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Isolation, Identification and Molecular Detection of *Brucella* spp., in Cattle and Buffaloes

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A B S T R A C T

The present study was carried out for the isolation, identification and molecular characterization of *Brucella* species. A total of 50 samples were collected from cattle and buffalo suffering from abortions and other reproductive disorders in and around Ludhiana, Punjab. Out of the 50 samples of fetal stomach contents (25), uterine discharges (10), vaginal mucus (8) and placenta (7) processed for isolation of *Brucella* of which a total of four isolate were obtained and identified biochemically. All the 4 isolates were typed as biotype 1. DNA was extracted from the solates and subjected to PCR using B4/B5 primer pair. All the isolates were positive by PCR and an amplicon size of 223bp was obtained.

Introduction

Brucellosis is a highly contagious and important zoonotic disease caused by *Brucella* spp. They are small, gram negative, non-motile, non-spore forming, facultative intracellular coccobacilli.

Transmission of the organism occurs mainly through contact with placenta, fetus, fetal fluids and vaginal discharges from an infected animal. Clinically, the disease is characterized by abortion in the third trimester of pregnancy. Infections may also cause stillborn or weak calves, retained placentas, reduced milk yield and orchitis and epididymitis in males. Confirmation in clinically affected animals is done by isolation and identification of the organism from aborted foetus, foetal membranes and vaginal mucus.

Current paper deals with isolation, identification and molecular detection of *Brucella* isolated from samples of aborted foetuses and maternal contents.

Materials and Methods

Samples (n=50) comprising foetal stomach contents, uterine discharges, vaginal mucus, placenta from cattle and buffalo suffering from abortions and other reproductive disorders in and around Ludhiana, Punjab were collected.

Isolation and identification

The samples were inoculated on BSM (*Brucella* specific medium). The inoculated plates were incubated microaerophilically at 37°C under 5-10% CO_2 for up to 3-5 days and were observed for growth. The isolates were identified based on the morphology, culture characteristics, various biochemical tests like oxidase, catalase, H_2S production, urease, nitrate reduction and indole, growth in the presence of dyes i.e. thionin ad basic fuchsin.

Molecular detection

DNA was extracted from the isolates obtained using hot cold lysis method. Confirmation of the *Brucella* isolates was done by genus specific PCR primers B4/B5 (Baily *et al.,* 1992) (Table 1). The contents and conditions of PCR are given in Table 2 & 3 respectively.

Results and Discussion

Out of the 50 samples collected from cattle (27) and buffalo (23), 4(8%) isolates of *Brucella* were obtained. The details of isolation are shown in the table 4. The isolates were detected on the basis of morphological and cultural characteristics. *Brucella* colonies were translucent, round, pinpoint, smooth, glistening, 1–2 mm in diameter, with smooth margins.

On Gram's staining, the isolates were identified as Gram negative, coccobacilli or rods whereas by MZN staining they appeared to be red with blue background. They were non-motile and did not show growth on McConkey's lactose agar (MLA).

All the four isolates were found positive for catalase, oxidase, urease, H_2S production and nitrate reduction test whereas all the isolates were negative for indole test (Table 5) and typed as biotype 1. The extracted DNA subjected to PCR revealed an Amplicon size of 223 bp in positive control as well as in all the four isolates (Fig. 1).

Out of the 50 samples collected 4(8%) isolates of *Brucella* were obtained. The current study is in agreement with earlier findings which reported 4% to 8% overall isolation rate of *Brucella* spp., (Ghodasara, 2008; Kanani, 2007) However, in contrast to these findings, isolation rate between 20% and 39% has been reported by Das *et al.,* 1990 and Pal and Jain, 1985. All isolates were oxidase and catalase positive as in corroboration with observations of Shome *et al.,* (1999) whereas, Piccininno *et al.,* (1978) identified one *B. abortus* that it was oxidase negative.

The four isolates of *B. abortus* grew in the presence of basic fuchsin (20μg/ml) but not in the presence of thionin (20μg/ml) and hence were typed as biotype 1. The results are in accordance with the findings of Shahzad *et al.,* (2014) who detected *B. abortus* biovar 1 from all the 30 isolates obtained. In contrary, Verma *et al.,* (2000) isolated *B. abortus* biotype 3 from two of seven aborted cows. Holstein Friesian, crossbreds and indigenous breeds of cattle and mixed and Murrah breeds of buffaloes in the age groups of 4-6 and 7-9 were taken into study of which Indigenous cow breed and Murrah breed of buffaloes were found to be most susceptible to brucellosis. Brucellosis in animals in the age group of 4-6 yrs was found to be more prevalent.

DNA was extracted from reference *B. abortus* S19 and from the suspected *Brucella* isolates.

The extracted DNA was subjected to PCR using *Brucella* genus specific primer pair B4/B5 targeting *bcsp*31 gene (Baily *et al.,* 1992). Amplicon size of 223 bp was detected in positive control as well as in all the four isolates (Fig. 1).

Table.1 Sequence of primers used for detection of genus *Brucella*

Name of the primers	Gene	Sequence (5'-3')	Size of the amplified product	Reference
B4 (F)	*bcsp*31	TGG CTC GGT TGC CAA TAT CAA	**223 bp**	Baily *et al* (1992)
B5 (R)		CGC GCT TGC CTT TCA GGT CTG		

Table.2 *Brucella* PCR reaction mixture for B4/B5 primer pair

S. No.	PCR components	Required concentration	Amount (µl)
1	H_2O (PCR grade)	Up to 25 µl	14.3
2	PCR Buffer (10X)	1X	2.5
3	$MgCl_2$ (25 mM)	1.5 mM	1.5
4	dNTPs (10 mM)	200µM	0.5
5	Primers (40pmol/µl each)	20 pmol each	0.5+0.5
6	*Taq* (5U/µl)	1 U	0.2
7	DNA template	~100 ng	5.0
8	**Total volume**		**25**

Table.3 *Brucella* PCR program by using B4/B5 primer pair

Stage	Step	Temperature (°C)	Duration	No. of cycles
1	Initial denaturation	94	5 min	1
2	Denaturation	94	60s	35
	Annealing	65	60s	
3	Extension	72	60s	
	Final extension	72	10 min	1

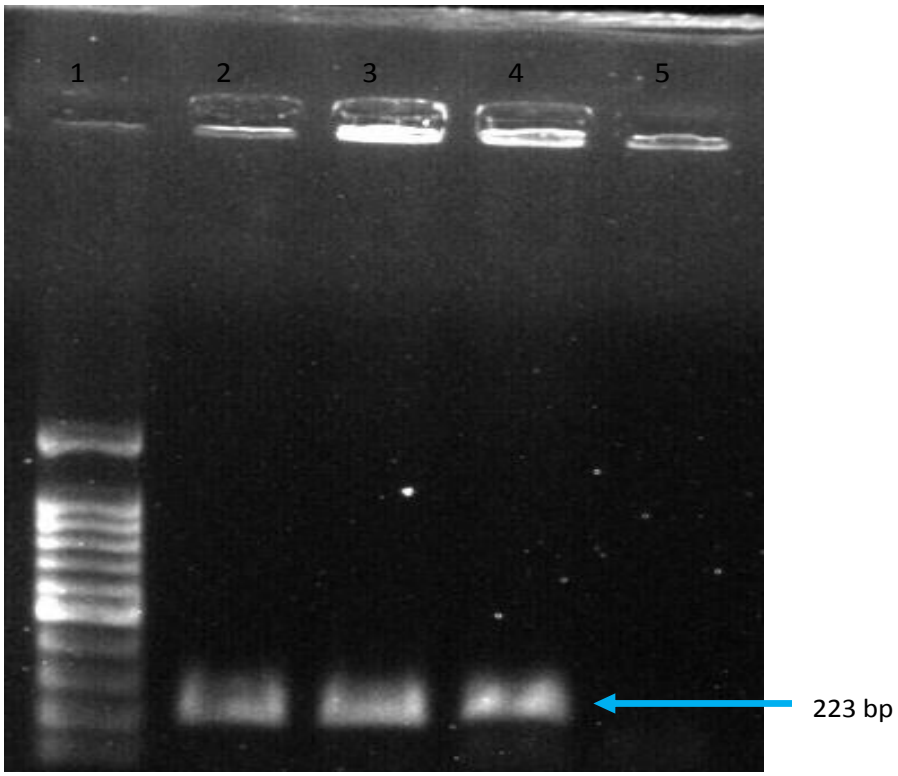
Table.4 Isolation of *Brucella* from different samples

Type of Sample	Cattle		Buffaloes	
	No. of samples processed	No. of samples positive for isolation	No. of samples processed	No. of samples positive for isolation
Foetal stomach content	11	02	14	01
Uterine discharge	7	-	3	-
Vaginal mucus and Vaginal discharge	5	-	3	-
Placenta	4	1	3	-
Total	**27**	**03**	**23**	**01**

Table.5 Biochemical characterization of *Brucella* spp.

S. No	Isolate no	Oxidase	Catalase	H_2S (TSI)	Urease	Nitrate reduction	Indole	Agglutination with antiserum	Growth in the presence of dyes		
									Thionin 20µg/ml	Basic fuschin 20µg/ml	Biotype
1	P1	+	+	+	+	+	_	+	_	+	1
2	P2	+	+	+	+	+	_	+	_	+	1
3	P3	+	+	+	+	+	_	+	_	+	1
4	P4	+	+	+	+	+	-	+	-	+	1

Fig.1 Gel electrophoresis of PCR amplified fragments from *Brucella* isolates using B4/B5 primer pair



Lane 1: DNA Ladder
Lane 2 & 3: *Brucella* field isolates
Lane 4: Positive control
Lane 5: Negative control

Kanani (2007) compared three pairs of primers amplifying three different fragments, a gene encoding a 31 kDa *B. abortus* antigen (primer B4/B5), a sequence 16S rRNA of *B. abortus* (primer F4/R2) and a gene encoding an *omp*2 (primer JPF/JPR) by testing 101 semen samples and found that B4/B5 primer pair was more sensitive followed by F4/R2 primer and JPF/JPR primer pair. Similarly, Jung *et al.*, (1998) carried out detection of *Brucella* by using *bcsp31* gene based B4/B5 primer. Navarro *et al.*, (2002) and Varasada (2003) used same primer pair for diagnosis of human brucellosis.

Based on the study conducted, foetal stomach content was found to be the best sample for isolation of *Brucella* spp. Indigenous breed of cattle and Murrah breed of buffaloes in the age group of 4-6 yrs were found to be most susceptible to brucellosis. Molecular detection based methods were found to be equally sensitive to isolation.

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