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Occurrence of Abortion Causing Organisms in Cattle and Buffaloes in Punjab Region and their Characterization

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A B S T R A C T

A total of 39 samples taken from 36 abortion cases of cows and buffaloes in the regions in and around Ludhiana were examined microbiologically for isolation of commonly prevalent specific bacteria causing abortions. Out of these, only 4 isolates belonging to economically important bacterial genera were obtained. Three of these isolates belonged to *Brucella* spp. and 1 isolate was of *Listeria* spp. Rest of the samples yielded non-specific bacteria like *E. coli*, *Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. etc. The isolates were obtained from animals which aborted in the last trimester of gestation. The *Brucella* spp. was isolated from foetal stomach contents and placenta (two from foetal stomach contents and one from placenta) and *Listeria* spp. was isolated from a placental sample. It is also concluded from this work that placenta and foetal stomach contents are the best specimens for isolation of *Brucella* spp. and *Listeria* spp.

Introduction

India is a known goldmine for its animal and livestock resources. It has the best germplasm for buffaloes and a rich gene pool for cattle; both in quantity and quality. A lot of means and measures have been employed to maintain and also increase the productivity of this sector, but diseases, especially production diseases, create a major obstruction. Reproductive disorders make up a major part of losses in production and most important among them are abortions. Abortions in cattle and buffaloes have a varied etiology, ranging from mechanical to infectious to nutritional causes. But infectious causes are of great concern and importance as far as increased incidences of infections are considered. Microbial infections, particularly the bacterial ones, have been major causes of abortions. Economically important bacteria which cause abortions and are prevalent in India are *Brucella* spp., *Listeria* spp., *Leptospira* spp. and *Campylobacter* spp. *Brucella* are Gram-negative, coccobacillary, non-motile, Modified Ziehl-Neelsen positive (stain red) organisms which cause abortion in last trimester of gestation. These are obligate parasites which have predilection for placenta of ungulates, foetal fluids and tissues and testes of bulls, rams, boars and dogs. *Listeria* are Gram-positive, coccobacillary, motile organisms which cause abortion in last trimester. A lot of methods and techniques

have been employed to detect the prevalence and to confirm the isolates of these organisms. The most commonly employed technique is Polymerase Chain Reaction (PCR) and its variants targeting specific gene sequences. These are being further explored in the form of Real-time PCR, Nested PCR etc.

Materials and Methods

Sample collection

A total of 39 samples (11 foetal stomach contents, 18 uterine discharges, 8 placenta and placental fluids and 2 vaginal secretions) were taken from 36 animals (21 cows and 15 buffaloes) (as given in Table 1) which suffered from abortion at different stages of gestation and were screened for the causative bacteria. All these samples were taken from cases coming in GADVASU clinical complex and from farms in and around Ludhiana. Samples were brought to Department of Veterinary Microbiology, GADVASU by keeping on ice and were processed immediately. If processing was delayed, the samples were kept at 4˚C.

Sample processing

The discharges and fluids were directly used for culture. In cases of tissue samples i.e. placenta, samples were triturated in sterilized Normal Saline Solution (NSS) aseptically using a pestle and mortar. The triturate was collected in a 15ml centrifuge tube, centrifuged at 500xg for 5 minutes. The supernatant was collected and was used for inoculation on Brain Heart Infusion (BHI) agar. For samples suspected for Brucellosis or Campylobacteriosis, Brucella Specific Medium (BSM) with supplements and antibiotics was used. For growth of aerobic or facultatively anaerobic bacteria, BHI plates were incubated at 37˚C under aerobic conditions for 24-48 hours. For capnophillic bacteria like *Brucella*, incubation was done for 48-72 hours in a candle jar at 37˚C to provide 5-10% CO_2 for growth. After incubation, the plates were checked for growth and their colony characters were studied. The colonies were stained with Gram's staining technique. Microscopic morphology of the bacteria was observed. Other specific staining technique like Modified Ziehl-Neelsen staining was also performed for specific organisms like *Brucella*. Staining and visualization was followed by a series of biochemical tests as given by Quinn *et al.,* (1994). For colonies suspected of *Brucella* spp., catalase, oxidase, indole, urease, hydrogen sulphide production and nitrate reduction tests were done and their results were observed and recorded. For colonies suspected of *Listeria* spp., catalase, oxidase, haemolysis on blood agar, CAMP test (with *Staphylococcus aureus*), nitrate reduction and sugar fermentation tests for Mannitol, Rhamnose and Xylose were carried out. For *Brucella* and *Listeria* isolates, additional molecular test i.e. PCR was applied for confirmation. DNA extraction was done by hot-cold lysis method (Dashti *et al.,* 2009). The PCR assay was carried out by using following primer pairs for *Brucella* spp. as shown in Table 2.

PCR Reaction mixture for *Brucella* spp. included 2.5 µl of (10X) PCR buffer, 0.5µl(20pmol/µl) of each primer, 1.5µl of (1.5mM) $MgCl_2$, 0.2µl of (1U) *Taq* DNA polymerase, 0.5µl of (200µM) dNTPs, 14.3µl of PCR grade water and 5µl of template DNA. The PCR conditions included an initial denaturation at 94°C for 5 min, followed by 35 cycles each of 94°C for 1 min (denaturation), 65°C for 1 min (annealing) and 72°C for 1 min (extension), and a final extension of 72°C for 10 minutes. *Brucella abortus* S19 strain culture available in the Department of Veterinary Microbiology, GADVASU, Ludhiana was used as reference strain for molecular work (positive control).

PCR assay was also carried out for *Listeria* spp. for the primer pair as mentioned in Table 3.

PCR reaction mixture (for one reaction) to confirm *Listeria* spp. was prepared in a volume of 25µl containing 12.5 µl master mix, 6.5µl nuclease free water, 0.5 µl (20pmol/µl) of each primer and 5µl of template DNA. PCR cycling conditions included initial denaturation at 94°C for 1 min, followed by 35 cycles each of 94°C for 30 seconds (denaturation), 53°C for 45 seconds (annealing) and 72°C for 45 seconds (extension), and a final extension of 72°C for 2 min.

Standard culture of *Listeria* spp. available in School of Public Health and Zoonoses, GADVASU, Ludhiana was used as reference strain for molecular work (positive control).The PCR products were analyzed using 1% agarose gel with 0.05µg/µl of ethidium bromide. It was electrophoresed at 90 volts/cm for 1 hour. Gene Ruler ladder plus 100 bp (MBI, Fermentas) was run for visualizing and comparing the relative molecular weight.

Results and Discussion

Out of total 39 abortion samples collected, 4 isolates of bacteria commonly and specifically involved in abortion were obtained. 3 samples were identified as *Brucella* spp. and 1 sample as *Listeria* spp. on the basis of colonial morphology and biochemical characteristics as given in Tables 3 and 4. All the *Brucella* isolates were Gram-negative organisms with coccobacillary structure. They were positive for nitrate reduction and production of hydrogen sulphide gas (on Triple Sugar Iron agar slant), oxidase and catalase enzymes. All the isolates were urease positive (Christensen's urea agar slope turned pink within 2 hours) and were negative for indole production too. The observations were in corroboration with the findings of Shareef (2006) and Abbas and Talei (2010). Kaur (2015) reported four isolates of *B. abortus* obtained from 100 reproductive samples (one from vaginal mucus, one from uterine discharge and two from foetal stomach contents).

Ramanatha and Gopal (1992), Batra *et al., (*1995), Chatterjee *et al., (*1995), Jeyaprakash *et al., (*1999), Shareef (2006) and Gift *et al.,* (2009) also reported *B. abortus* to show similar biochemical characteristics which are typical of *B. abortus*. One (1) isolate of *Listeria* obtained showed up as Gram- positive and coccobacillary bacteria. Upon being subjected to biochemical tests, the isolate gave positive reactions for catalase and CAMP test and showed β-haemolysis on blood agar.

It gave negative reactions for oxidase test and nitrate reduction test. Carbohydrate utilization tests revealed that the bacteria used Rhamnose for its growth but Mannitol and Xylose were not utilized. A worker Gasanov *et al.,* (2005) reported that suspected bacteria are usually classified as *Listeria* if they display characteristics like Gram-positive rods, aerobic and facultatively anaerobic, non-spore forming, catalase-positive, oxidase-negative and fermentative in sugars. These findings were also recorded for the *Listeria* isolate obtained in this study.

On performing PCR assays, 3 isolates of *Brucella* spp. and 1 isolate of *Listeria* spp. were confirmed molecularly (Figure 1 and 2). The reference DNA used in the study gave positive amplification band of 223 bp and 370 bp for *Brucella* spp. and *Listeria* spp. respectively. This suggests that the PCR assay used was specific and sensitive for the targeted organism and could be used further to detect these organisms in different samples from infected animals.

Table.1 Sample collection and distribution among cows and buffaloes

Type of sample collected	Cow	Buffalo
Foetal stomach contents	06	05
Uterine discharge	09	09
Placenta/placental fluid	06	02
Vaginal secretions	02	00

Table.2 Primer sequence used for PCR of *Brucella* spp.

Name of the primers	Gene	Sequence (5'-3')	Size of the amplified product	Reference
B4 (F)	*bcsp31*	TGG CTC GGT TGC CAA TAT CAA	223 bp	Baily *et al.*, (1992)
B5 (R)		CGC GCT TGC CTT TCA GGT CTG		

Table.3 Primer pair used for PCR of *Listeria* spp.

Name of the primers	Gene	Sequence (5'-3')	Size of the amplified product	Reference
F	*prs*	GCT GAA GAG ATT GCG AAA GAA G	370 bp	Doumith *et al.*, (2004)
R		CAA AGA AAC CTT GGA TTT GCG G		

Table.4 Biochemical tests employed for *Brucella* spp.

	S. No.	Oxidase	Catalase	H_2S (TSI)	Urease	Nitrate reduction	Indole
	B1	+	+	+	+	+	_
	B2	+	+	+	+	+	_
	B3	+	+	+	+	+	_

TSI-Triple Sugar Iron, H_2S-Hydrogen sulphide

Table.5 Biochemical tests employed for *Listeria* spp.

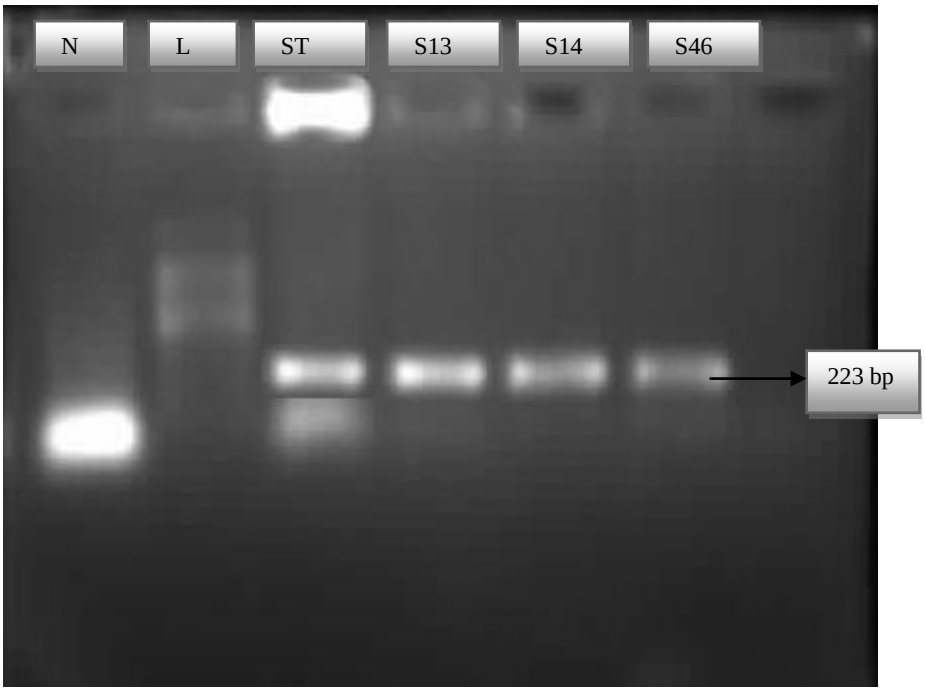
S. no.	Catalase	Oxidase	Haemolysis	CAMP test (*S. aureus*)	Fermentation tests			Nitrate reduction
					Ma	Rh	Xy	
L1	+ve	-ve	+ ve (β-haemolysis)	+ ve	-ve	+ve	-ve	-ve

CAMP test- Christie, Atkins, and Munch-Peterson test; Ma –Mannitol, Rh-Rhamnose, Xy-Xylose

Table.6 Type of samples from which *Brucella* spp. and *Listeria* spp. have been isolated

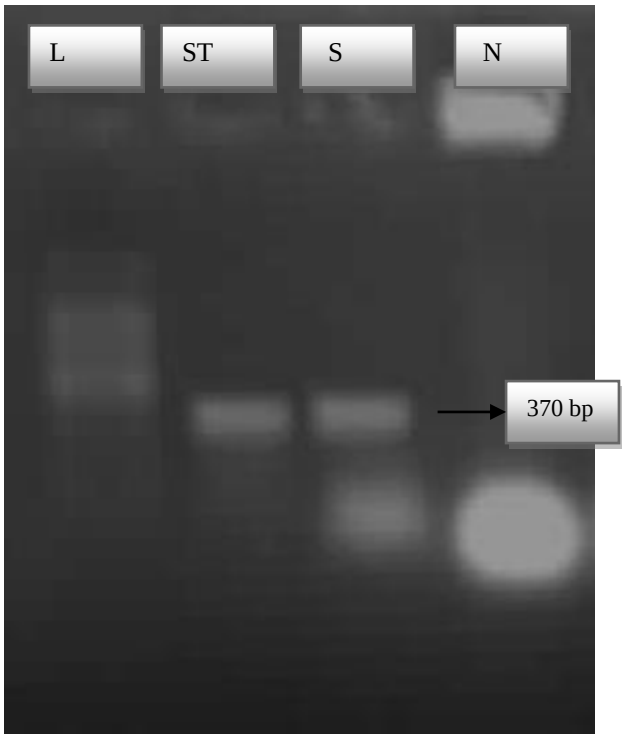
Type of sample collected	Brucella	Listeria
Foetal stomach contents	02	00
Uterine discharge	00	00
Placenta/placental fluid	00	01
Vaginal secretions	00	00

Fig.1 PCR assay of *Brucella* spp. showing confirmed isolates



Lane L – Ladder; Lane N – Negative control; Lane ST – Standard *Brucella*S19 strain; Lane S13, S 14 & S46 – *Brucella* isolates

Fig.2 PCR assay of *Listeria* spp. showing confirmed isolate



Lane L – Ladder; Lane N – Negative control; Lane ST – Standard for *Listeria* spp.; Lane S28 – *Listeria* isolate

Yu and Nielsen (2010) reported that PCR-based methods are more useful and practical than conventional methods used to identify *Brucella spp*. Another worker Cerekci *et al.,* (2011) compared multiplex real-time-polymerase chain reaction (M-RT-PCR) and conventional biotyping for the differentiation of three Brucella species. The isolates were identified at genus level by conventional microbiological methods and classified using the classical Brucella species biotyping scheme based on CO2 requirement for growth, urease activity, H2S production, sensitivity to basic fuchsin and thionin (20 and 40 µg/ml), lysis by Tbilisi and Berkeley phages, and agglutination with monospecific antisera for A and M antigens. For the identification of Brucella spp., the primers and probes which targeted the bcsp31 gene were used. It was stated that the M-RT-PCR assay could be a valuable tool for the rapid detection and differentiation of Brucella species in clinical samples which usually have very low bacterial load. Table 5 shows the type of samples which yielded the isolates.

Kaur (2015) also reported that foetal stomach content was found to be the best sample for isolation of *B. abortus*. Aborted foetus is one of the best samples to isolate *Brucella* from cattle and buffaloes (Nielsen and Duncan 1990) (Table 6). Šteingolde *et al.,* (2013) reported 44 cases among 186 total cases of cattle abortion. All abortion cases were observed in the second and the third trimester of the gestation which is alike the present study where *Listeria* was also isolated from a case of abortion at 8 months.

4 isolates of economically important and specific abortion causing bacteria were obtained. Three of these isolates belonged to *Brucella* spp. and one to *Listeria* spp. Foetal stomach contents and placenta were found to be the best samples for isolation of *Brucella* spp.

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