



Prevalence of bovine brucellosis in a dairy herd, Uttarakhand, India

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Brucellosis is a disease of livestock, caused by host adapted species of Gram-negative intracellular bacteria of the genus *Brucella* (Mantur *et al.* 2007). It is associated with lower milk yields (20–25%, ILRI 2012), in India it causes economic losses of Rs. 350 million (PD-ADMAS 2012). A considerable high prevalence (17% to 22.18%) of bovine brucellosis has been reported in Indian dairy herds (Chahota *et al.* 2003, Trangadia *et al.* 2009). Shedding of *Brucella* in milk presents a potential threat to the new-born calves (Vassalos *et al.* 2009). Collection of milk and its use instead of blood represents an important advantage in sampling from lactating animals. Diagnostic tests such as MRT and ELISA are widely used for the detection of anti-*Brucella* antibody in milk. MRT is the most extensively used test for the screening and monitoring of brucellosis in dairy cattle (Alton *et al.* 1988).

Isolation of the causative agent is the most accepted tool for confirmatory diagnosis but, it is time-consuming and has reduced sensitivity in chronic infection. Handling of suspected clinical samples will require the containment level for group 3 pathogens (OIE 2012). Molecular approaches are more precise and can be used reliably for detection and characterization of organisms without cultivation. In addition, it is more sensitive than traditional culture (Amin *et al.* 2001) and serological (Cortez *et al.* 2001) method. It has been applied to detect *Brucella* DNA in varieties of clinical samples viz. aborted fetuses, maternal tissues, blood, milk and semen (Cortez *et al.* 2001).

Aim of the present study was to detect *Brucella* antibodies in milk using both MRT and ELISA. Conserved 16S rRNA of *Brucella* was also targeted in DNA isolated from milk in simple PCR format. Milk samples were collected from the lactating cattle having the history of abortion ever in past

nine year. The study was carried out to evaluate the reason behind the decreased milk production and abortion.

A total of 246 (158 Crossbred and 88 Indigenous Sahiwal breed) milk samples from lactating cattle were collected from Organized Dairy Farm, Pantnagar, India. Approximately, 15 ml milk was collected in duplicate; one set was used for detection of antibodies (MRT and ELISA) and another for antigen detection (PCR). The Abortus Bang Ring Antigen and AniGen *B. Brucella* Ab ELISA kit (i-ELISA or Milk-ELISA) were procured from IVRI, Izatnagar (India) and Bionote, Korea, respectively.

Milk ring test: MRT was performed on individual milk samples according to the method described in OIE (2012). Dark pink ring above the white milk column was taken as positive whereas pink colour of the underlying milk exceeds that of the cream layer as negative.

Indirect enzyme linked immunosorbent assay: Milk-ELISA was performed by using AniGen *B. Brucella* Ab ELISA kit. Method mentioned in manual provided with kit was followed. Positive and negative samples were determined based on Percent positivity (% P) value. The samples given % P value ≥ 15 were taken as positive whereas samples with $< 15\%$ P value as negative. It was calculated as follows:

$$\% P = \frac{\text{OD of milk samples}}{\text{Average OD of standard positive control milk}} \times 100$$

Polymerase chain reaction: Specific DNA sequence of 905 bp, which belong to 16S rRNA of *Brucella abortus* (Dorsch *et al.* 1989) was targeted for amplification. Oligonucleotides (F4, 5'-TCGAGCGCCCGCAAGGGG-3' and R2, 5'-AACCATAGTGTCTCCACTAA-3') required for amplification of earlier said sequence were taken from published literature (Romero *et al.* 1995). Method described for DNA extraction from milk (Romero and Lopez-Goni 1999) and PCR (Romero *et al.* 1995) were followed as mentioned in their respective literatures.

Various studies have been conducted in India to establish the prevalence of the brucellosis in bovines (Sandhu *et al.* 2001, Singh *et al.* 2004, Sharma *et al.* 2007) but these studies

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were based mainly on non-random sampling techniques. Hence the information gathered from such surveys cannot be extrapolated to apply to state or national bovine populations.

Prevalence: The periodical surveillance of brucellosis in dairy herd using MRT for pooled milk and ELISA for random or herd screening has been suggested (PD-ADMAS 2012). In the present study, overall 23.98% and 28.04% of milk samples were positive for brucellosis respectively, by MRT and milk-ELISA (Table 1). High proportion of *Brucella* positive animal was anticipated to samples collection, as samples belongs to animal having history of suspected infection. In similar study (Punjab, India), 22.47% and 11.86% animals were found positive by Avidin-biotin milk-ELISA and MRT, respectively (Gumber *et al.* 2004). Almost half of the cattle (private and government farm) screened in Egypt were brucellosis positive by both MRT and ELISA (Ibrahim *et al.* 2012). However, Kangethe *et al.* (2000) recorded the overall prevalence in Kenya at the consumer level were 4.9% and 3.9% by milk-ELISA and MRT respectively, whereas at the informal market level, it was 2.4% and 3.4% respectively. Similarly, in Quetta, 3% cattle and 8.5% buffaloes were found positive for brucellosis in MRT and i-ELISA, respectively (Shafee *et al.* 2011).

Brucellosis is widely prevalent throughout the world, particularly among dairy cattle (Radostits *et al.* 2007). It is generally higher among dairy cattle than range cattle due to the intensive farming practices being followed (Langomi *et al.* 2000). The milk-ELISA is a not only sensitive and specific test, but also able to distinguish between infected and

vaccinated animals (OIE 2012). It is highly correlated with MRT (Bonfoh *et al.* 2002), even in organized and unorganized farms (Chand *et al.* 2004) and eliminates false-positive MRT samples (Boraker *et al.* 1981).

Among the breed, significantly ($p < 0.05$) higher proportion of crossbred cattle (28.48% and 33.54%) under study were positive for brucellosis in comparison to indigenous breed (15.91% and 18.18%), respectively by MRT and milk-ELISA (Table 1). Exotic germplasm in crossbred cattle make vulnerable to pathogen and hence likely to suffer from disease (Aulakh *et al.* 2008) in comparison to local breed (Kiputa *et al.* 2008).

Brucellosis is a disease of the sexually mature animals, its predilection site is the reproductive tract, especially the gravid uterus. Allantoic factors viz. erythritol, steroid hormones and other substances stimulate the growth of most of the *Brucella* (Radolf 1994). In the present study, 33.71% and 38.20% of animals in age group 6 to <9 years age groups were positive for brucellosis followed by ≥ 9 years age group (20.24% and 22.62%) and 3 to <6 years (16.44% and 21.92%) respectively, by MRT and ELISA (Table 1). Statistically, these differences were significant ($p < 0.05$) among these age groups. Earlier different workers (Bekele *et al.* 2000, Sharma 2000, Silva *et al.* 2000, Kazi *et al.* 2005) have also reported higher proportions of positive reactors in older animals. However, Rahman *et al.* (1997) didn't observe such difference in regard to age. Susceptibility to disease seems to increase with age, but actually, it is associated with sexual maturity of animals irrespective of age (Radostits *et al.* 2007).

Molecular diagnosis: Antigenic detection in terms of DNA

Table 1. Prevalence of *Brucella* antibodies in cattle milk among breed, age and sensitivity and specificity of MRT with respect to ELISA

Breed	No. of milk samples tested	Positive samples			
		MRT	ELISA		
Crossbred	158	45 (28.48) ^a	53 (33.54) ^a		
Indigenous breed (Sahiwal)	88	14 (15.91) ^a	16 (18.18) ^a		
Total	246	59 (23.98)	69 (28.04)		
Age					
3—< 6 yrs	73	12 (16.44) ^a	16 (21.92) ^a		
6—< 9 yrs	89	30 (33.71) ^{ab}	34 (38.20) ^{ab}		
≥9 yrs	84	17 (20.24) ^b	19 (22.62) ^b		
Total	246	59 (23.98)	69 (28.04)		
Sensitivity and specificity					
Test	ELISA		Total	Sensitivity	Specificity
	Positive	Negative			
MRT	Positive	54	5	78.26%	97.17%
	Negative	187	172		
	Total	69	177		

Figures in parentheses indicate percentage, ^{a, b} Figures having same superscripts are significant at 5% level of significance for respective tests.

of *B. abortus* (Darla and Bricker 2000) or isolation of an organism itself (Bale and Kumi-Diaka 1981) is always more reliable than the antibody detection. Presence of *B. abortus* DNA in the milk of lactating cows suggests that organism is excreted in the milk (live or dead), which may be the source of infection to consumers. In the present study, 31 (12.60%) out of the 246 milk samples were given 905 bp amplicon of 16S rRNA, which is specific for *B. abortus*. Sometimes it was impossible to isolate *Brucella* from clinical samples even animals were seropositive (De *et al.* 1989). Therefore, level of infection in farm animals widely varies among different husbandry set up (Langomi *et al.* 2000, Patel 2007).

Sensitivity and specificity: Considering ELISA as a gold standard test, the sensitivity and specificity of MRT were 78.26% and 97.17%, respectively (Table 1). Sensitivity and specificity of ELISA recorded by different workers were higher than MRT (Vanzini *et al.* 2001, Rivera *et al.* 2003, Chand *et al.* 2004). MRT often gives false results, and hence its sensitivity and specificity have been found lower than ELISA (OIE 2012). However, its specificity is comparable to milk-ELISA (Gumber *et al.* 2004).

Comparison of MRT, milk-ELISA and PCR for diagnosis of brucellosis: Out of 246 cattle tested, 69 (28.05%) and 31 (12.60%) cattle were shown antibodies and *Brucella* DNA in their milk (Fig. 1), respectively. Only 29 milk-ELISA positive cattle were confirmed to excrete *Brucella* in their milk. However, DNA was also detected in two milk-ELISA negative cattle. In culture-positive animals, milk-ELISA had higher sensitivity than PCR, but agreement between these two tests was excellent (0.91) (Romero *et al.* 1995). However, in *Brucella*-free cattle, specificities of both tests were 100% (Romero *et al.* 1995). Most of the earlier studies indicate superiority of PCR technique over serological or cultural methods (Leal-Klevezas *et al.* 2000, Cortez *et al.* 2001, Gupta *et al.* 2006) but Evangelista *et al.* (2005) found that PCR was less sensitive than the serological methods.

In the present study, brucellosis is the cause of abortion in cattle, and hence decreases the milk production of dairy

cattle. Sexual maturity of animals is one of the determinants for brucellosis irrespective of age. Prevalence of brucellosis in an organized dairy herd was higher than endemic level. PCR simultaneously with MRT and/or milk-ELISA must be employed to diagnose *Brucella* infection to know the actual infection level. The PCR technique could provide a useful diagnostic tool, as it is rapid, automated, efficient, sensitive and specific. It is further required to identify strains and biotypes of *Brucella* to know the epidemiology of infection.

SUMMARY

Brucellosis is the disease of animals, which accounts significant losses to livestock industry. It has socio-economic impacts, especially in the countries in which rural income relies on livestock breeding and dairy products. Milk of infected animals presents a potential threat to the new-born calves. Reliable tests to screen the herd are Milk Ring Test (MRT) and milk-ELISA. Total 246 milk samples of cattle of an organized dairy farm were screened for brucellosis by MRT, milk-ELISA and PCR, targeted to 16S rRNA of bacterial genome. Overall 23.98% and 28.05% cattle under study were positive, respectively by MRT and milk-ELISA. Among the breed, comparatively more number of crossbred cattle (28.48% and 33.54%) were infected with *Brucella* than indigenous cattle (15.91% and 18.18%), respectively by MRT and milk-ELISA. However, analysis of samples with respect to age of animals revealed, maximum numbers of infected cases were found in age group 6 to <9 years followed by ≥9 years and 3 to <6 years, respectively by both tests. Molecular diagnosis in terms of *B. abortus* specific genome indicated, 12.60% animals screened were positive by PCR. But, most of PCR positive samples were also positive in milk-ELISA except; two samples were found negative in milk-ELISA.

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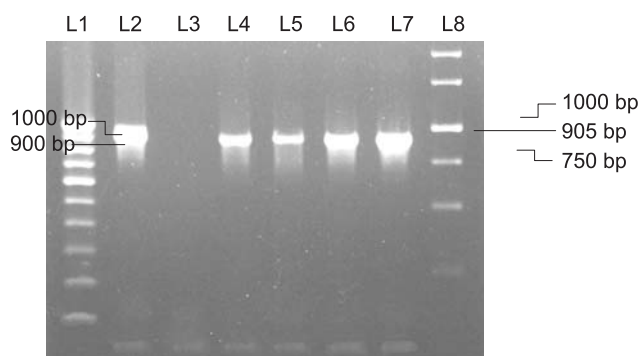


Fig. 1. Species specific amplicons of *B. abortus* 16S rRNA gene. L1, 100 bp DNA ladder; L2, Positive control, L3 Negative control; L4–L7, 905 bp amplified product from cattle milk and 8 DNA ladder 1000 bp.

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