

Original Research Article

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Isolation and Molecular Characterization of *Brucella melitensis* from Abortion Storms in Sheep with its Zoonotic Implications

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ABSTRACT

Outbreak of brucellosis has been recorded in sheep flocks in Udaipur district of Rajasthan, India. Total 15 abortions were recorded in two flocks of 115 sheep. Samples from aborted fetus, retained placenta colostrum and sera samples were collected. Sera samples were tested by Rose Bengal plate agglutination test. Culture and isolation were done on selective media. All tested sera samples were found positive for brucellosis while *Brucella melitensis* was isolated from fetal abomasal content and placenta samples. Three isolated strains were subjected to molecular characterization by Real time PCR using BCSP31 gene specific oligonucleotide primer pair in real time PCR with SYBR Green chemistry. Further these isolates were confirmed by Taqman probe chemistry with IS711 gene. Species level characterization was done by Bruce ladder PCR. Outbreak of Brucellosis has been confirmed. Three strains were isolated and by molecular characterization found to be *Brucella melitensis*. All sheep examined found serologically positive for brucellosis. This is a primary report of culture confirmation of *Brucella melitensis* outbreak in southern Rajasthan. Study show the occurrence of *Brucella melitensis* in the area which is of major zoonotic concern. Here we are reporting first time isolation and molecular confirmation of *Brucella melitensis* in sheep from Udaipur district of Rajasthan

Keywords

Brucellosis, Real time PCR, *Brucella melitensis*, Sheep, Udaipur, Rajasthan

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Introduction

Brucellosis has worldwide distribution and is more common in countries with poor animal and public health programme. *Brucella melitensis* is most virulent species of *Brucella*

genus and has zoonotic potential (Aparcio, 2013). Goats are classical natural host of *B. melitensis* together with sheep as its preferred host with main clinical signs of abortion, retention of placenta, still birth and infertility (Blasco and Molina-Flores, 2011).

Brucellosis remains an important contagious

disease with zoonotic significance. It affects approximately 500,000 people annually globally (Aparcio, 2013). It is endemic in many parts of world including India, in developing country like India this disease remains highly neglected but it is endemic and important zoonoses (Aparcio, 2013; Blasco and Molina-Flores, 2011 and Kumar, 2010). The disease is responsible for heavy economic losses due to abortions, still birth and infertility. Economic losses due to Brucellosis in India have been estimated USD 3.4 Million annually (Singh *et al.*, 2015). Brucellosis in sheep and goat is primarily caused by *Brucella melitensis*, while in cattle the primary causative agent is *B. abortus* (Aparcio, 2013). The most reliable and gold standard methods for diagnosis and confirmation of the disease is bacterial isolation and identification (Chand *et al.*, 2012). Further its molecular characterization by PCR from culture leads to confirmation and species level characterization of the causative agents (Chand *et al.*, 2012).

Present paper report storm of abortions caused by *Brucella melitensis* its culture and molecular confirmation in sheep flocks from a village of Udaipur district of Rajasthan. The results contribute to better understanding of epidemiological distribution of *Brucella melitensis* and are important in development of prevention and control strategies. In hilly tract of Arawali region farmers have close association with sheep and goats, with it these sheep and goat share pasture land of village with other livestock. So the infected sheep may act as a potential source of infection to human beings as well as other livestock.

To the best of our knowledge, present paper is a primary report of culture, isolation and molecular characterization of *Brucella melitensis* from storms of abortion in sheep flocks from Southern Rajasthan.

Materials and Methods

Ethical approval: Sample collection and disease diagnosis is part of routine work of regional disease diagnostic centre Department of animal Husbandry Udaipur, hence no ethical approval required

Description of village, animal husbandry and the sheep flock

Village Mading Pura of Bhinder tehsil involved in this study is located about 60 KM away from Udaipur city of Rajasthan state. The flocks under consideration were owned by two persons, Mr. Mangu Lal and Mr. Sambhu Lal Gadri of Mading Pura village. These two flocks consist of 55 and 60 sheep of Sonadi breed respectively out of which 26 male and rest were female.

The total number of sheep in the village is 160. These sheep are maintained by open grazing method with one time a day concentrate (barley) feeding. These sheep share pasture with cattle and buffalo of the village. The poor country men keep small flock up to 60 sheep, for their bread and butter, animals are sold for meat purpose and wool is sheared two times in a year. In this way these sheep are moving bank for the farmers.

History of abortions

First abortion was observed in the month of August 2015 followed by a storm of abortions and 15 abortions were recorded by owners. All these abortions were followed by retention of placenta and metritis. In this way, the abortions continued and owner reported at regional disease diagnostic center, (RDDC) department of animal husbandry (DAH), Udaipur, Rajasthan. A team of Assistant professors from department of veterinary microbiology, veterinary pathology, College

of veterinary and animal science Navania, Udaipur and Veterinary officers from RDDC visited the village and samples were collected.

Collection and handling of blood samples

Approximately 7 ml venous blood sample was collected from sheep having history of abortion and from all adult male sheep in flock (total 26). The blood samples were allowed to clot for 15-20 minutes and were brought at department of Veterinary Microbiology, C.V.A.S Navania, on ice for serological testing. Serum was separated by centrifugation at 3000 RPM for 10 minutes. Sera samples were stored at - 20 degree centigrade till further use.

Collection and handling of vaginal swab and fetal abomasal content

Vaginal swabs were collected from recently aborted sheep. Fetal abomasal content and heart blood were collected by postmortem of available fetus. And milk samples collected from all sheep aborted in recent past (in Aug and Sept 2015). Detail of sample shown in table 1.

Serological test

For serological testing Rose Bengal Plate Agglutination Test (RBPT), was performed as per protocol supplied by manufacturer (IVRI BP Lab UP, INDIA).

Bacterial culture

All samples were sent to Department of Animal Biotechnology and Microbiology laboratory, College of Veterinary Sciences and Animal Husbandry (A center of Network project on Brucellosis), Dantiwada Gujarat for culture and isolation. Samples were streaked on Brucella selective agar (Himedia cat. No. M 0074), supplemented with horse serum

(Himedia cat. No. RM 1239-100ml) antibiotic and anti-fungal (Ready mix of Himedia cat. No. FD 005) Containing per ml Bacitracin 25µg, polymixin 5 IU; Cyclohexamide 100µg and Nalidixic acid 5µg and nystatin 100 µg. and inoculated into Brucella selective broth tubes

The inoculated plate and broth tubes were incubated in Maclintose anaerobic cultivation Jar with candle light in it to improve CO₂ concentration and one set incubated aerobically. After five days incubation, broth tubes had fungal contamination and hence discarded after autoclaving. Petri plate had smooth bluish colonies with translucent moist and 2-4 mm in diameter were considered suspected colonies.

Primary characterization was carried out by catalase test, oxidase test and modified ZN staining as described (Quinn *et al.*, 2000). Positive colonies were transferred to glycerol BHI broth and stored at -20 degree centigrade till further use. These suspected cultures were used for molecular characterization by PCR methods.

Molecular characterization

DNA extraction – DNA extraction was carried out using DNAeasy blood and tissue kit (Qiagen cat. no. 69540 and 69506) as per the standard protocol provided by the manufacturer, described here briefly – One ml of Broth culture was centrifuged at 300g and re-suspended in 200 µl PBS and proteinase K 20 µl was added. To it 200µl buffer Al mix was added and mixed thoroughly by vortexing and 200 µl ethanol (96%V/V) was added and was mixed thoroughly by vortexing. Mixture was transferred to DNase mini spin column and placed in a 2 ml collection tube it was centrifuged at 6000 g for 1 min and flow was discarded. The spin column was placed in a new 2 ml collection tube and 500µl Buffer

(AW1) was added to it and centrifuged at 6000 g for 1 min and flow was discarded. The spin column was placed in a new 2 ml collection tube and 500 µl Buffer (AW2) was added and centrifuged for 3 minutes at 20,000 g. The spin column was Transferred to new 2 ml micro centrifuge tube and DNA was eluted by adding 200 µl buffer (AE) to the center of the spin column membrane. It was incubated for one minute at room temperature and centrifuged at 6000 g.

Molecular characterization and identification of *Brucella* genus was first done using BCSP31 gene specific oligonucleotide primer pair in real time PCR with SYBR Green chemistry (Baily *et al.*, 1992). The reaction mixture as well as PCR cycle conditions follows as per Table 2. This reaction showed melting value (T_m) 87⁰ C directly matches with positive control. Then again *Brucella* genus level molecular characterization was carried out by real time PCR for conformation on extracted genomic DNA with Taqman probe chemistry with IS711 gene (Newby *et al.*, 2003) and this reaction mixture as well as PCR cycle conditions according to Table 3.

Amplification and fluorescence detection were done in real time PCR (Applied Biosystem 7500 Real time PCR system) with samples, positive and negative control included. When the cycle threshold (CT) values were evaluated as positive 18.75, 19.10 and 20 as per DNA concentration of samples in this real time PCR was designed for detection of genus level characterization as it has genus specific probe only.

Brucella species confirmation has been carried out on all three isolates found positive in real time PCR analysis using Bruce ladder multiplex PCR assay, selected primer as per described (Gracia-yoldi *et al.*, 2006) and PCR reaction mixture as well as thermal cycle conditions were provided as per Table 4, after

than run PCR product in 2% gel electrophoresis for observation of amplified product.

Results and Discussion

Outbreak of Brucellosis has been confirmed by serological tests, culture and isolation. Three strains of *Brucella melitensis* were isolated and confirmed by PCR methods. Study show the occurrence of *Brucella melitensis* in the area which is of major zoonotic concern.

Bacterial isolation

A total three isolates were obtained from Twenty eight samples collected and confirmed as *Brucella melitensis*. Two isolate were obtained from vaginal swabs while one isolate was from fetal abomasal content. Isolates could not be recovered from milk/colostrum.

Identification of *Brucella melitensis* isolates

All *Brucella* strains were identified as *Brucella melitensis* by biochemical characteristics i.e. Modified ZN positive, No CO₂ requirements for growth and oxidase positive. Twenty six out of 115 sheep were found positive in RBPT. Three isolate of *Brucella melitensis* were obtained from sample collected. All isolates have typical colonial appearance viz. translucent bluish smooth colonies. With modified ZN positive staining and Catalase and Oxidase test positive.

Real time PCR and Bruce ladder PCR for confirmation

Further Genus level identification was carried out by Real time PCR and species level identification carried out by Bruce ladder multiplex PCR as described and confirmed as *Brucella melitensis* further for biotyping the

culture were sent to Indian Veterinary Research Institute U.P. India.

Storm of abortions due to brucellosis leads to rapid spread of pathogen among animals and humans so it needs immediate attention. Storm of abortions in cattle as well as sheep and goat have been reported by many workers from all over the world (Blasco and Molina-Flores, 2011; Chand *et al.*, 2012; Chahota *et al.*, 2003; Sharma *et al.*, 2008 and Al-Garadi *et al.*, 2011). Many studies of sero-prevalence indicate the occurrence of brucellosis in India and abroad (Chahota *et al.*, 2003; Kumar *et al.*, 1997; Lone *et al.*, 2013; Suryawanshi *et al.*, 2014; Mantur and Amarnath, 2008; Mugizi *et al.*, 2015). Sero-prevalence reports from Rajasthan indicate the presence of the disease (Leyla *et al.*, 2003). But there is not a single report of culture confirmation of *B. melitensis* from Southern Rajasthan.

In our study we observed 15 abortions in two flocks of 115 sheep. We could obtain Three *Brucella melitensis* isolates, two from vaginal swabs and one from fetal abomasal contents. Isolation of *Brucella melitensis* form vaginal

swab is in accordance of above researchers from India (Chand *et al.*, 2012; Sharma *et al.*, 2008) and abroad (Sharma *et al.*, 2008; Al-Garadi *et al.*, 2011). It was also observed that vaginal swab samples were relatively easy to obtain and process for bacterial isolation in laboratory. whereas *Brucella melitensis* culture from Fetal abomasal content was not reported by above cited researchers (except Chand P. *et al.*, who reported two isolate in 2004 and one isolate in 2006 and 23) fetal abomasal content is most reliable source for pure culture of etiological agent from abortion cases (Sonawane *et al.*, 2011). So obtaining pure culture from fetal abomasal content is significant. Though excretion of *Brucella* from milk is well documented and reported (Chand *et al.*, 2012). But we could not recover any isolate from milk/colostrum. This might be due to variation in culture media we used and/or centrifugation procedure described elsewhere was not used by us (Chand *et al.*, 2012). Orchitis and epididymitis have been reported in affected Ram (Chand *et al.*, 2012). But in our study we examined more than 20 Rams and could not find Orchititis/epididymitis in any Ram of affected flock.

Table.1 Animals and samples detail

Sex	Group	Number of animal examined	Number of animal positive by serological test	Culture	
				Sample collected	Positive
			RBPT		
Male	Adult	14	1	Nil	Nil
	Sub-adult	12	Negative	Nil	Nil
	Lamb	12	Negative	Nil	Nil
Female	Adult	43	25	14	3
	Sub-adult	34	Negative	Nil	Nil
	Lamb	-	Negative	Nil	Nil
Total		115	26	14	3

Table.2 PCR reaction mixtures and cycle condition for SYBR Green chemistry with B4/B5 primer pair

Genus specific primers :					
Sr. No	Primer	Forward/ Reverse	Sequence (5'-3')	Product size (bp)	Reference
1.	B4 (<i>BCSP31</i>)	Forward	TGG CTC GGT TGC CAA TAT CAA	223 bp	(7)
2.	B5 (<i>BCSP31</i>)	Reverse	CGC GCT TGC CTT TCA GGT CTG		
PCR reaction mixture					
Sr. No	Components		Aliquot(μl)		
1.	PCR Master Mix (2X)		12.5 μl		
2.	Forward Primer (10 pmol/ul)		1 μl		
3.	Reverse Primer (10 pmol/ul)		1 μl		
4.	Template DNA		2 μl		
5.	Nuclease free water		8.5 μl		
Conditions of thermal cycling primer pair B4/B5					
Steps	Temperature		Duration	Number of cycle	
Initial Denaturation	93°C		5 min.	1 cycles	
Denaturation	95°C		30min.	40 cycles	
Annealing	55°C		30 sec.		
Extension	72°C		30 min.		
Melt curve condition	95°C		15 sec	1hr	
	60° C		15 sec		

Table.3 TaqMan probe based Real Time PCR for Molecular characterization of Brucella

TaqMan probe based Real Time PCR (8)			
Sr. No.	Components	Aliquot(μl)	
1.	Universal PCR Master Mix without UNG (2X)	12 μl	
2.	Forward Primer (10 pmol/ μl)	1 μl	
3.	Reverse Primer (10 pmol/ μl)	1 μl	
4.	Template DNA	2 μl	
5.	Nuclease free water	5 μl	
6.	50X IPC DNA	0.5 μl	
7.	10X IPC mixture	2.5 μl	
8.	Probe(20pmol/ μl)	1 μl	
Thermal cycle condition for probe based assay			
Steps	Temperature	Duration	Number of cycle
Pre holding stage	95°C	10 min.	1 cycles
Cycling stage	95°C	30sec	40 cycles
	60°C	50sec	
Post PCR read	60°C	1 min.	1 cycles

Table.4 PCR reaction mixture and thermal cycle condition for Bruce ladder multiplex PCR

Bruce ladder (multiplex PCR) for species identification			
Sr. No.	Components	Aliquot(µl)	
1.	PCR Master Mix (2X)	15 µl	
2.	Primer Mix (6.25Pmol/µl)	2µl	
3	Template DNA	2 µl	
4	Nuclease free water	10 µl	
5	10mg/ml MgCl2	1 µl	
Bruce ladder thermal cycle condition			
Steps	Temperature	Duration	Number of cycle
Initial Denaturation	95°C	5 min.	1 cycles
Denaturation	90°C	1 min.	35 cycles
Annealing	60°C	1min	
Extension	72°C	2 min.	
Final extension	72°C	10 min.	1 cycles

Adopting a new methodology after primary characterization by preliminary tests viz Modified ZN, Oxidase positive colonies were isolated and subjected to real time PCR. All three isolates were confirmed using BCSP31 gene specific oligonucleotide primer pair in real time PCR with SYBR Green chemistry (Baily *et al.*, 1992). To clear the doubt in results it was further confirmed using Taqman probe chemistry with IS711 gene (Newby *et al.*, 2003). This method not only reduces the handling of most zoonotic strain of *Brucella* in laboratory but also reduce the chance of aerosol formation in the environment. Confirmation of *Brucella melitensis* species was carried out by Bruce ladder PCR as described.

All examined sera samples were found positive in Rose Bangle Plate agglutination Test seroprevalence study conducted by many researchers from India (Chahota *et al.*, 2003; Kumar *et al.*, 1997; Lone *et al.*, 2013; Suryawanshi *et al.*, 2014; Mantur and Amarnath, 2008; Mugizi *et al.*, 2015) but culture confirmation is very scanty (Chand *et al.*, 2012; Sharma *et al.*, 2008). Culture confirmation and molecular characterization

of *Brucella* spp. documented well abroad (Al-Garadi *et al.*, 2011, Sonawane *et al.*, 2011). And a few report of seroprevalence brucellosis from Rajasthan (Leyla *et al.*, 2003), however up to our best knowledge there is not a single report of culture, isolation and molecular confirmation of *Brucella melitensis* by real time PCR from Rajasthan. This is a major hurdle in determining the species and biovar prevalent in the area. Isolation and molecular characterization up to species level of the pathogen plays a major role in determining the prevention and control strategy. Successful isolation and species level molecular characterization of *Brucella melitensis* is therefore very significant. Zoonotic importance of *Brucella melitensis* is well documented (Renukaradhya *et al.*, 2002; Smits and Manzoor, 2005). In our study both the owners of the flock were having history of orchitis and infertility. It also indicates the zoonotic potential of the organism isolated from the flock. The situation needs immediate prevention and control intervention. The reports of investigation were handed over to Department of Animal Husbandry, Udaipur Rajasthan for necessary preventive and therapeutic measures.

Source of infection in the flock although could not be ascertain yet sero-prevalence report of brucellosis in semiarid Rajasthan (Sonawane *et al.*, 2011) give a clue regarding spread of the disease. Sero-positive animal though not abort but spread the organism at the time of parturition (Renukaradhya *et al.*, 2002) so previously infected animal may act as potential carrier throughout its life (Smits and Manzoor, 2005). This spread might be due to migratory sheep. Because these migratory flocks have tract of movement from desert of Thar (arid and semiarid Rajasthan) to Madhya Pradesh state and back annually. In between these sheep share common pasture of native sheep and they stay there for few days. So these migratory sheep may be a source of infection in the area. For further clarification, study about current status of brucellosis in migratory sheep flock needed.

Conclusion: Primary report of isolation and molecular characterization of *Brucella melitensis* from abortion storm among sheep flocks from Udaipur district of Rajasthan.

Twenty six out of 115 sheep were found positive in RBPT. Three isolate of *Brucella melitensis* were obtained from sample collected. All isolates have typical colonial appearance viz. translucent bluish smooth colonies.

With modified ZN positive staining and Catalase and Oxidase test positive. Genus level identification was carried out by Real time PCR and species level identification carried out by Bruce ladder multiplex PCR as described. And confirmed as *Brucella melitensis* further for biotyping the culture were sent to Indian Veterinary Research Institute U.P. India.

Competing interests

Authors have no competing interests.

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