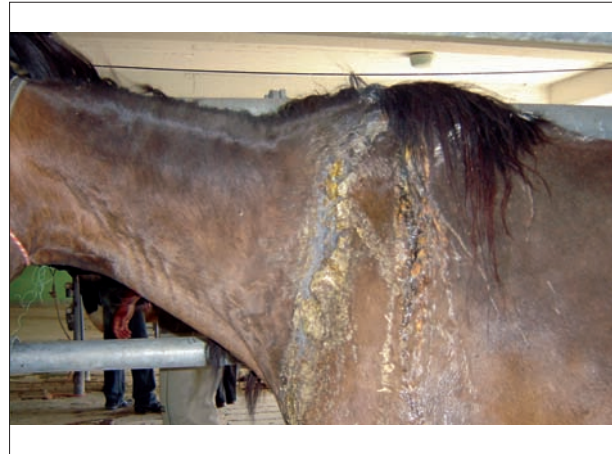


Figure 2. A case of fistulous withers showing straw-colored, viscid exudates.

Acknowledgements

We greatly appreciate the financial support received from Shiraz University.

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سرواپیدمیولوژی و تشخیص مولکولی عفونت بروسلایی در اسب‌های استان فارس (جنوب ایران)

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(دریافت مقاله: ۱۱ آبان ماه ۱۳۹۱، پذیرش نهایی: ۶ دی ماه ۱۳۹۱)

چکیده

زمینه مطالعه: بروسلوز بیماری مشترک انسان و دام بوده و در سراسر جهان در میان انسانها و همچنین حیوانات شایع است. سرو پروالانس بروسلوز در حیوانات مختلف در ایران گزارش شده است، اما گزارشات در مورد اسب بسیار محدود است. **هدف:** هدف از این تحقیق تعیین سرو پروالانس این ارگانیزم در منطقه جنوب ایران و مشخص نمودن عوامل خطر مرتبط با این بیماری می باشد. **روش کار:** ۳۱۲ اسب در منطقه به صورت تصادفی انتخاب شدند. آنتی بادی های سرم از نظر وجود آنتی بادی علیه بروسلابا استفاده از آزمون های RBP، SAT و ۲ME مورد بررسی قرار گرفت. علاوه بر این از روش PCR برای تشخیص موارد بالینی مشکوک به بروسلابا استفاده گردید. **نتایج:** بیشتر موارد مثبت سرمی به بروسلاد در این مطالعه بدون علائم کلینیکی بودند. شیوع واقعی سرمی به بروسلوز به ترتیب ۹/۹، ۸ و ۷٪ توسط SAT، RBPT و ۲ME گزارش شد. سن، جنس و سابقه تماس با نشخوارکنندگان، هیچ تاثیری بر شیوع سرمی بروسلوز در اسبها نداشت. تمام اسب‌ها با سابقه علائم بالینی (۳/۲٪) دارای نتایج مثبت با آزمون های RBPT، SAT و ۲ME بودند. در PCR، یکی از سه اسب با فیستول جدوگاه، محصول ۴۵۰ جفت بازی مختص به سویه فیلدی بروسلار بر اساس تکثیر ناحیه ژنی wbo نشان داد. **نتیجه گیری نهایی:** این مطالعه شیوع سرمی بروسلوز اسب در مناطق مختلف استان فارس را مشخص نمود و نشان داده شد که روش PCR ممکن است در تشخیص اسب های مشکوک به بروسلوز و همراه با تظاهرات بالینی آن مفید باشد.

واژه‌های کلیدی: اسب، عفونت بروسلایی، واکنش های زنجیره ای پلی مرز

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