Development of a multiplex polymerase chain reaction assay for differentiation of field strain isolates and vaccine strains S19 and RB51 of *Brucella* in Iran

Sharifi Yazdi, H.[1]; Khazraiinia, P.[2]; Zahraei Salehi, T.[3*] and Behroozikhah, A. M.[4]

[1]Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [3]Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [4]Department of Brucella Vaccine Research and Production, Razi Vaccine and Serum Research Institute, Karadj, Iran

[*]**Correspondence:** T. Zahraei Salehi, Department of Microbiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. E-mail: tsalehi@ut.ac.ir

(Received 16 Jan 2007; revised version 10 Apr 2007; accepted 15 Apr 2007)

Summary

Bovine brucellosis is a zoonotic disease distributed worldwide and characterized by abortion and reduced fertility in cows. Since brucellosis eradication programme in Iran uses vaccination, test, slaughter and quarantine as control measures, it is essential to distinguish vaccine strains from strains that cause infections among vaccinated cattle herds. We developed and evaluated a multiplex polymerase chain reaction (PCR) assay to identify and differentiate the vaccine strains from wild field isolates, including all *Brucella* types usually found in cattle in Iran. Two pair primers were used to amplify *eri* and *wbo* regions of DNA sequences those strain-specific targets for *B. abortus* S19 and RB51 vaccine strains. This multiplex PCR method evaluated with DNA from reference strains, two vaccine strains and 29 field strains of *Brucella*. The results showed that the multiplex PCR can differentiate *Brucella* isolates into three categories: strain 19 (S19), strain RB51 and field strains. This PCR assay was successfully used, compared with traditional method to differentiate of S19 and RB51 from field *Brucella* isolates.

Key words: Multiplex PCR, *Brucella abortus*, S19, RB51, Iran

Introduction

Bovine brucellosis is a zoonotic disease distributed worldwide and characterized by abortion and reduced fertility in cows. The disease is caused by *Brucella abortus*, which is able to survive and multiply within the tissues of the reticuloendothelial system (Samartino and Enright, 1993).

Vaccination with live, attenuated *B. abortus* strains has been effective in preventing *B. abortus* infection and abortion in cattle. Until recently, strain 19 (S19), a naturally smooth and attenuated strain of *B. abortus*, had been used as the vaccine for cattle brucellosis. There are several disadvantages with adult S19 vaccination such as cross reactivity in diagnostic tests and abortion in pregnant cattle (Nagy *et al*., 1967; Diaz *et al*., 1968; Nicoletti, 1990; Sutherland and Searson, 1990). So vaccine strain RB51 was developed and vaccination with this strain is as effective as vaccination with S19 in protecting cattle against brucellosis (Cheville *et al*., 1993, 1996). Vaccine strain RB51 (S-RB51) is an attenuated, rough organism which essentially lacks the O-side chain of the LPS (Schurig *et al*., 1991). The lack of O-side chain means that this vaccine can be given one or several times without inducing antibodies that interfere with conventional diagnostic tests (Schurig *et al*., 1991). Recently, vaccine S-RB51 being replaced S19 in some commercial herds in Iran. Brucellosis vaccination programme in Iran involves mandatory vaccination of 4–12-month-old female calves (full dose) and

adult (reduced dose) in four provinces (Tehran, Isfahan, Qom, Qazvin) with RB51, and the use of S19 vaccine (full dose) in 3–6-month-old female calves in other areas.

Since the brucellosis eradication programme in Iran uses vaccination, test, slaughter and quarantine as control measures, it is essential to distinguish vaccine strains from strains that cause infections in vaccinated cattle herds. Although bacteriological characterization and biotyping process of *Brucella* organisms is reliable and usually applied to distinguish vaccine strains, this process also have some disadvantages. First, the length of the process from clinical specimen to definitive identification takes time—typically, two weeks. Second, the tests are complex and must be performed in an authorized laboratory by highly-skilled personnel. Some of the characteristics are subjective, such as colony morphology, and require the trained eye and an experienced hand. Third, the zoonotic nature of most *Brucella* species is a potential hazard for laboratory personnel who must manipulate the infectious agent during testing. Finally, the results are not always definitive (Bricker, 2002). Therefore, a rapid test which is highly sensitive and highly specific is needed more than ever. Hence, we designed a multiplex PCR assay to distinguish S19 and RB51 vaccine strains from all field strains of *Brucella* isolated in Iran.

Materials and Methods

Samples

In addition to reference and vaccine strains (S19 and RB51), several field isolated biovars and strains of *B. abortus* and *B. melitensis* which usually found in cattle in Iran were tested. Bacterial strains and biovars used in this study are listed in Table 1. Bacterial strains which isolated in Microbiology Laboratory of Faculty of Veterinary Medicine of Tehran University, consisted of 25 strains including, *B. abortus* biovar 3 (n = 21), *B. melitensis* biovar 1 (n = 3) and *B. melitensis* biovar 2 (n = 1). These bacteria were isolated from milk or supramammary lymph nodes of several cows which were naturally infected with *Brucella*. These samples collected from cows when transported to slaughterhouses in Tehran province. Also, we tested four additional *Brucella* biovars (1, 2, 5 and 7) which were isolated from cases with brucellosis in low frequency in Iran by Razi Vaccine and Serum Research Institute (since 1972 until 2000).

Table 1: *Brucella* reference, vaccine and field strains that used in this study

Bacterium	Biovar	Strain	No. of strains
B. abortus[*]	1	S99 (Reference)	1
B. abortus[*]	1	544 (Reference)	1
B. abortus[*]	1	RB51 (Vaccine)	1
B. abortus[*]	1	S19 (Vaccine)	1
B. abortus[*]	1	Field strain	1
B. abortus[*]	2	Field strain	1
B. abortus[**]	3	Field strain	21
B. abortus[*]	5	Field strain	1
B. abortus[*]	7	Field strain	1
B. melitensis[*]	1	16M (Reference)	1
B. melitensis[**]	1	Field strain	3
B. melitensis[**]	2	Field strain	1

[*]These strains provided by Razi Vaccine and Serum Research Institute of Iran. [**]These strains isolated in Laboratory of Microbiology of Faculty of Veterinary Medicine, University of Tehran

Bacteriological analysis

Brucella species, biovars and vaccine strains were identified by morphological, serological and conventional biochemical tests, growth in the presence of thionine, fuchsin, rifampicin, benzylpenicillin and i-erythritol. The strains were tested by monospecific A, M and R antisera and phage lysis with Tibilissi (*Tb*) phage according to the standard procedures (Alton *et al*., 1988; Schurig *et al*., 1991).

Genomic DNA extraction

A medium-sized bacterial colony suspended in 200 µl of sterile distilled water, incubated in a boiling water bath for 15 min, and centrifuged for five min at 10,000 × g, and then two µl of the supernatant was used as template DNA for the polymerase chain reaction (PCR) amplification (Holmes and Quiqley, 1981).

Primers

We used two pairs of strain-specific primers to show differences between vaccine strains (S19 and RB51) and field strains of *Brucella*. Our multiplex PCR assay contained the following primers: wbo1 5´-GCC AAC CAA CCC AAA TGC TCA CAA-3´ and wbo3 5´-TTA AGC GCT GAT GCC ATT TCC TTC AC-3´ for RB51 detection (Vemulapalli *et al*., 1999); eri1 5´-GCG CCG CGA AGA ACT TAT CAA-3´and eri2 5´-CGC CAT GTT AGC GGC GGT GA-3´ for S19 detection (Bricker and Halling, 1995; Ewalt and Bricker, 2000).

These primers were designed previously based on the following strain-specific genetic differences for two PCR assays separately: a) a *wboA* gene disruption by an IS*711* element in the *B. abortus* RB51 (Vemulapalli *et al*., 1999); and b) a 702-bp deletion in the *eri* operon (essential for erythritol catabolism) in the *B. abortus* S19 (Sangari *et al*., 1994). All oligonucleotide primers used in this study were synthesized by Cinnagen Co. in Iran.

DNA amplification and detection of PCR product

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 $MgCl_2$, 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 95°C for seven min, the PCR profile was as follows: 30 cycles of 45 s of template denaturation at 95°C; 60 s of primer annealing at 64°C and 45 s of primer extension at 72°C; with a final extension at 72°C for six min. The presence of PCR products was determined by electrophoresis of 10 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 were visualized by staining with ethidium bromide (0.5 µg/ml) under UV light. Images were captured on a computer. Sterile water was used as the negative control. At least each sample was tested in duplicate.

Specificity of primers

We tested the specificity and cross reactivity of this strain-specific multiplex PCR assay on several bacterial species that known to be etiologic agent of mastitis in cows (*Staphylococcus aureus*, *Streptococcus* spp. and *Escherichia coli*) and bacteria that are phylogenetically and serologically related to *Brucella* such as *Agrobacterium* spp. and *Yersinia enterocolitica* O:9, respectively.

Results

DNA was successfully extracted from all *Brucella* reference, vaccine and field strains. When the two pairs of primers were used together in the reaction, different sizes of fragments were amplified. As expected, wbo1 and wbo2 produced ~1,300-bp fragment from RB51 and ~400-bp fragment from other field strains. Primers eri1 and eri2 amplified a 178-bp fragment from the field strains and RB51 genomic DNA but none from that of S19 strain (Fig. 1).

This multiplex PCR correctly identified all of bacteria listed in Table 1 as wild-type

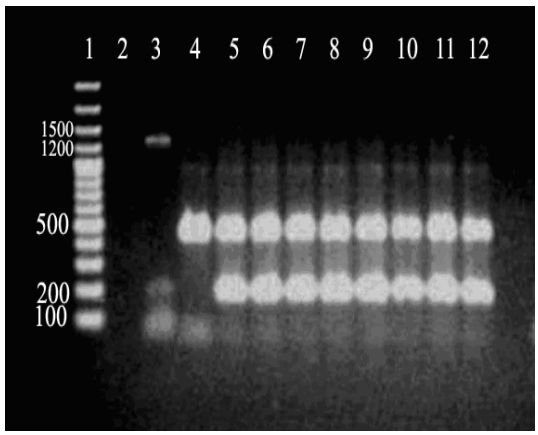

Fig. 1: Electrophoretic analysis (1.5% agarose gel) of DNA amplified fragments from different *Brucella* strains. DNA was amplified by multiplex PCR. Lane 1: Marker 100 base pair; Lane 2: negative control; Lane 3: *B. abortus* strain RB51; Lane 4: *B. abortus* strain S19; Lane 5: *B. abortus* 544; Lane 6: *B. abortus* S99; Lane 7: *B. abortus* biovar 1; Lane 8: *B. abortus* biovar 2; Lane 9: *B. abortus* biovar 3; Lane 10: *B. abortus* biovar 5; Lane 11: *B. abortus* biovar 7 and Lane 12: *B. melitensis* 16 M. Primer-dimer complexes of less than 100 bp were occasionally observed in the reaction mixtures

field strains or *Brucella* vaccine strains (RB51/S19) compared with traditional microbiological and biotyping methods.

No amplified products were detectable when the template was genomic DNA from bacteria that are closely related to *Brucella* such as *Agrobacterium* spp. No products could also be amplified from the genomic DNAs of *Y. enterocolitica* O:9 which synthesizes O antigen that is identical to that of *Brucella*. In addition, the result of PCR on several bacterial species that known to be etiologic agent of mastitis in cows (*S. aureus*, *Streptococcus* spp. and *E. coli*) was negative and no cross reactions was observed.

Discussion

Brucellosis, caused by species of the Gram-negative bacterium *Brucella*, continues to be a problem for humans and animals throughout the world (Alton *et al*., 1988). For some purposes, such as diagnosis of human brucellosis or contamination of food products a genus-specific PCR assay is sufficient. However, there are many instances where the *Brucella* strains and biovars discrimination is important. For example, in brucellosis eradication programms and appropriate action for each epidemiological source of infection it is necessary to differentiate the vaccine strains from the field strains (Bricker, 2002). Accurate typing of *Brucella* is critical for eradication and control of disease-causing organisms. The classical method of identification of the species and biovars of *Brucella* strains requires a minimum of five days. Application of molecular assays may usefully provide high sensitivity and specificity as well as speed for genotyping.

The multiplex PCR in *Brucella* was first used in the USA (Bricker and Halling, 1994) exploiting the multi-copy element IS*711* (Halling *et al*., 1993), also known as IS*6501* (Ouahrani *et al*., 1993). The PCR assay was named "AMOS" which is an acronym for the *Brucella* species it can identify. AMOS-PCR can identify and differentiate *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis*. As more *Brucella* gene sequences were published (Sangari *et al*., 1994; Vemulapalli *et al*., 1999), this PCR assay was modified to distinguish the two commonly used vaccine strains: S19 and RB51 among wild field strain by strain-specific primers (Bricker and Halling, 1995; Ewalt and Bricker, 2000).

Use of the modified AMOS technique (Bricker and Halling, 1995) in Iran for diagnostic purposes is not suitable, because of the difference between the *Brucella* species in our country and strains present in the USA. For instance, *B. abortus* biovar 3 which is the most frequent biovar existing in Iran (Zowghi and Ebadi, 1982) cannot be detected by this method (Bricker and Halling, 1994; Adone *et al*., 2001). In addition, unresolved bands ranging from 600- to 700-bp were reported from RB51 in this method (Adone *et al*., 2001).

We developed a multiplex PCR assay that can identify and differentiate two vaccine strains from field isolates and biovars (Table 1). Originally, PCR assay that distinguished RB51 from other strains, performed with combinations of primers wbo1, wbo2 and wbo3, whereas in our multiplex PCR only the reaction mixtures contained wbo1 and wbo3 primers with two additional primers of eri1 and eri2 from modified AMOS-PCR to distinguish RB51 and S16 vaccine strains from other strains (Bricker and Halling, 1995; Vemulapalli *et al*., 1999; Ewalt and Bricker, 2000). These four primers employed in a multiplex PCR to amplify *eri* and *wbo* regions of DNA sequences of those vaccine strain-specific targets for S19 and RB51, respectively and produced PCR amplified fragments of different sizes which make it easy for analysis on agarose gel. These primers were chosen to allow a more distribution of amplicon sizes for easier analysis. In addition, combination of these primers allowed us to have an internal control for S19 strain that producing a 400-bp fragment in this multiplex PCR assay. Field strains of *Brucella* amplified two DNA fragments of 178- and 400-bp, while the oligonucleotide primers, allowed the amplification of 178-bp plus a 1300-bp fragment specific for RB51. In addition, the identification of S19 is based on the presence of a 400-bp fragment as the only product and the absence of amplification of a 178-bp fragment. So identification of S19 is based on a PCR primer pair which amplifies a short sequence

(178-bp) of the *eri* gene, present in all *Brucella* strains except *B. abortus* S19 (Sangari *et al*., 1994; Bricker and Halling, 1995). In a study which employed eight-pair primers to differentiate all *Brucella* species and vaccine strains, these two regions of gene (*eri* and *wbo*) were also used to distinguish vaccine strains (Garcia-Yoldi *et al*., 2006).

Based on these results, we recommend the PCR assay with primers wbo1, wbo3, eri1 and eri2 to distinguish strain RB51 and S19 from all other *Brucella* strains. The assay described in this report has several advantages over the current microbiological methods used to identify *Brucella* species. A major advantage is the speed with which the assay can be performed, i.e., within less than one working day this method provides useful, early information to make a decision. In addition, the bacteria can be killed and sent for identification. This is very important because *Brucella* is a human pathogen.

The comparison of the conventional methods and multiplex PCR results from all bacteria for the diagnosis of *B. abortus* vaccine or *Brucella* field strains, showed 100% agreement between both tests. Samples were correctly identified as either *Brucella* field strain; *B. abortus* vaccine strain RB51 or *B. abortus* vaccine strain S19. This PCR assay has provided methods for the direct analysis of cultured bacterial samples. In future studies, this PCR method can be used as a direct test for differentiation of post-vaccine infection from field infection in clinical sample such as tissues, semen, milk and blood of cattle. However, it is necessary to remove PCR inhibiting components and needs extensive sample preparation (Leal-Klevezas *et al*., 1995). The main advantage of the assay is its ability to differentiate two vaccine strains which are used in Iran from field isolates. This PCR is rapid, very specific and easily adapted to high volume demands. As long as careful attention is given to avoid contamination, the method is very reliable and usually highly reproducible at any properly-equipped laboratory so this assay can be used by National Veterinary Services Laboratories, where most of the suspected veterinary samples are submitted for identification of causative organisms. This multiplex PCR assay has the ability to quickly verify the presence or absence of RB51 and S19 among *Brucella* isolates from clinical samples.

Acknowledgements

This project was kindly funded by the grants of Veterinary Faculty, University of Tehran and The Organization of Research and Study of Basij. The authors wish to express sincere gratitude to Mr. M. Gaffari and Mr. I. Ashrafi.

References

Adone, R; Ciuchini, F; La Rosa, G; Marianelli, C and Muscillo, M (2001). Use of polymerase chain reaction to identify *Brucella abortus* strain RB51 among *Brucella* field isolates from cattle in Italy. J. Vet. Med. B. 48: 107-113.

Alton, GG; Jones, LM; Angus, RD and Verger, JM (1988). *Techniques for the brucellosis laboratory*. 1st. Edn., Institut National de la Recherche Agronomique (INRA), 147, Paris, France. PP: 13-61.

Bricker, BJ (2002). PCR as a diagnostic tool for brucellosis. Vet. Microbiol., 90: 435-446.

Bricker, BJ and Halling, SM (1994). Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. J. Clin. Microbiol., 32: 2660-2666.

Bricker, BJ and Halling, SM (1995). Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. J. Clin. Microbiol., 33: 1640-1642.

Cheville, NF; Olsen, SC; Jensen, AE; Stevens, MG and Palmer, MV (1996). Effects of age at vaccination on efficacy of *Brucella abortus* strain RB51 to protect cattle against brucellosis. Am. J. Vet. Res., 57: 1153-1156.

Cheville, NF; Stevens, MG; Jensen, AE; Tatum, FM and Halling, SM (1993). Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of *Brucella abortus*. Am. J. Vet. Res., 54: 1591-1597.

Diaz, R; Jones, LM and Leons, D (1968). Surface antigens of smooth brucellae. J. Bacteriol., 96: 893-901.

Ewalt, DR and Bricker, BJ (2000). Validation of the abbreviated *Brucella* AMOS PCR as a rapid screening method for differentiation of *Brucella abortus* field strain isolates and the

vaccine strains, 19 and RB51. J. Clin. Microbiol., 38: 3085-3086.

Garcia-Yoldi, D; Marin, CM; de Miguel, MJ; Munoz, PM; Vizmanos, JL and Lopez-Goni, I (2006). Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. Clin. Chem., 52: 779-781.

Halling, SM; Tatum, FM and Bricker, BJ (1993). Sequence and characterization of an insertion sequence, IS*711*, from *Brucella ovis*. Gene. 133: 123-127.

Holmes, DS and Quiqley, M (1981). A rapid boiling method for the preparation of bacterial plasmid. Anal. Biochem., 114: 193-197.

Leal-Klevezas, DS; Martinez-Vazquez, IO; Lopez-Merino, A and Martinez-Soriano, JP (1995). Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. J. Clin. Microbiol., 33: 3087-3090.

Nagy, LK; Hignett, PG and Ironside, CJT (1967). Bovine brucellosis: a study of an adult vaccinated infected herd. Vet. Rec., 81: 140-144.

Nicoletti, P (1990). Vaccination. In: Nielsen, K and Duncan, JR (Eds.), *Animal brucellosis*. (1st. Edn.), CRC Press, Boca Raton, FL. PP: 283-299.

Ouahrani, S; Michaux, S; Sri Widada, J; Bourg, G; Tournebize, R; Ramuz, M and Liautard, JP (1993). Identification and sequence analysis of IS6501, an insertion sequence in *Brucella* spp.: relationship between genomic structure and the number of IS6501 copies. J. Gen. Microbiol., 139: 3265-3273.

Samartino, LE and Enright, FM (1993). Pathogenesis of abortion in bovine brucellosis. Comp. Immunol. Microbiol. Infect. Dis., 16: 95-101.

Sangari, FJ; Garcia-Lobo, JM and Aguero, J (1994). The *Brucella abortus* vaccine strain S19 carries a deletion in the erythritol catabolic genes. FEMS Microbiol. Lett., 121: 337-342.

Schurig, GG; Roop, RM; Bagchi, T; Boyle, S; Buhrman, D and Sriranganathan, N (1991). Biological properties of RB51: a stable rough strain of *Brucella abortus*. Vet. Microbiol., 28: 171-188.

Sutherland, SS and Searson, J (1990). The immune response to *Brucella abortus*: the humoral response. In: Nielsen, K and Duncan, JR (Eds.), *Animal brucellosis*. (1st. Edn.), CRC Press, Boca Raton, FL. PP: 65-81 (Chapter 3).

Vemulapalli, R; McQuiston, JR; Schurig, GG; Sriranganathan, N; Halling, SM and Boyle, SM (1999). Identification of an IS*711* element interrupting the *wbo*A gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51 from other *Brucella* species and strains. Clin. Diagn. Lab. Immunol., 6: 760-764.

Zowghi, E and Ebadi, A (1982). Typing of *Brucella* strains isolated in Iran. Arch. Razi Inst., 23: 109-114.