

## Development of 16S rRNA Polymerase Chain Reaction and its Comparison with BCSP31 Polymerase Chain Reaction for Identification of *Brucella*

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(Received: 20 July 2023; Revised: 02 September 2023; Accepted: 03 October 2023; Published: 15 October 2023)  
(Published by Research Trend)

**ABSTRACT:** The present study was carried out to develop a 16S rRNA-based polymerase chain reaction (PCR) assay for the identification of *Brucella* isolates at the genus level and evaluate its efficiency by comparing it against the BCSP31 PCR assay. Oligonucleotide primers specific for the 16S rRNA gene of *Brucella* were designed and compared with published primers specific for BCSP31 for the identification of *Brucella* at the genus level. A total of 46 *Brucella* isolates (11 standard isolates and 35 *Brucella melitensis* clinical isolates) were used for amplification with both the primers. The sensitivity and specificity of the primer were also evaluated. Both the PCR assays demonstrated specificity in the identification of *Brucella*, but the 16S rRNA PCR assay exhibited lower sensitivity compared to the BCSP31 gene-specific PCR assay. The 16S rRNA PCR assay is suitable for genus-level identification of *Brucella* isolates, although further validation is needed for its application in direct clinical sample detection.

**Keywords:** *Brucella*, Diagnosis, PCR, 16S rRNA, BCSP31.

## INTRODUCTION

Brucellosis is an important zoonotic disease caused by *Brucella* spp., a Gram-negative facultative intracellular bacterium. The disease is responsible for causing serious economic loss to the livestock industry through reproductive and productive loss (Londhe *et al.*, 2010; Dadar *et al.*, 2021; Khurana *et al.*, 2021). The genus *Brucella* comprises 35 species that affect terrestrial, amphibian, and marine animals (LSPN.dsmz.de, 2023). *Brucella melitensis*, *Brucella abortus*, *Brucella suis*, and *Brucella canis* are important from the zoonotic point of view, of which *B. melitensis* is the most virulent species (Acha and Szyfres 2006; Fugier *et al.*, 2007; Rajagunalan *et al.*, 2013; Deng *et al.*, 2019; Dadar *et al.*, 2021). The disease is transmitted from cattle, sheep, goats, pigs, and camels (Gupte and Kaur 2015). Humans acquire brucellosis by consuming unpasteurized milk or through contact with secretions or discharges from infected animals (Khurana *et al.*, 2021; Tulu, 2022; Islam *et al.*, 2023). Conventionally, *Brucella* has been identified in the laboratory by isolation and confirmation by biochemical tests, which is considered the 'gold standard' (Gupte and Kaur 2015; Di Bonaventura *et al.*, 2021). Even with the use of commercial bacterial identification systems, several

reports of misidentification of *Brucella* isolates as other related bacteria are available (Elsaghir and James 2003; Horvat *et al.*, 2011). The sensitivity of isolation of *Brucella* is reported to be low as it depends on the stage of the disease (Navarro *et al.*, 2004). These procedures are labor-intensive and time-consuming. They also require proper containment facilities and expertise. The isolation procedures might require multiple sub-culturing and requires 5 to 7 days to obtain results (Yagupsky *et al.*, 2019). The procedures also pose a significant risk of infection to laboratory personnel in both clinical and research settings. Brucellosis is the most common laboratory-acquired infection, worldwide approximately one-fourth of all lab-acquired bacterial infections (Horvat *et al.*, 2011). Serological tests cannot be used for early detection as well as recurrent infection, and these tests are also associated with the inherent defect of antibody cross-reaction with organisms like *Stenotrophomonas maltophilia*, *Escherichia coli* O157:H7, *Yersinia enterocolitica*, *Francisella tularensis*, and *Bartonella* (Khurana *et al.*, 2021).

Polymerase chain reaction (PCR)-based methods are very useful in the identification of *Brucella* from culture and clinical samples, even at the strain level (Yu and Nielsen 2010). These methods are also sensitive

and rapid in providing results in a few hours (Navarro *et al.*, 2004; Becker and Toun 2021). These are also helpful in large-scale screening and identification of *Brucella*, as they tend to be simple and adequate (Mukherjee *et al.*, 2007). Numerous PCR assays targeting various conserved genes of *Brucella* have been published for the identification of *Brucella* at the genus level. The important genes targeted are *BCSP31*, *16S rRNA*, *IS711*, *BMEI1162*, *BMEI10466*, *alkB*, *eryC*, and *per* (Khurana *et al.*, 2021). Minhas *et al.* (2013) developed and evaluated *omp22* gene based PCR assay for the diagnosis of brucellosis. Nyarku *et al.* (2020) developed a real time PCR assay targeting *16S-23S rDNA ITS* region for genus specific identification of *Brucella* and compared it with *bcsp31* based assay and culture based method. Becker and Toun (2021) evaluated *IS711* and *BCSP31* gene for the detection of brucellosis cases in qPCR format in this, *IS711* showed lower detection limit compared to *BCSP31*. The *BCSP31* gene is the most commonly used target, as it is present as a single-copy gene in the genome of *Brucella*, and it confers higher sensitivity than other genes (Ghodasara *et al.*, 2010; Al-Dahouk *et al.*, 2013; Khurana *et al.*, 2021). The *16S rRNA* gene is highly conserved, and all *Brucella* species have identical sequences (Bricker *et al.*, 2000; Al-Dahouk *et al.*, 2013). In the present study, we designed primers targeting the *16S rRNA* gene and evaluated their efficacy by comparing them with *BCSP31* gene-specific primers for the detection of both reference and field isolates of *Brucella*.

## MATERIALS AND METHODS

**Brucella isolates.** A total of 46 *Brucella* isolates, comprising both reference (11) and field *B. melitensis* (35) isolates, were available in the *Brucella* Laboratory, Division of Veterinary Public Health, ICAR-Indian Veterinary Research Institute, India (Table 1).

**Primers.** Published primers specific for the *BCSP31* gene (Serpe *et al.*, 1999) were used in this study. While primers specific for the *16S rRNA* gene of *Brucella* spp. were designed based on the available nucleotide sequences on the GenBank database and got synthesized commercially (Table 2).

**Genomic DNA isolation.** The isolates were subcultured on tryptic soy agar slants, incubated at 37°C for 72 h, and a loopful of the culture was used for genomic DNA extraction using the DNA easy blood and tissue kit (Qiagen) as per the manufacturer's instructions. Extracted DNA was quantified by measuring absorbance at 260 nm and stored at -20°C until use.

**PCR amplification.** The PCR was standardized, and amplification of both the genes was carried out for all 46 isolates. The PCR was performed in a 25 µl volume reaction mixture consisting of DreamTaq Green PCR (2x) master mix (12.5 µl), 1 µl of each primer (10 pmol/µl), DNA template (2 µl), and 8.5 µl of nuclease-free water. The cycling conditions employed for *BCSP31* gene amplification were as follows: initial denaturation (94°C for 5 min) followed by 25 cycles of denaturation at 94°C for 45 s, annealing at 53.5°C for

45 s, and extension at 72°C for 60 s; later, a final extension step was carried out at 72°C for 60 s. In the case of *16S rRNA*, similar cycling conditions were used except for the annealing temperature (50°C). All the amplifications were carried out in the Mastercycler Nexus GSX7 (Eppendorf). After amplification, amplicons were analyzed on 1% agarose gel with ethidium bromide (0.5 µg/ml) and documented using an Alpha Imager gel documentation system. For the determination of the detection limit of the PCR assays, 10-fold dilution of the *B. melitensis* 16M DNA was made and used as a template. For confirming the specificity of both *BCSP31* and *16S rRNA* primers, template DNA obtained from other bacteria like *Ochrobactrum anthropi*, *Campylobacter coli*, *Campylobacter jejuni*, *Salmonella Typhimurium*, and *Pasteurella multocida* was used.

## RESULTS AND DISCUSSION

Brucellosis is an important zoonotic disease affecting approximately 5,00,000 humans annually, posing a serious threat to public health (Deng *et al.*, 2019; Khurana *et al.*, 2021). Accurate diagnosis of brucellosis by bacteriological methods is impractical for regular screening of large numbers of samples (Mukherjee *et al.*, 2007; Gupte and Kaur 2015; Di Bonaventura *et al.*, 2021). The use of DNA-based methods for identification has overcome the difficulties of culture-based methods, facilitating accurate and rapid identification of the isolates (Navarro *et al.*, 2004; Londhe *et al.*, 2010; Becker and Toun 2021). Numerous PCR-based assays have been developed for the identification of *Brucella* at the genus level (Mukherjee *et al.*, 2007; Ghodasara *et al.*, 2010; Al-Dahouk *et al.*, 2013; Gupte and Kaur 2015). The present study focused on the development of a *16S rRNA* gene-based PCR assay for accurate identification of *Brucella* spp. and comparing its efficacy with that of the commonly used *BCSP31* gene-based PCR assay. The PCR was standardized for both the gene targets using DNA extracted from 11 standard isolates of five different *Brucella* species. Both the PCR assays yielded single amplicons of expected size for *BCSP31* and *16S rRNA*, 443 bp and 850 bp, respectively. Screening of all 35 *B. melitensis* isolates was carried out with both *BCSP31* and *16S rRNA* gene-specific PCR assays. Both the PCR assays produced specific amplicons of expected molecular weight with all the *Brucella* isolates (Fig. 1 and 2). To determine the lowest detection limit of *Brucella* DNA, *B. melitensis* 16M DNA was diluted 10-fold, and PCR was performed using both *BCSP31* and *16S rRNA*-specific primers. Using *BCSP31* primers, the detection limit was found to be 30.6 pg (Fig. 3), while for *16S rRNA*, the detection limit was low at 3.06 ng only (Fig. 4). Neither the *BCSP31* nor the *16S rRNA* PCR protocols produced amplicons with templates from other bacteria tested: *Ochrobactrum anthropi*, *Campylobacter coli*, *C. jejuni*, *Salmonella Typhimurium*, and *Pasteurella multocida*. Comparison of the two PCR assays revealed no differences in specificity; however, the *BCSP31* assay showed higher sensitivity than the *16S rRNA* assay. Yu and Nielsen

(2010) also reported that *BCSP31*-specific primers offer better sensitivity compared to primers targeting other genes of *Brucella*. Similar results have also been reported by Mukherjee *et al.* (2007). Garshasbi *et al.* (2014) also reported that the *BCSP31*-based PCR assay is more sensitive than the *IS711*-based PCR assay in the detection of *Brucella* DNA in the serum of infected

patients. This lower sensitivity of the *16S rRNA* assay might limit the use of this primer in routine screening of samples, as low bacterial loads in different stages of infection could be missed. But this primer needs to be evaluated in further studies using clinical isolates of other species of *Brucella*.

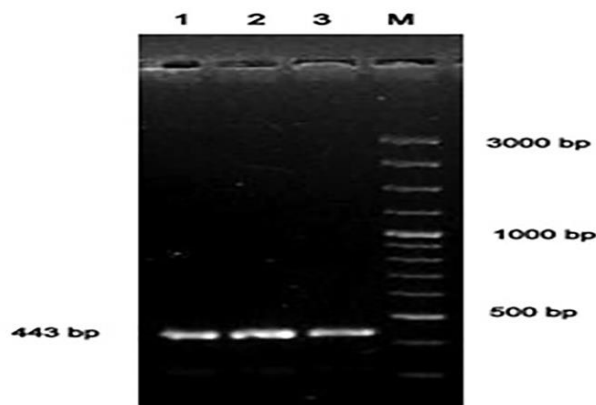
**Table 1: Details of the primes used in the present study.**

Target gene	Name	Sequences (5'-3')	Amplicon	Reference(s)
<i>BCSP31</i>	BRU-UP	GGGCAAGGTGGAAGATTT	443 bp	Serpe <i>et al.</i> (1999)
	BRU-LOW	CGGCAAGGGTCGGTGTTT		
<i>16S rRNA</i>	<i>Bru-16S</i> ID-F	GTGCGACTGATTATAGCCAT	850 bp	Present study
	<i>Bru-16S</i> ID-R	AATGTTTAATGCGTTAGC TGC		

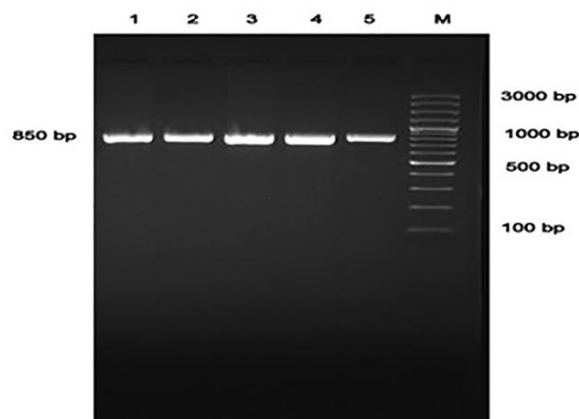
**Table 2: Details of the *Brucella* isolates used in the present study.**

Isolate number	<i>Brucella</i> species
16M	<i>B. melitensis</i>
Isfahan	<i>B. melitensis</i>
B115	<i>B. melitensis</i>
Rev 1	<i>B. melitensis</i>
544	<i>B. abortus</i>
S99	<i>B. abortus</i>
S19	<i>B. abortus</i>
1119-R	<i>B. abortus</i>
1330	<i>B. suis</i>
63/290	<i>B. ovis</i>
MEX 51	<i>B. canis</i>
M06/VPH	<i>B. abortus</i>
1/VPH	<i>B. melitensis</i>
100/VPH	<i>B. melitensis</i>
101/VPH	<i>B. melitensis</i>
104a/VPH	<i>B. melitensis</i>
104b/VPH	<i>B. melitensis</i>
134BM_VPH	<i>B. melitensis</i>
27/VPH	<i>B. melitensis</i>
30/VPH	<i>B. melitensis</i>
36b/VPH	<i>B. melitensis</i>
36c/VPH	<i>B. melitensis</i>
42/VPH	<i>B. melitensis</i>
47/VPH	<i>B. melitensis</i>
47a(31)/VPH	<i>B. melitensis</i>
52/VPH	<i>B. melitensis</i>
53/VPH	<i>B. melitensis</i>
73/VPH	<i>B. melitensis</i>
77/VPH	<i>B. melitensis</i>
80/VPH	<i>B. melitensis</i>
82/VPH	<i>B. melitensis</i>
83/VPH	<i>B. melitensis</i>
86/VPH	<i>B. melitensis</i>
86/VPH	<i>B. melitensis</i>
88/VPH	<i>B. melitensis</i>
89/VPH	<i>B. melitensis</i>
89/VPH	<i>B. melitensis</i>
92/VPH	<i>B. melitensis</i>
93/VPH	<i>B. melitensis</i>
94/VPH	<i>B. melitensis</i>
95/VPH	<i>B. melitensis</i>
97/VPH	<i>B. melitensis</i>
98/VPH	<i>B. melitensis</i>
99/VPH	<i>B. melitensis</i>
Br 175	<i>B. melitensis</i>
Br 341	<i>B. melitensis</i>

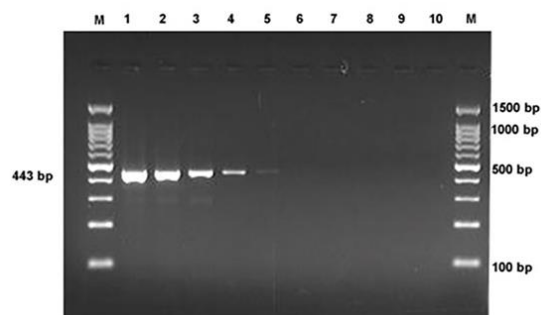
*B. melitensis*=*Brucella melitensis*, *B. abortus*=*Brucella abortus*, *B. suis*=*Brucella suis*, *B. ovis*=*Brucella ovis*, *B. canis*=*Brucella canis*



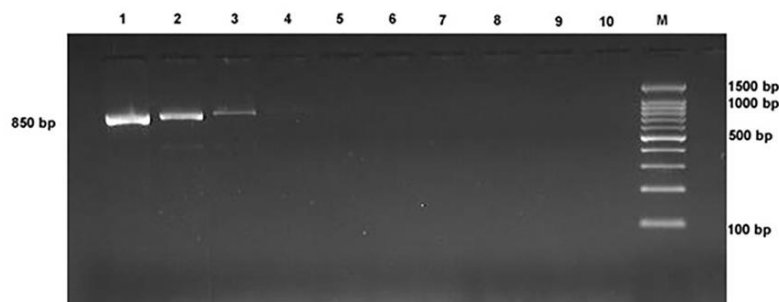
**Fig. 1.** Agarose gel showing *BCSP31* specific amplicons, Lane M: 100 bp ladder, Lane 1, 2, 3: *Brucella* isolates.



**Fig. 2.** Agarose gel showing *16S rRNA* specific amplicons, Lane M: 100 bp ladder, Lane 1, 2, 3: *Brucella* isolates.



**Fig. 3.** Analytical sensitivity *BCSP31* polymerase chain reaction assay, each lane contains 10 fold dilutions of DNA of *B. melitensis* 16M. Lane M: 100 bp ladder, Lane 1: 306 ng, Lane 2: 30.6 ng, Lane 3: 3.06 ng, Lane 4: 306 pg, Lane 5: 30.6 pg, Lane 6: 3.06 pg, Lane 7: 306 fg, Lane 8: 30.6 fg, Lane 9: 3.06 fg, Lane 10: 306 ag.



**Fig. 4.** Analytical sensitivity *16S rRNA* polymerase chain reaction assay, each lane contains 10 fold dilutions of DNA of *B. melitensis* 16M. Lane M: 100 bp ladder, Lane 1: 306 ng, Lane 2: 30.6 ng, Lane 3: 3.06 ng, Lane 4: 306 pg, Lane 5: 30.6 pg, Lane 6: 3.06 pg, Lane 7: 306 fg, Lane 8: 30.6 fg, Lane 9: 3.06 fg, Lane 10: 306 ag.

## CONCLUSIONS

This study developed a PCR assay targeting the *16S rRNA* gene and evaluated its utility in screening *Brucella* isolates, demonstrating high specificity but

lower sensitivity compared to *BCSP31*-targeted assays. Despite their lower sensitivity, these primers remain valuable for screening suspected *Brucella* isolates.



## FUTURE SCOPE

The PCR assay targeting the *16S rRNA* gene must be evaluated in a much higher number of samples and for the detection of *Brucella* spp. directly on different types of clinical samples for integration into routine diagnostic workflows.

**Acknowledgement.** The authors are thankful to the Director, ICAR-Indian Veterinary Research Institute, Izatnagar, for providing necessary facilities to carry out this research work. The fellowship received by SR in the form of ICAR-SRF is also duly acknowledged.

**Conflict of Interest.** None.

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**How to cite this article:** S. Rajagunalan, Soni Doimari, S. Murugavel and D.K. Singh (2023). Development of *16S rRNA* Polymerase Chain Reaction and its Comparison with *BCSP31* Polymerase Chain Reaction for Identification of *Brucella*. *Biological Forum – An International Journal*, 15(10): 1714-1718.