



## Research Article

# Comparison of Milk Ring Test; Serum Plate Agglutination Test and Polymerase Chain Reaction for the Detection of Bovine Brucellosis

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### ABSTRACT

In the following study, comparative efficacy of Milk Ring Test (MRT), Serum Plate Agglutination Test (SPAT) was determined by calculating the sensitivities and specificities for detection of Brucellosis in bovines from district Peshawar of Khyber PakhtunKhwa, Pakistan. Considering Polymerase Chain Reaction (PCR) as a gold standard diagnostic test, MRT showed very low sensitivity (4.8%), while its specificity was 90.9 %, likewise, SPAT also showed low sensitivity (41 %), while its specificity was 66.7% in bovines. Statistically, high specificities and low sensitivities of MRT and SPAT in bovines recommended the poor efficacy of these tests, when used individually in contrast to PCR. As PCR is more reliable test, while considering its sensitivity for antigen detection as compare to antibody detection, therefore, PCR may be used as a routine screening test in clinical practices in farms animals for detection of Brucellosis.

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### INTRODUCTION

Brucellosis is considered the most contagious and zoonotic bacterial infection of livestock worldwide (Munir *et al.*, 2010). *Brucella abortus* and *Brucella melitensis* are the principal cause of brucellosis in bovines (Radostits *et al.*, 2000; Karaca *et al.*, 2007). These *brucella* species also cause brucellosis in humans (Gul *et al.*, 2007). In animals, brucellosis frequently results in abortion, birth of weak calves, death of young stock, infertility in males and reduced milk yield in females (Maadi *et al.*, 2011; Abubakar *et al.*, 2011).

There is neither a single diagnostic test available by which a bacterium can be identified as *Brucella* nor any signs and symptoms of brucellosis are specific, therefore, a combination of cultural, serological and molecular methods is necessary for accurate diagnosis. However, all these methods have serious limitations (Poester *et al.*, 2010; Abubakar *et al.*, 2011). Accurate diagnosis of brucellosis requires *Brucella* isolation and detection in the laboratory, which is impractical for regular screening of large populations and could not be used as criteria for control and eradication of disease. Serological tests are relatively easy to perform and provide a practical advantage in detecting the prevalence of *Brucella* infection (Abubakar *et al.*, 2011). The use of the Polymerase Chain Reaction (PCR) to identify *Brucella* DNA at genus, species and even biovar levels has becoming extended to improve diagnostic tests (Poester *et al.*, 2010; Chimana *et al.*, 2010).

In diagnostic laboratories, sera usually are screened with any simple serological test of high sensitivity. For milk samples, *Brucella* milk ring test is used for screening and monitoring the dairy herds at regular intervals (Chimana *et al.*, 2010). Rarely, the clinical samples after initial screening by conventional

methods are subjected to PCR for confirmation of disease (Abubakar *et al.*, 2011).

In the agriculture based economy of Pakistan livestock accounts for 37.5 percent of agricultural value-addition and about 9.4% of its GDP. Brucellosis infection has a considerable impact on human and animal health as well as on socioeconomic factors where rural income relies mainly on land cultivation and domestic animals farming and people usually live in very close proximity with their livestock (Maadi *et al.*, 2011; Shafee *et al.*, 2011). People practice mixed crop-livestock farms where usually breed 90% buffaloes and 10% cattle, for dairy products and milk production (Afzal and Naqvi, 2004).

In Pakistan, the incidence of brucellosis is increasing day by day, particularly in large dairy herds. Several studies have reported the high incidence rates of brucellosis in livestock farms in government and private sector in different districts and provinces of Pakistan (Abubakar *et al.*, 2010; Hamidullah *et al.*, 2009; Iftikhar *et al.*, 2008; Mukhtar and Kokab, 2008; Omer *et al.*, 2010; Rabab *et al.*, 2000; Shafee *et al.*, 2011). Brucellosis can be controlled in Pakistan by routine screening of domestic livestock and animal vaccination programs (Abubakar *et al.*, 2011). In Pakistan, veterinarians mostly rely on the conventional screening tests due to the lack of more specific diagnostic facilities and financial limitations. Rose Bengal Precipitation Test (RBPT), Serum Plate Agglutination Test (SPAT) and Milk Ring Test (MRT) are the most commonly performed tests at both government and private livestock laboratories in Pakistan (Asif *et al.*, 2009; Gul *et al.*, 2007).

This study was carried to evaluate Milk Ring Test, Serum Plate Agglutination Test and Polymerase chain reaction (PCR) for the detection of brucellosis in milk and serum samples of bovines, collected from private farms located in the district Peshawar of Khyber PakhtunKhwa region of Pakistan.

**MATERIALS AND METHODS**

In this study, 142 milk and blood samples from bovines were collected (70 cattle and 72 buffaloes) using the simple random sampling from different private farms in district Peshawar of Khyber Pakhtunkhwa region in Pakistan.

**Initial Screening Tests on Milk and Serum Samples of Bovines**

The serum samples were subjected to SPAT for screening *Brucella* antibodies as described by (Alton *et al.*, 1975). The results of agglutination were recorded. A titer of 1:80 or above was considered positive for brucella infection. Milk ring test was conducted on milk samples as described by (Alton *et al.*, 1988). The positive samples were differentiated on the basis of blue ring present on the top of milk after overnight reaction.

**DNA Extraction and PCR**

DNA was extracted from same serum and milk samples by using DNA isolation kit (Shanghai ZJ Bio- Tech Co., Ltd. China), then PCR assay was performed in a total reaction volume of 50µl (Shanghai ZJ Bio- Tech Co., Ltd. China), according to supplier’s manual. This kit contains a reaction mix for the specific amplification of *Brucella* DNA. The amplification was performed in a DNA thermal cycler (Multi-gene Labnet international Inc.USA). Initial denaturation was carried out at 94 °C for 2 minutes, and then for 35 cycles the sample DNA was denatured at 93 °C for 15 seconds, annealed at 55°C for 30 seconds, and extended at 72°C for 30 seconds. The final

incubation was done at 72 °C for 10 minutes. For positive controls, DNA extracted from *Brucella abortus* and *Brucella melitensis* strains, obtained from veterinary research institute, Peshawar was used. However, for negative control, distilled water was used. The PCR products were resolved and analyzed by using 1.5% of agarose gel electrophoresis and photographed on UV photo-documentation system (Multi-gene Labnet international Inc.USA). The clear bands of *Brucella* species DNA were considered as positive results.

**STATISTICAL ANALYSIS**

Considering PCR as a gold standard diagnostic test, relative sensitivity, specificity, positive and negative predictive values of RBPT and SPAT were calculated by using the MedCalc Software (Version 12.3.0).

**RESULTS**

The prevalence rate of Brucellosis detected in cattle and buffaloes in district Peshawar, using MRT, SPAT and PCR are given in Table 1. The overall prevalence rate detected by SPAT (38.7%) was greater than MRT (8.4%) when a total of 142 milk and serum samples of bovines were tested by MRT and SPAT. Moreover, serum samples were found more positive by PCR than milk samples. But PCR gave more positive results than MRT and less positive results than SPAT (Table 1).

Table 1: Prevalence of brucellosis in cattle and buffaloes by using MRT, SPAT and PCR

Species	No. of Milk Samples	Positive Milk Samples		No. of blood Samples	Positive Serum Samples	
		MRT	PCR		SPAT	PCR
Cattle	70	05 (7.1%)	7 (10%)	70	26 (37.1%)	18 (25.7%)
Buffaloes	72	07 (9.7%)	16 (22%)	72	29 (40.3%)	21 (29.2%)
Total	142	12 (8.4%)	23 (16%)	142	55 (38.7%)	39 (27%)

Table 2: The relation between PCR results with MRT and SPAT

Test	Results	PCR		Total	Test	Results	PCR		Total
		Positive	Negative				Positive	Negative	
MRT	Positive	01 (0.7%)	11(7.8%)	12 (8.4%)	SPAT	Positive	39(27.5%)	16(11.3%)	55(38.7%)
	Negative	20 (14.1%)	110 (77.5%)	130 (91.6%)		Negative	55(38.7%)	32(22.5%)	87(61.3%)
	Total	21	121	142		Total	94	48	142

Tests	Compared with	Sensitivity %	Specificity %	PPV %	NPV%
MRT	PCR	4.8	90.9	8.3	84.6
SPAT	PCR	41	66.7	70.9	36.8

Table 3: Sensitivity, Specificity, Positive and Negative Predictive Values of MRT and SPAT.

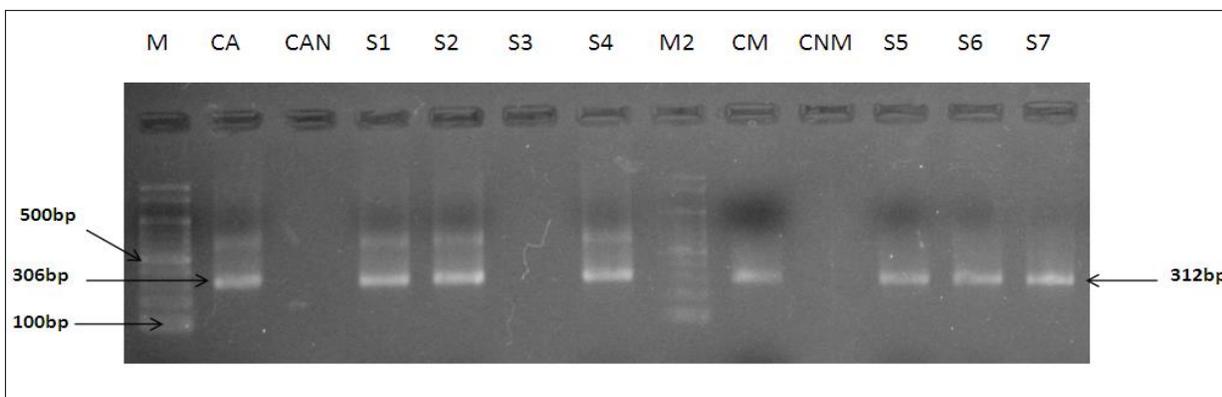


Figure 1: Electrophoresis pattern of PCR products 306 bp for brucella abortus in image on left side and 312bp for brucella melitensis in image on right side.[ M (Marker or ladder 100bp); CA (positive control for *B. abortus*); CAN (negative control for *B. abortus*); S1 (sample1 positive); S2 (sample2 positive); S3 (sample3 Negative); S4 (sample4 positive); M2 (Marker or ladder 100bp); CA (positive control for *B. melitensis*); CNM (negative control for *B. melitensis*); S5 (sample1 positive); S6 (sample2 positive); S7 (sample3 positive)].

It was also found that 0.7 % milk samples were positive to both MRT and PCR and 77.5 % milk samples were negative to both tests. Similarly, 27.5% serum samples were positive to both SPAT and PCR and 22.5 % serum samples were negative to both tests (Table 2).

Statistically, MRT and SPAT were found less sensitive and more specific in detecting *Brucella* species antibodies. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value of MRT and SPAT, when compared with PCR are given in Table 3. When PCR was used to detect *Brucella* species DNA in milk and serum samples of bovines, it gave much positive results than MRT and SPAT. PCR-amplified products of 306bp and 312bp were clearly visualized on agarose gel electrophoresis for *Brucella abortus* and *Brucella Melitensis* respectively (Figure 1).

## DISCUSSION

Brucellosis has been recognized as an important zoonotic disease as it concerned with both animal and human health. As this disease make vulnerable economic losses to the country particularly to livestock industry, therefore, definitive diagnosis means (conventional and molecular techniques) are required for its effective eradication.

Currently, veterinary diagnostic laboratories utilize Milk Ring Test for diagnosis of brucellosis in bovine milk samples, which indirectly identifies *Brucella* species in the host (Chimana *et al.*, 2010). In present study, all collected milk samples from bovines were tested by MRT and PCR for diagnosis of brucellosis. The prevalence rate of Brucellosis was 8.4% when using MRT lower than the rate obtained when PCR was used as 16% reacted positively. This difference could be attributed to the fact that the MRT test results may be false-negative when the milk samples contain small quantities of IgM and IgA antibodies, or due to deficiency of the fat clustering factors (O'Leary *et al.*, 2006). In contrast, sensitivity of PCR for detection of *Brucella* genome is extremely high as it could detect *Brucella* genome from 30fg of total DNA (Rabab *et al.*, 2000). In a study PCR was used for diagnosis of Brucellosis in bovines and showed considerable increase sensitivity as compared to MRT (Al-Mariri and Haj-Mahmoud, 2010). Similarly, in another study (Akhtar *et al.*, 2010) diagnostic efficacy of RBPT, MRT and PCR for brucellosis infection was evaluated in cattle and buffaloes. The antigenic detection of *Brucella* using PCR gave more positive results than conventional RBPT and MRT. However, in the present study when the two tests MRT and PCR were used together to evaluate the prevalence, only 0.7 % of the milk samples were positive and 77.5% were negative to both tests.

The SPAT is one of standard serological screening tests, used for the diagnosis of brucellosis (Memish and Balkhy, 2004). During this study, the prevalence rate of Brucellosis was 38.7% when using SPAT higher than the rate obtained when PCR was used, 27% reacted positively, which was contrary to that reported previously (Asif *et al.*, 2009). Moreover, serological tests like RBPT and SPAT give false-positive results due to cross-reactions with other gram negative bacteria including *Salmonella*, *E. coli*, *Yersinia enterocolitica*, *Vibrio cholerae* etc. (Al-Attas *et al.*, 2000). When the two tests SPAT and PCR were used together to evaluate the prevalence, 27.5% of the blood samples were positive in both SPAT and PCR and 22.5% were negative to both tests.

In this study, the antibodies detection of *Brucella* using MRT (8.4%) and SPAT (38.7%) was compared in milk and serum samples respectively. The SPAT test showed more positive results in serum samples as compared MRT in milk samples. This difference may be due to the fact that milk

proteins hamper the *Brucella* antibodies isolation (Akhtar *et al.*, 2010).

Statistically, the MRT showed very low sensitivity (4.8%), while its specificity was 90.9 % as compared to 70% sensitivity and 80% specificity in bovines reported earlier (Al-Mariri and Haj-Mahmoud, 2010). The SPAT also showed low sensitivity (41 %), while its specificity was 66.7% in bovines. High specificities and low sensitivities of MRT and SPAT in bovines suggested the poor efficacy of both tests used individually as compare to PCR. The PCR is currently used for the diagnosis of many diseases including Brucellosis, as PCR is more reliable and sensitive due to its ability for antigenic detection than antibody detection (Akhtar *et al.*, 2010). In present study, the calculated positive and negative predictive values of MRT and SPAT in bovines were in correspondence with their specificity and sensitivity values respectively. However, when PCR was evaluated for its diagnostic efficacy for detection of *Brucella* species in milk and serum samples, PCR gave more positive results on serum samples than on milk samples. These findings are consistent with the results and suggestions given in another study (Akhtar *et al.*, 2010). Therefore, the combination of conventional routine screening tests (MRT and SPAT) along with serum-PCR may be recommended in livestock diagnostic laboratories.

The inconsistent results in terms of the sensitivity and specificity of MRT and SPAT in bovines suggested that these tests may be used for routine screening of herds but not for confirmatory diagnosis of brucellosis in individual animals. Therefore, after the initial screening by MRT and SPAT a further confirmation through PCR is needed for accurate diagnosis of Brucellosis in livestock diagnostic laboratories. Furthermore, As PCR is more reliable test, when its sensitivity is considered for antigen detection in clinical samples than antibody detection, it may be included as a routine screening test in clinical practice in farms animals irrespective of high cost as compared to conventional tests in order to reduce economic losses in Pakistan.

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