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ISOLATION, BIOTYPING AND ANTIBIOGRAM EVALUATION OF *Brucella abortus* IN CATTLE AND BUFFALOES FROM PUNJAB (INDIA)

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ABSTRACT

The present study was based on the isolation, identification and biochemical characterization of *Brucella* species, the causal pathogen of brucellosis, from the samples taken from cattle and buffaloes in Punjab (India). Out of the 84 samples of vaginal mucus (35), foetal stomach content (31), foetal membranes (11) and uterine discharges (7), collected from aborted cattle (29) and buffaloes (55), 9 (10.7%) were positive through isolation. Of these 9 isolates, one isolate was typed as biotype 2, one as biotype 3 and seven as biotype 1. Prevalence of *Brucella abortus* through isolation in buffaloes and cattle was 10.9 and 10.3%, respectively. Maximum number of *B. abortus* isolations in cattle (25.9%) and buffaloes (41.2%) were obtained from the animals in age group of 4-6 years. All the brucellosis positive animals had history of abortion in 6-9th month of gestation. Antimicrobial sensitivity revealed that *B. abortus* was sensitive to doxycycline, oxytetracyclin, ampicillin, gentamicin, enrofloxacin, streptomycin, neomycin, chloramphenicol, norfloxacin and kanamycin but resistant to penicillin, cephalothin, cotrimoxazole and erythromycin.

Key words: Antimicrobial sensitivity, biotypes, *Brucella abortus*, brucellosis, buffalo, cattle

INTRODUCTION

Bovine brucellosis is major economic threat, caused by *Brucella abortus*. There is no specific treatment against brucellosis and the affected animals need to be culled. But in India due to religious belief slaughtering of cattle is prohibited therefore segregation of animals is opted. Segregated animals are again a major source of infection. The epidemiological survey of disease in India was done by Dhand *et al.* (2005) and isolation of *Brucella* species done by Aulakh *et al.* (2008) in Punjab, but due to the changes in global environment and variation in the geographical distribution of organism, there is need to isolate and identify the pattern of sensitivity in Punjab (India). The conventional tests *viz.*, rose-Bengal plate test, serum tube agglutination test, complement fixation test and enzyme immunoassay are commonly used in the diagnosis of brucellosis. Their diagnostic values may be questionable on individual basis, because of cross-reacting antibodies but screening herds with any of these tests remain ideal. Isolation of causative organism is "gold standard" for confirmatory diagnosis of brucellosis. It has the advantage of detecting the organisms directly. The present study was aimed at the isolation, biotyping and antibiogram evaluation of *Brucella abortus* in cattle and buffaloe of Punjab (India).

MATERIALS AND METHODS

Vaginal mucus, placenta, cotyledons and foetal stomach contents of aborting cattle and buffaloes (84 samples) were collected from different farms in and around Ludhiana, Punjab. The samples were inoculated on selective brain heart infusion (BHI) agar (Himedia) medium which contained 7-10%

defebrinated sheep blood, 100 mg L^{-1} of cycloheximide (fungistat), 6,000 units L^{-1} of polymyxin B (active against Gram -ve bacteria), 10 mg L^{-1} of vancomycin (active against Gram +ve bacteria) and 5 mg L^{-1} of trimethoprim (active against *Proteus* species) (Quinn *et al.*, 1994). For maintenance of culture, solid *Brucella* agar base supplemented with 1% *Brucella* selective medium was used.

Vaginal mucus samples were diluted with equal volume of PBS (pH 7.2) and inoculated on selective media for isolation. The samples of placenta were washed in PBS and flamed to sterilize the surface followed by homogenization in a pestle and mortar, and homogenate inoculated on cultural medium. The samples of foetal stomach contents and uterine discharges were inoculated directly onto selective cultural medium. The plates were incubated at 37°C under microaerophilic environment in candle jar for 3-5 days. The colonies were checked for their morphology, staining pattern, slide agglutination with anti-*Brucella abortus* (IVRI, Izzatnagar) serum, urease, catalase and oxidase tests for the identification of *B. abortus*.

The biotyping was done by incorporating the dyes thionine (blue) and basic fuchsin (red) separately in trypticase soya agar @ 20 µg ml^{-1} each (Quinn *et al*., 1994). The medium was prepared by heating 0.1% solution of either dye in boiling water bath for 20 min. and then adding it to the required amount of autoclaved agar. The dye was mixed with agar and poured onto petriplates. A sterile swab was used to inoculate dye medium with a suspension of test strain. Two cultures were tested per plate, plates incubated at 37ºC for 3-5 days and examined for growth. The antimicrobial susceptibility test was performed using Mueller-Hinton agar plates supplemented with 5% sheep blood serum. Colonies (3-4) were incubated in tryptone soya broth overnight for moderate turbidity (10^5-10^6 cells ml^{-1}), checked by McFurland nephrometer, inoculated on Mueller-Hinton agar and incubated for 3-4 days. Antimicrobial agents (Himedia) *viz.*, doxycycline, oxytetracyclin, enrofloxacin, penicillin, neomycin, amoxicillin, ampicillin, streptomycin, gentamicin, cephalothin, chloramphenicol, kanamycin, erythromycin and cotrimoxazole were used. All media and supplements were procured and prepared as per the manufacturer's recommendations (HiMedia, Mumbai).

RESULTS AND DISCUSSION

In present study, of the 84 samples of foetal stomach contents, foetal membranes and vaginal mucus processed for the isolation of *Brucella* species, nine (10.7%) were positive. All the animals positive for brucellosis aborted at 6 to 9th month of gestation. In buffaloes, of the 20 foetal stomach contents, *B. abortus* was isolated from 4 samples. Out of 19 samples of vaginal mucus and 6 uterine discharges, one was positive in both samples. Similarly in cattle, of the 11 foetal stomach contents and one uterine discharge two and one were positive for *B. abortus,* respectively (Fig. 1). None of the foetal membrane samples from cattle and buffaloes were positive. Prevalence of brucellosis in cattle and buffaloes was 10.3 and 10.9%, respectively (Table 1). Prevalence of *B. abortus* in relation to animal age was categorized into three groups (Table 2; Fig. 2). In less than 4 years age group, out of 8 cattle one was positive for *Brucella* whereas out of 13 buffaloes one was positive. In 4-6 year age group, out of 15 cattle two were positive and out of 24 buffaloes four were positive. Thus of the 84 samples (55 samples from buffaloes and 29 from cattle) prevalence of *B. abortus* in <4 years age-group of cattle was higher (12.5%) than buffaloes (7.6%) while in 4-6 years age-group the prevalence was higher in buffaloes (16.6%) than in cattle (13.3%). In 6-8 year age-group out of six cattle one was positive whereas of 18 the buffaloes three were positive. Serological evidences are suggestive of high endemicity of brucellosis in India with national average as high as 5.0% in cattle, 3.0% in buffalo, 7.9% in sheep and 2.2% in goat (Renukaradhya *et al.*, 2002). In Punjab the incidence of brucellosis in buffalo and cattle have been reported to be 13.4% and 9.9%, respectively (Dhand *et al.*, 2005) while in 2008 the disease prevalence in buffalo and cattle increased to 16.4 and 20.7%, respectively, with overall apparent prevalence of brucellosis 18.2% (Aulakh, 2008). Mehra *et al.* (2000) found no difference in the prevalence of brucellosis with respect to age whereas Kubuafor *et al.* (2000) found a significant increase in brucellosis with age. Mathias *et al.* (1998) found prevalence rate of brucellosis around 18.6% in cattle and buffaloes aged 6 years or more. Tome *et al.* (1987) found that out of 568 cattle of

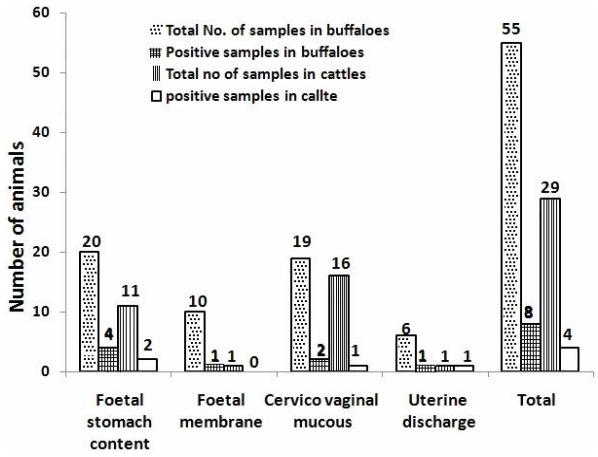

Fig. 1: Prevalence of *Brucella abortus* in different samples in cattle and buffaloes

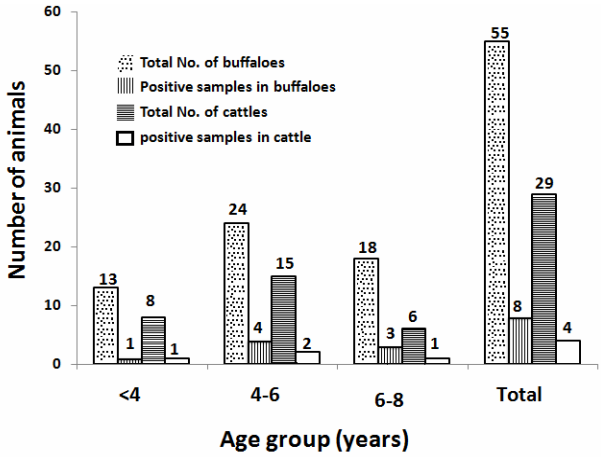

Fig. 2: Prevalence of Brucellosis in different age groups of cattle and buffaloes

Table 1: Prevalence of *B. abortus* in different samples from cattle and buffaloes

Type of sample	Cattle		Buffalo	
	No. of animals	No. of isolates	No. of animals	No. of isolates
Foetal abomasal contents	11	2 (18.2)	20	4 (20.0)
Foetal membranes	1	0 (00.0)	10	1 (10.0)
Cervico-vaginal mucus	16	0 (00.0)	19	1 (05.2)
Uterine discharge	1	1 (100)	6	0 (00.0)
Total	29	3 (10.3)	55	6 (10.9)

over 24 months of age, 20.6% were positive for brucellosis. Fosgate *et al.* (2002) found that median age for *B. abortus* positive cattle and buffaloes was five years.

The isolates were confirmed by Gram staining, modified Ziehl Neelsen staining and by biochemical tests. Out of 9 isolates, only one isolate did not produce H_2S by lead acetate strip method. Low success in isolation may be due to slow growing fastidious nature of the organism (Alton *et al.*, 1975). In addition there are higher number of commensal bacteria and low number of *Brucella* in foetal membranes and vaginal mucus samples, which made isolations difficult.

B. abortus isolates were biotyped on the basis of growth in presence of dyes. *B. abortus* isolates were grown in presence of basic fuchsin (20 µg ml^{-1}) and thionine (20 µg ml^{-1}) and grouped into biotype 3. The isolates which did not grow in presence of basic fuchsin and thionine were grouped into biotype 2 whereas the isolates which grew in presence of basic fuchsin but not in presence of thionine were grouped into biotype 1. One (11.1%) *B. abortus* isolate was typed as biotype 2, one (11.1%) as biotype 3 and 7 (77.7%) as biotype 1. Megid *et al.* (2005) successfully isolated *B. abortus* biotype 1, biotype 2 and biotype 3 from aborted cattle and buffalo fetuses. Ocholi *et al.* (2004) identified 25 isolates and all of them were typed as *B. abortus* biotype 1 whereas Rathore *et al.* (2002) identified 96.9% isolates as *B. abortus* biotype 1. Out of 7 aborted cows Verma *et al.* (2000) isolated *B. abortus* biotype 3 from two. All the isolates were found positive by agglutination test using standard antiserum (IVRI, Izzatnagar).

Table 2: Prevalence of *B. abortus* with relation to age

Age group (years)	Cattle		Buffalo	
	No. of animals	No. of isolates	No. of animals	No. of isolates
< 4	8	1 (12.5)	13	1 (07.6)
4 – 6	15	2 (13.3)	24	4 (16.6)
6 – 8	6	1 (16.6)	18	3 (16.6)
Total	29	4 (13.7)	55	8 (14.5)

Table 3: Zone of inhibition of antibiotics against *Brucella abortus*

Antibiotics	Zone of inhibition (mm)	Sensitivity pattern
Doxycycline	28.0	100.0
Oxytetracyclin	26.0	100.0
Ampicillin	23.5	88.8
Gentamicin	22.0	88.8
Enrofloxacin	22.0	88.8
Streptomycin	20.0	77.7
Neomycin	21.0	77.7
Chloramphenicol	19.5	77.7
Kanamycin	21.0	66.6
Amoxicillin	21.0	44.4
Erythromycin	10.0	33.3
Cotrimoxazole	07.0	22.2
Cephalothin	05.5	22.2
Penicillin	0.0	0.0

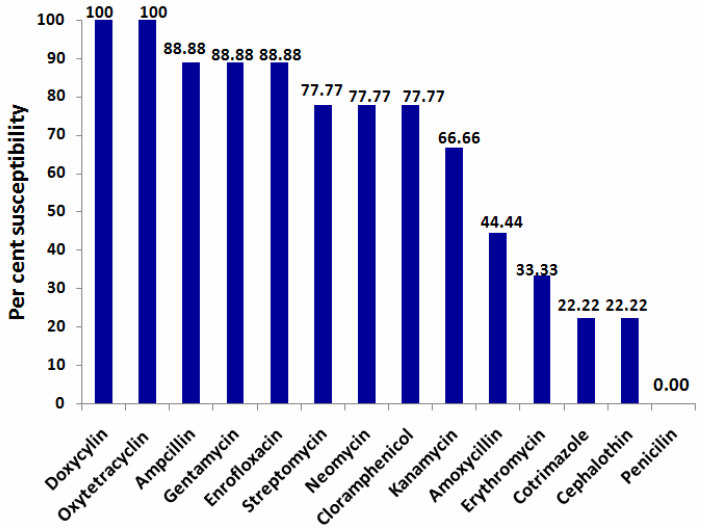

Fig. 3: Sensitivity of antimicrobial agents against *B. abortus*

B. abortus was highly sensitive for doxycyclin and oxytetracyclin and sensitivity gradually decreased for ampicillin, gentamicin, enrofloxacin, streptomycin, neomycin and chloramphenicol; and showed resistance to penicillin (Fig. 3, Table 3). Jeyaprakash *et al.* (1999) observed that *B. abortus* was resistant to penicillin and highly sensitive to chloramphenicol, streptomycin and cotrimoxazole whereas Mzizi and Madsen (1994) found that all 24 strains of *B. abortus* sensitive to ampicillin, cotrimoxazole and chloramphenicol and all but one strain resistant to cephalothin. Three strains were sensitive to tetracyclin. Baykam *et al.* (2004) determined MIC_{50} and MIC_{90} values of doxycycline, rifampicin, ciprofloxacin, trimethoprim-sulphamethoxazole and ceftriaxone for 42 blood isolates of *Brucella* species and reported that doxycycline had the lowest and rifampicin the highest MIC_{50} values.

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