

## Original Research Article

<https://doi.org/10.20546/ijcmas.2019.801.065>

## Molecular Characterization of *Brucella melitensis* Field Isolates by Bruce-Ladder Multiplex PCR

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### ABSTRACT

#### Keywords

Brucellosis,  
*Brucella melitensis*,  
Bruce-ladder  
multiplex PCR and  
Amplification  
profile

#### Article Info

Accepted:  
07 December 2018  
Available Online:  
10 January 2019

Brucellosis still remains an infectious, highly contagious and re-emerging endemic zoonosis especially in the Mediterranean and Middle-East regions of the world involving many countries including India where it constitutes occupational hazard (Shakerian *et al.*, 2013 and Khamesipour *et al.*, 2014). It also poses a serious threat to livestock economy by causing abortion, loss of offspring, infertility and reduction in milk yield. The prevalence of brucellosis in animal reservoirs is an evidence of its prevalence in human population and control of animal brucellosis is the key to its control in humans. Early diagnosis is essential to minimise the spread of the disease besides public health importance. In the present study molecular characterization of five *Brucella melitensis* isolates was carried out through Bruce-ladder multiplex PCR and compared with *Brucella abortus*, *Brucella melitensis* and *Brucella suis* vaccine and challenge strains. The amplification profile confirmed the isolates as *Brucella melitensis* and there was a significant difference among these field isolates with that of reference vaccine strains and the amplicons of all the field isolates were similar to amplicons of reference challenge strain, *Brucella melitensis* 16M.

### Introduction

Brucellosis in India is very common but often a neglected disease (Renukaradhy *et al.*, 2002). Caprine and ovine brucellosis caused by *B. melitensis* is widespread in India due to frequent mixing of flocks while grazing and

trading (Smits and Kadri, 2005) and is the major cause of abortion in small ruminants imposing economic loss due to an adverse effect on total animal protein supplies and severe hazard to human health (Abeer *et al.*, 2003).

The human brucellosis results mainly due to

consumption of contaminated milk, meat, exposure to animal reservoirs. Due to high rates of brucellosis in sheep and goats, socioeconomic status of the risk group and higher pathogenicity of *B. melitensis* among all the *Brucella* species, makes this organism being most frequently isolated from humans (OIE, 2008).

Since, there is no licensed human vaccine and *B. melitensis* primarily infect sheep and goats, the prevention of human brucellosis largely depends upon the prevention and control of ovine and caprine brucellosis by prophylactic and sanitary measures in endemic areas (OIE, 2009). An accurate diagnostic and typing procedure for identification of the different species is of great epidemiological importance to institute control strategies.

Several multiplex PCRs have been described for identification of *Brucella* at the species level and partly at the biovar level using different primer combinations. The multiplex PCR, called AMOS PCR for *Brucella abortus*, *B. melitensis*, *B. ovis* and *B. suis*, was published in 1994 where they have used five primers to identify *Brucella* at the species level (Bricker and Halling, 1994). This method could detect selected biovars of 4 species of *Brucella*; biovars 1, 2, and 4 of *B. abortus*; all 3 biovars of *B. melitensis*; biovar 1 of *B. suis*; and biovar 1 of *B. ovis* but unable to differentiate individual biovars within a species. The PCR was used to evaluate animal field samples and was found to be in 100 per cent agreement with the conventional biotyping methods.

A multiplex PCR using 8 multi-locus variable number tandem repeat analysis (MLVA) primers was able to distinguish *B. melitensis* from other *Brucella* species and allowed strain typing (Rees *et al.*, 2009). More recently, a multiplex PCR assay (Bruce-ladder) has been used to identify all *Brucella* sp. at genus level,

including 6 six terrestrial species, the marine species of *Brucella*, and the vaccine strains S19, RB51, and Rev. 1 (Lopez-goni *et al.*, 2008). Based on the Bruce-ladder PCR, an improved multiplex PCR was developed that differentiates all 9 currently recognized *Brucella* species, including the recently described species *B. microti*, *B. inopinata*, *B. ceti* and *B. pinnipedialis*. The method was used to identify all known *Brucella* strains and their biotypes in one test (Mayer-scholl *et al.*, 2010).

The present study reports the molecular characterization of five *Brucella melitensis* isolates which recovered from 18 different sheep and goat farms in Karnataka suspected to have brucellosis which were confirmed by AMOS multiplex PCR and compared with reference vaccine and challenge strains of *Brucellae* by employing Bruce-ladder multiplex PCR.

## Materials and Methods

### Strains and growth conditions

The reference bacterial strains viz., *Brucella abortus* S19, *B. abortus* 544, *Brucella melitensis* Rev 1, *Brucella melitensis* 16M and *B. suis* 1330, mono-specific A and M antiserum, *Pasteurella multocida* P52 were procured from Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh. The five field isolates recovered from 18 different brucellosis suspected sheep and goats farms located in four different districts of Karnataka, India, were confirmed by Biochemical tests, group specific and species specific PCR.

*Brucella* strains were grown in *Brucella* selective broth (Difco) at 37°C for 48 h. *Pasteurella multocida* P52 was grown on standard nutrient broth at 37°C for 24 h. Genomic DNA was isolated from reference

strains and suspected *Brucella* cultures using Genomic DNA extraction kit from M/s Qiagen, USA as per manufacturer's instructions. The purity of the DNA extracted was assessed using Qubit assays M/s Invitrogen, Singapore as per manufacturer's instructions.

### **Bruce-ladder multiplex PCR**

The Bruce-ladder multiplex PCR assay target for identical genes conserved for each species of *Brucella* was carried out using 8 pairs of oligonucleotides primers and thermal cycling conditions as per the method described by Lopez-Goni *et al.*, (2008).

The Bruce-ladder is a new multiplex PCR assay for differentiating the various *Brucella* species and strains in their high degree of genetic homology. Bruce-ladder identification was based on the of eight amplicons, 1682 bp, 450 bp, 1071 bp, 794 bp, 587 bp, 272 bp 218 bp and 152 bp by PCR.

Briefly, the Bruce-ladder multiplex PCR was performed with a reaction mixture containing 15 ng of extracted DNA of each strain, 2X Dream taq master mix 12.5µl, 0.3 µl of each individual 10 µM primers and made the final volume of 25 µl with nuclease free water. The thermal cycling conditions include an initial denaturation at 94 °C for 7 min, followed by 25 cycles of template denaturation at 94 °C for 60 s, primer annealing at 64 °C for 45 s and primer extension at 72 °C for 3 min, followed by a final extension at 72 °C for 10 min. The amplified products were resolved by electrophoresis using a 1.5 per cent agarose gel and followed by staining with ethidium bromide.

### **Results and Discussion**

The amplification profile for *Brucella* field isolates and reference strains obtained were shown in Figure 1. The reference strain

yielded amplicons as described by (Garcia-Yoldi *et al.*, 2006) and Lopez-Goni *et al.*, (2008). The amplicon size, DNA target and sources of genetic differences is listed in the Table 1.

The Bruce-ladder multiplex PCR targeting DNA from *Brucella abortus* 544 strain yielded five fragments, of 1,682, 794, 587, 450 and 152 bp (Lane 8; Fig. 1) in size, an additional amplicon of 1071 bp was noticed in *B. melitensis* 16M (Lane 7). Similarly, *B. suis*1330 yielded an extra amplicon of 272 bp size. Whereas the vaccine strain, *B. abortus* S19 did not yield 587 bp fragments which is otherwise common to all *Brucella* strains tested, Finally *B. melitensis* Rev 1 vaccine strain yielded an additional fragment of 218 bp, which readily distinguished *B. melitensis* Rev 1 from other *B. melitensis* strains (Fig. 1). The amplicon 218 bp accounts for gene encoding the ribosomal protein S12, *rpsL*. In Rev 1 vaccine strain there is chromosomally acquired streptomycin resistance is frequently due to mutations in the gene encoding the ribosomal protein S12, *rpsL*. Nucleotide sequencing revealed one mutation in the *rpsL* gene of vaccine strain Rev.1 compared to that of reference strain 16M leading to an amino acid Pro-to-Leu change at codon position 91 (Pro91Leu) (Cloeckaert *et al.*, 2002).

All the field isolates yielded similar banding pattern as *B. melitensis* 16M. There is lack of additional fragment 218 bp in all the field isolates too, which confirms streptomycin susceptibility in these strains. The assay could differentiate field isolate from reference strain and vaccine strain, the *B. melitensis* Rev 1. In the present investigation too, this technique yielded lucid results, in differentiating *B. melitensis* field isolates from *B. melitensis* Rev 1, *B. abortus* S19, *B. melitensis* 16M, *B. abortus* 544 and *B. suis* 1330 (Fig. 1). In the present study all the five isolates recovered from *Brucella* affected sheep and goats were confirmed as *B. melitensis* which revealed a

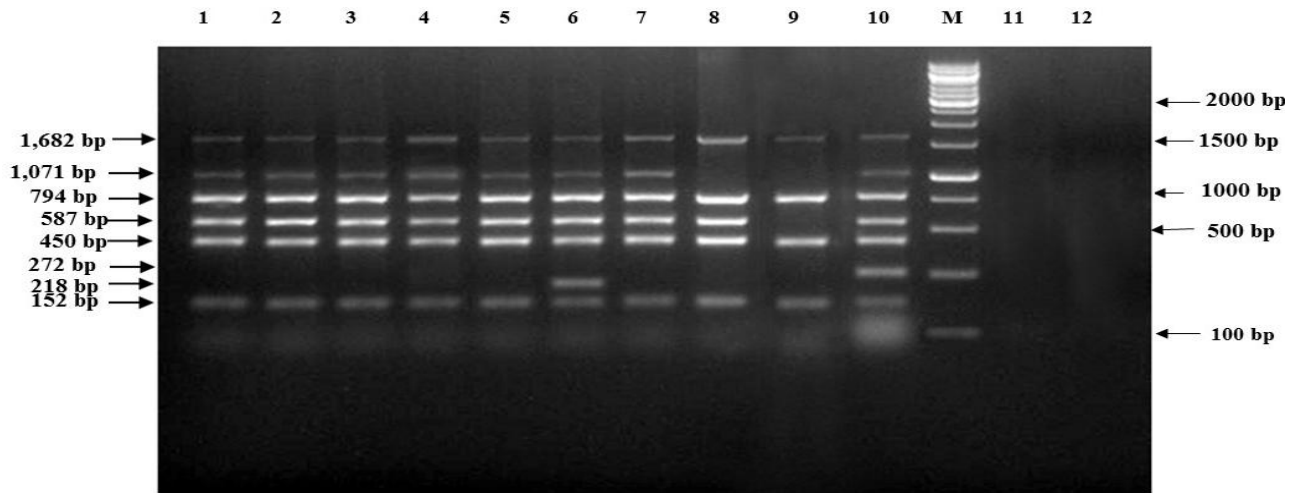
banding pattern similar to challenge strain *B. melitensis* 16M. Ovine and Caprine brucellosis caused by *B. melitensis* is widespread in India and its relative importance varies with the geographical region influenced by husbandry practices, availability of susceptible animal

population, frequent mixing of flocks during grazing and inter-state trade of animals. The major advantage of the Bruce-ladder PCR assay was that it could identify and differentiate all the *Brucella* species and the vaccine strains in one go.

**Table.1** Oligonucleotides used in Bruce-ladder multiplex PCR Assay with target gene and target protein amplified (Lo´pez-Gon˜i et al., 2008)

Primer	Sequence (5'-3')	Amplicon Size (bp)	DNA target	Source of genetic differences
<b>BMEI0998f</b>	ATC C TA TTG CCC CGA TAA GG	1,682	<i>Gene WboA</i> Glycosyltransferase	IS711 insertion in BMEI0998 in <i>B. abortus</i> RB51 and deletion of 15,079 bp in BMEI0993–BMEI1012 in <i>B. ovis</i>
<b>BMEI0997r</b>	GCT TCG CAT TTT CAC TGT AGC			
<b>BMEI0535f</b>	GCG CAT TCT TCG GTT ATG AA	450	<i>gene bp26</i> Immunodominant antigen,	IS711 insertion in BMEI0535– BMEI0536 in <i>Brucella</i> strains isolated from marine mammals
<b>BMEI0536r</b>	CGC AGG CGA AAA CAG CTA TAA			
<b>BMEII0843f</b>	TTT ACA CAG GCA ATC CAG CA	1071	<i>gene omp31</i> Outer membrane protein,	Deletion of 25,061 bp in BMEII826–BMEII0850 in <i>B. abortus</i>
<b>BMEII0844r</b>	GCG TCC AGT TGT TGT TGA TG			
<b>BMEI1436f</b>	ACG CAG ACG ACC TTC GGT AT	794	Polysaccharidde deacetylase	Deletion of 976 bp in BMEI1435 in <i>B. canis</i>
<b>BMEI1435r</b>	TTT ATC CAT CGC CCT GTC AC			
<b>BMEII0428f</b>	GCC GCT ATT ATG TGG ACT GG	587	<i>gene eryC</i> Erythritol catabolism, (erythrulose-1 phosphate dehydrogenas)	Deletion of 702 bp in BMEII0427–BMEII0428 in <i>B. abortus</i> S19
<b>BMEII0428r</b>	AAT GAC TTC ACG GTC GTT CG			
<b>BR0953f</b>	GGA ACA CTA CGC CAC CTT GT	272	ABC transporter binding protein	Deletion of 2,653 bp in BR0951– BR0955 in <i>B. melitensis</i> and <i>B. abortus</i>
<b>BR0953r</b>	GAT GGA GCA AAC GCT GAA G			
<b>BMEI0752f</b>	CAG GCA AAC CCT CAG AAG C	218	<i>gene rpsL</i> Ribosomal protein S12,	Point mutation in BMEI0752 in <i>B. melitensis</i> Rev.1
<b>BMEI0752r</b>	GAT GTG GTA ACG CAC ACC AA			
<b>BMEII0987f</b>	CGC AGA CAG TGA CCA TCA AA	152	Transcriptional regulator, CRP family	Deletion of 2,203 bp in BMEII0986–BMEII0988 in <i>B. neotomae</i>
<b>BMEII0987r</b>	GTA TTC AGC CCC CGT TAC CT			

**Fig.1** Amplification profile of *Brucella* field isolates and reference strains by Bruce- ladder multiplex PCR



**Lanes 1 to 5 : *B. melitensis* field isolates,**  
**Lane 6 : *B. melitensis* Rev 1**  
**Lane 7 : *B. melitensis* 16M**  
**Lane 8 : *B. abortus* 544**  
**Lane 9 : *B. abortus* S19**  
**Lane 10 : *B. suis* 1330**  
**Lane M : 3 kb ladder.**  
**Lane 11 : *Pasteurella multocida* P52 DNA (Negative control)**  
**Lane 12 : No template control**

Furthermore, it can also detect DNA from *B. canis*, *B. neotomae*, *B. abortus* biovars 3, 5, 6, and 9, and *B. suis* biovars 2, 3, 4, and 5 as reported by Lopez-Goni *et al.*, (2008). This method was used to identify 7 epidemiologically-linked clusters of *B. melitensis* and the source of a laboratory-acquired infection. The assay was found to be practical for technical and economical reasons.

In conclusion, in the present study all the five isolates recovered from *Brucella* affected sheep and goats were further identified and confirmed as *B. melitensis* which were resulted banding pattern similar to challenge strain *B. melitensis* 16M. Further the study ruled out the infections that might have resulted due to any of the vaccine strains. Since there is no effective vaccine against sheep and goat brucellosis in India, extensive

studies need to be carried out by including different geographical areas of the country with varied endemicity to establish the prevalence of *B. melitensis* infections for instituting the effective control strategies to minimise the disease and intern further spread to public.

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#### How to cite this article:

Sumathi, B.R., B.M. Veeregowda, S.M. Byregowda, D. Rathnamma, Shrikrishna Isloor, Rajeswari Shome and Narayanaswamy, H.D. 2019. Molecular Characterization of *Brucella melitensis* Field Isolates by Bruce- Ladder Multiplex PCR. *Int.J.Curr.Microbiol.App.Sci*. 8(01): 585-590. doi: <https://doi.org/10.20546/ijcmas.2019.801.065>