Int.J.Curr.Microbiol.App.Sci (2019) *8*(1): 2973-2979

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 01 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.801.316

Molecular Characterization of *Brucella melitensis* Detected from Aborted Sheep

Vinay Kumar*, Aman Kumar, Sushila Maan, Nitish Bansal and Trilok Nanda

Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125 004, India

**Corresponding author*

Keywords

Brucella melitensis, Sheep, Real time PCR, Sequencing

Article Info

Accepted:
20 December 2018
Available Online:
10 January 2019

A B S T R A C T

Brucellosis is one of the world's major zoonoses that affect wide range of domesticated as well as wild animals. Despite the eradication program of brucellosis, the disease is still endemic among cattle, buffaloes, sheep and goats. In many developing countries including India, *Brucella melitensis* is of much concern due to heavy economic losses to animal husbandry sector as well as humans. The present study was carried out to detect and characterize *B. melitensis* from biological samples (blood, vaginal swabs and aborted foetal material from 8 animals) collected from a disease outbreak in sheep flock using TaqMan probe based real time PCR (qPCR) assay and nucleotide sequencing. Out of 20 biological samples, 6 (30%) were found positive for *Brucella spp*. specific and *Brucella melitensis* specific qPCR assay. Further *B. melitensis* was confirmed by sequencing of the 1343 bp of 16S rRNA gene generated in conventional PCR.

Introduction

Brucellosis is a contagious, infectious and communicable disease caused by bacteria of genus *Brucella*, which is small, gram-negative coccobacillus (*Brucellaeceae* family). Besides ongoing eradication programs, brucellosis is the most widespread zoonosis that infects mainly cattle, buffalo, bison, sheep, goats, and pigs. It can transfer from animals to humans by direct contact with blood, placenta, fetuses, or uterine secretions or through consumption of infected and raw animal products (especially milk and milk products) (Gupta *et al.*, 2006).The genus *Brucella* is composed of nine recognised species, out of which, six are the "classical" members (*B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*) (Cutler *et al.*, 2005).

Brucella melitensis is the main causative agent responsible for caprine and ovine brucellosis. It leads to significant financial losses in animal husbandry due to abortion and fertility problems in sheep and goats (Pappas *et al.*, 2005; Seleem *et al.*, 2010). The clinical manifestations of brucellosis in goat and sheep are similar to those in cattle in regards to decrease in fertility, abortion, stillbirth, decrease in milk production and reproductive

failure (Gupta *et al.,* 2006). *B. melitensis* also affects humans and causes a serious, debilitating and sometimes chronic disease that can affect a variety of organs.

Brucellosis has an important world-wide impact on animal industries and human health. Control measures are based on prevention and eradication. Although in some developed countries this disease have been eradicated by the combination of strict veterinary hygiene measures, surveillance programs and improved food safety measures, it remains endemic in large areas (Habtamu *et al.,* 2013). Brucellosis is still an uncontrolled serious public health problem in many developing countries including India (Acha and Szyfres, 2003; Saleem *et al.,* 2004; Minas, 2006; WHO, 2006).

The huge economic losses to animal husbandry (Singh *et al.,* 2015) due to brucellosis in sheep and goats demand the use of sensitive and rapid diagnostic methods for proper detection and implementation of control strategies for *Brucella* spp. and *B. melitensis*. At present, brucellosis in goats is either diagnosed by isolation of *Brucella* from clinical samples or the detection of antibody in serum (Alton *et al.,* 1988). However, these methods are not fully satisfactory and are lengthy and labour intensive and also have reduced sensitivity in chronic infections. Bacteriological isolation is time consuming and hazardous procedure as it is associated with a high risk of laboratory acquired infection (Gupta *et al.,* 2006; Kumar *et al.,* 2015). Serological methods are easy to perform but are inconclusive because cross-reaction with other bacteria can give false negative results (Alton *et al.,* 1988; OIE, 2009).

Nucleic acid based techniques (PCR-based) have the potential to be simple, fast, less hazardous, usually more sensitive and efficient in detecting *Brucella* (Bricker, 2002). Real-time PCR is rapid, fast and more sensitive over conventional PCR as real-time PCR results can be evaluated without gel electrophoresis which ultimately results in reduced experiment time and increased throughput (Kumar *et al.,* 2015). The present study was aimed for molecular detection and characterisation of *Brucella* spp. from clinical samples collected during a field investigation from a flock of sheep by employing IS711 and BMEII0466 gene based real time PCR assay (Kumar *et al.,* 2015; 2017).

Materials and Methods

Collection of samples

Blood samples, vaginal/cervical swab samples were collected aseptically from affected sheep (n=10) having clinical signs of brucellosis from different herds in Bure village (28.9463° N, 75.7556° E), Hisar District of Haryana. The main clinical manifestations in sheep were reproductive failures, heavy number of abortions and birth of weak offspring. Abortion generally occurred during the last 2 months of pregnancy.

Extraction of genomic DNA

Genomic DNA was extracted from collected samples by using Purelink DNA isolation kit (Invitrogen, USA) as per manufacturer's protocol. Briefly, 400µl of sample (blood) was taken in a 1.5µl sterile micro centrifuge tube. Vaginal swabs and cervical swabs were firstly dissolved in sterile PBS and then total of 400µl sample was taken in a 1.5µl sterile micro centrifuge tube. Then, 400µl of genomic lysis/binding buffer and 40µl proteinase K was added, mixed and incubated at 55°C for 30 min in water bath. After that 400µl of absolute ethanol was added in the solution and the solution was vortexed and transferred in silica based spin column. The

spin column was centrifuged at 13000 rpm for 2 min. After washing with 500µl of wash buffer 1 and wash buffer II sequentially, 30µl of elution buffer was added in the column and centrifuged at 13000 rpm for 3 min to elute the DNA. The concentration and purity of the extracted DNA was measured spectrophotometrically (BIO-RAD, India) by measuring the wavelength at A_{260} and A_{280} and their purity was assessed by taking the 260/280 ratio (Sambrook and Russel, 2001). The obtained DNA was used as template in conventional PCR as well as real time PCR.

Real time PCR amplification

The extracted genomic DNA was subjected to real time PCR for amplification of *Brucella* spp. specific gene and *Brucella melitensis* specific gene using the primer and probe shown in table 1. The real time PCR assay was performed in a total reaction volume of 25 µl consisting of 12.5 µl of 2X TaqMan Fast Universal Master Mix (ABI, USA), 1µl (0.8µM) of each forward primer, reverse primer and probe, and 4µl of extracted DNA as template. The amplification and fluorescence detection was performed on Step One Plus (ABI, USA) real time PCR system using the thermal conditions as: preheating at 50^{o}C for 2 min, initial denaturation at 95^{o}C for 10 min, 40 cycles of denaturation at 95^{o}C for 15 sec, annealing and extension at 60^{o}C for 1 min.

PCR amplification of 16S rRNA and sequence analysis

The extracted DNA was also used in PCR (Veriti, ABI, USA) for amplification of 16S rRNA gene of *Brucella* species using published primers as shown in table 1. The PCR assay was performed in a total reaction volume of 50 µl consisting of 25 µl of 2X High Fidelity Phusion Master Mix (NEB, UK), 2 µl of forward and reverse primer, 2 µl of DMSO, and 12 µl of extracted DNA as template. The cyclic conditions used in PCR were: Initial denaturation at 98°C for 45 sec, and 35 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec followed by final elongation at 72°C for 5 min. The PCR products were run on 1.5% agarose gel, stained with ethidium bromide and visualized under gel documentation system (BIO-RAD, India). The PCR products were purified using QIAGEN gel extraction kit (USA) as per manufacturer's protocol and the purified PCR products were sequenced from both ends using forward and reverse primers in separate reaction by dideoxy chain termination method in ABI 3730 sequencer (ABI, USA).

The alignment of DNA sequences was performed by using Bioedit software along with the reference sequence of *B. melitensis* 16M. The BLAST analysis of the aligned sequences was also done to compare with other reported sequences of *Brucella* species.

Results and Discussion

Real time PCR results

Six samples out of twenty (30%) were showing significant fluorescence with good Ct values ranging from 15 to 32 in real time PCR as shown in figure 1, indicating the presence of *Brucella* spp. in the clinical samples. All the six samples showing significant fluorescence with good Ct values ranging from 20 to 34 in real time PCR as shown in figure 2, indicating the presence of *Brucella melitensis* in the clinical samples. No significant fluorescence was observed in the negative control.

Molecular characterization

The PCR results for 16S rRNA amplification showed amplification of 1343 bp product with LPW primer set as shown in figure 3. No amplification was observed in negative

control. Sequencing analysis of 16S rRNA based primers was done for 2 samples using dideoxy chain termination method and the obtained sequences were aligned and matched with *B. melitensis* 16M reference sequence. The BLAST analysis of deduced sequences of 16S rRNA revealed 100 per cent similarity with 16S rRNA of *Brucella melitensis* 16M (biotype1).

Table.1 Primer and probe for sequencing and detection of *Brucella melitensis*

Primer pair	Primer sequence(5'-3')	Product size (bp)	References
***Brucella*IS711 F**	GACATATTCAAAGTCCGGCGTAT	102	Kumar *et al.*, 2015
***Brucella*IS711 R**	CAAATGGACAGCGGTTCATGC		
***Brucella*IS711 P**	VIC- CCTTTCCCATACACCGGCGTGCGACC-BHQ1		
***Brucella melitensis* F**	GAAAGAAGCGGCGAAATGGT	78	Kumar *et al.*, 2017
***Brucella melitensis* R**	ATTGAAACTGCCGATGCGATATTG		
***Brucella melitensis* P**	JOE-CAGCTTGCCGCCGA TCAGGGCTTTGCGCC- BHQ1		
LPW F	AGTTTGATCCTGGCTCAG	1343	Woo *et al.*, 2003
LPW R	AGGCCCGGGAACGTATTCAC		

Fig.1 Amplification plot of field samples (n=6) positive with *Brucella* spp. specific q PCR

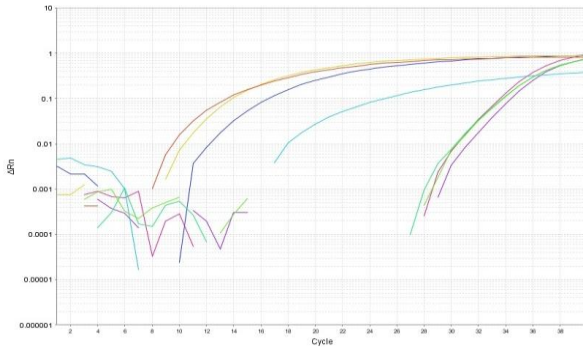


Fig.2 Amplification plot of field samples (n=6) positive with *Brucella melitensis* specific q PCR

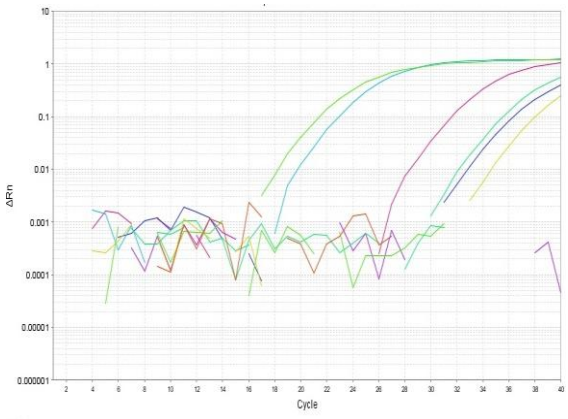
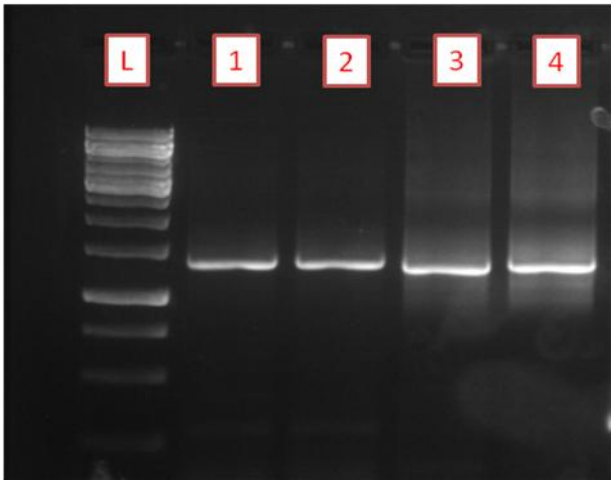


Fig.3 Agarose gel electrophoresis of conventional PCR of 16s RNA specific products generated using High Fidelity Phusion Taq DNA polymerase: L- 1Kb DNA Ladder, 1,2,3 and 4- 1343bp band



Brucellosis is still an emerging disease since the discovery of *Brucella melitensis* by Sir David Bruce in 1887. In tropical countries as well as in subtropical regions, Brucellosis remains the most widespread and important zoonotic disease (Gul and Khan, 2007). Identification and isolation of the bacterial species from an outbreak is essential to know the exact incidence and severity of a disease in that region and also to plan epidemiological studies, control and eradication programmes in that particular region. However, in our study, *Brucella* organisms were not isolated due to the fact that, *Brucella* culturing is hazardous, and the technique is restricted to few laboratories. Isolation rate is very low even in experienced laboratories (Wareth *et al.,* 2014). Sometimes, there is probability of presence of few organisms in the samples or due to unhygienic sample collection procedures the sample may be heavily contaminated which reduces the chances of successful isolation of *Brucella.* Negative culture results may sometimes also show the infection with *Brucella* due to contamination (Bercovich, 1998). Also, the antibody titers against *Brucella* start rising by 1–2 weeks after infection which may lead to failure of early serological diagnosis from freshly aborted animals (Poester *et al.,* 2010), therefore circulating *Brucella* DNA can detected earlier by molecular techniques. Thus, the diagnostic window of *Brucella* serology should be complemented by bacteriological or molecular diagnosis (Marianelli *et al.,* 2008). Now a day, molecular confirmation by PCR targeting different genes has become the most common approach for early detection of bacterial isolates from an outbreak (Gee *et al.,* 2004 and Herman and De Ridder, 1992).

In the present investigation of a suspected outbreak of ovine brucellosis, both molecular detection by real time PCR (q PCR) and sequencing procedures of the causative agent were employed. Out of a total number of 20 clinical samples of sheep subjected to nucleic acid isolation and q PCR detection, 6 (30%) samples were found positive by IS711 based spp. identification q PCR assay (Kumar *et al.,* 2015) and BMEII0466 (Kumar *et al.,* 2017) gene based *Brucella melitensis* specific q PCR assay.

These results are in accordance with the previous reports published by Wareth *et al.*, 2015; Habtamu *et al.*, 2013 and Gupta *et al.*, 2006.

Sequence analysis of 16S rRNA gene can be extensively used for molecular detection or taxonomic analysis of different bacterial species (Woo *et al.*, 2003). It has been reported that 16S rRNA gene sequencing is a reliable tool for rapid genus level identification of *Brucella* (Fitch *et al.*, 1990). In this study 16S rRNA gene based primers were used to produce 1343bp product and was sequenced. The sequence analysis of the generated products by BLAST shows 100 % similarities with published sequences of *Brucella melitensis*. These results are in accordance with the previous studies done by Barua *et al.*, (2016) and Habtamu *et al.*, (2013).

Brucellosis is an important but neglected disease in India. The disease may be overlooked and misdiagnosed because of the difficult diagnosis and lack of experience with the laboratory testing. It has also been estimated that the true incidence of brucellosis may be 25 times higher than the reported incidence due to misdiagnosis and underreporting (Smith and Kadri, 2005). Therefore, to study the epidemiological surveillance, public health importance and early diagnosis of *Brucella* spp. the fast, reliable, sensitive and easy to perform molecular assays are urgently needed. Therefore, there is an urgent need for the strict implementation of a control policy not only for cattle but also for small ruminants. In conclusion, real time PCR based assays may be useful in early diagnosis of *Brucella* infection not in cattle but also in small ruminants in quick time and can replace existing lengthy and laborious laboratory tests which require skilled personnel and courage to handle the zoonotic organism.

References

Acha, N.P. and Szyfres, B. 2003. Zoonoses and communicable diseases common to man and animals. 3rd edn. Pan American Health Organization (PAHO), Washington, DC.

Alton, G.G., Jones, L.M., Angus, R.D., Verger, J.M. 1988. Techniques for the brucellosis laboratory. *Institut Nacional de la Recherche Agronomique*, Paris.

Barua, A., Kumar, A., Thavaselvam, D., Mangalgi, S., Prakash, A., Tiwari, S., Arora, S. And Sathyaseelan, K. 2016. Isolation & characterization of *Brucella melitensis* isolated from patients suspected for human brucellosis in India. *Indian J. Med. Res.* 143: 652-658.

Bercovich, Z. 1998. Maintenance of *Brucella abortus*- free herds: a review with emphasis on the epidemiology and the problems in diagnosing brucellosis in areas of low prevalence. *Vet. Q.* 20(3): 81-88.

Bricker, B.J. 2002. PCR as a diagnostic tool for brucellosis. *Vet. Microbiol*. 90: 435-446.

Cutler, S.J, Whatmore, A.M and Commander, N.J. 2005. Brucellosis – New aspect of an old disease. *J. Appl. Microbial.* 98(6): 1270-1281.

Fitch, T.A., Bearden, S.W., Sowa, B.A. and Marquis, H. 1990. Genetic variation at the omp2 porin locus of the brucellae: species specific markers. *Mol. Microbiol*. 4: 1135-1142.

Gee, J.E., De, B.K., Levett, P.N., Whitney, A.M., Novak, R.T. and Popovic, T. 2004. Use of 16 S rRNA genes sequencing for rapid confirmatory identification of *Brucella* isolates. *J. Clin. Microbiol.* 42: 3649-3654.

Gul, S.T. and Khan A. 2007. Epidemiology and Epizootology of Brucellosis: A Review. *Pakistan Vet. J*. 27(3): 145-151.

Gupta, V.K., Verma, D.K., Rout, P.K., Singh, S.V. and Vihan, V.S. 2006. Polymerase chain reaction (PCR) for detection of *Brucella melitensis* in goat milk. *Small Rumin.Res.* 65: 79-84.

Habtamu, T.T., Rathore, R., Dhama, K. and Karthik, K. 2013. Isolation and molecular

detection of *Brucella melitensis* from disease outbreak in sheep and *B. abortus* from cattle farm by *IS711* and *omp2a* gene based PCR. *Int. J. Current. Res.* 5(7): 1920-1925.

Herman, L. and De Ridder, H. 1992. Identification of *Brucella* spp. by using the polymerase chain reaction. *Appl. Environ. Microbiol*. 58: 2099-2101.

Kumar, V., Kumar, A., Debnath, A., Batra, K., Bansal, N., Gupta, A.K., Tiwari, A. and Maan, S. 2015. Development of real time PCR assay for diagnosis of *Brucella* spp. *Haryana Vet.* 54(2): 99-102.

Kumar, V., Maan, S., Kumar, A., Batra, K., Chaudhary, D., Dalal, A., Gupta, A.K., Bansal, N., Sheoran, N. and Maan, N.S. 2017. Real time PCR assay for differentiation of *Brucella abortus* and *Brucella melitensis. Indian J. Anim. Res.* doi:10.18805/ijar.v0iOF.8464.

Marianelli, C., Martucciello, A., Tarantino, M., Vecchio, R., Iovane, G. and Galiero, G. 2008. Evaluation of molecular methods for the detection of *Brucella* species in water buffalo milk. *J. Dairy Sci*. 91: 3779-3786.

Minas, A. 2006. Control and eradication of brucellosis in small ruminants. *Small Rum. Res*., 62: 101-107.

OIE, 2009. Terrestrial Animal Health Code Brucellosis, www.oie.int/

Pappas, G., Akritidis, N. and Tsianos, E.V. 2005. Brucellosis- Reply. *New Engl. J. Med*. 353(10): 1072.

Poester, F.P., Nielsen, K., Samartino, L.E. and Yu, W.L. 2010. Diagnosis of brucellosis. *Open Vet. Sci.* **4**: 46-60.

Saleem, A.N., Rhaymah, M.S. and Shamoon, G.N. 2004. Isolation and seroprevalence of ovine brucellosis. *Iraq. J. Vet. Sci.* 18: 31-38.

Sambrook, J. and Russell, D.W. 2001. Molecular cloning: A laboratory manual. (3rd edn.), Cold spring harbor laboratory press, New York, USA.

Seleem, M.N., Boyle, S.M. and Sriranganathan, N. 2010. Brucellosis: a re-emerging zoonosis. *Vet Microbiol*. 140(3-4): 392-398.

Singh, B.B., Dhand, N.K. and Gill, J.P.S. 2015. Economic losses occurring due to brucellosis in Indian livestock populations. *Prev. Vet. Med.* 119(3-4): 211-215.

Smith, H.L. and Kadri, S.M. 2005. Brucellosis in India: a deceptive infectious disease. *Indian J. Med. Res*. 122: 375-384.

W.H.O. 2006. Brucellosis in humans and animals. WHO Press, World Health Organization, Avenue Appia, Geneva, Switzerland.

Wareth, G., Hikal, A., Refai, M., Melzer, F., Roesler, U. and Neubauer, H. 2014. Animal brucellosis in Egypt. *J. Infect. Dev. Ctries.* 8(11): 1365–1373.

Wareth, G., Melzer, F., Tomaso, H., Roesler, U. and Neubauer, H. 2015. Detection of *Brucella abortus* DNA in aborted goats and sheep in Egypt by real time PCR. *BMC Res. Notes* 8: 212-217.

Woo, P.C.Y., Ng, K.H.L., Lau, S.K.P., Yip, K.T., Fung, A.M.Y., Leung, K.W., Tam, D.M.W., Que, T.L. and Yuen, K.Y. 2003. Usefulness of the microseq 500 16s ribosomal DNA-based bacterial identification system for identification of clinically significant bacterial isolates with ambiguous biochemical profiles. *J. Clin. Microbiol*. 41(5): 1996-2001.

How to cite this article:

Vinay Kumar, Aman Kumar, Sushila Maan, Nitish Bansal and Trilok Nanda. 2019. Molecular Characterization of *Brucella melitensis* Detected from Aborted Sheep. *Int.J.Curr.Microbiol.App.Sci.* 8(01): 2973-2979. doi: https://doi.org/10.20546/ijcmas.2019.801.316