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RESEARCH ARTICLE

Milk Indirect-ELISA and Milk Ring Test for Screening of Brucellosis in Buffaloes, Goats and Bulk Tank Milk Samples Collected from Two Districts of Punjab, Pakistan

Tayyaba Israr Khan[1], Syed Ehtisham-ul-Haque[1*], Usman Waheed[1], Iahtasham Khan[2], Muhammad Younus[3] and Shahzad Ali[4]

[1]Department of Pathobiology (Microbiology section); [2]Department of Clinical Sciences (Epidemiology and Public Health section); [3]Department of Pathobiology (Pathology section), College of Veterinary and Animal Sciences, Jhang, Pakistan; [4]Department of Wild Life and Ecology (Zoological Section), University of Veterinary and Animal Sciences (Ravi Campus) Pattoki
*Corresponding author: ehtishamsyed@uvas.edu.pk

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A B S T R A C T

Bovine and caprine brucellosis caused by *Brucella abortus* and *Brucella melitensis*, are serious zoonotic problem. In this study, the prevalence and risk factors of brucellosis were studied at herd level in two Districts of Punjab. A total of 300 milk samples from buffaloes, goats and bulk tank milk (BTM) were analyzed through milk ring test (MRT), and through a commercially available Milk Indirect ELISA (i-ELISA) kit detecting both *B. abortus* and *B. melitensis* antibodies. Higher prevalence was found in goats (76%) followed by BTM samples (42%) and buffalo samples (15%) as tested though Milk i-ELISA. Diagnostic sensitivity (DSe) and specificity (DSp) of MRT was evaluated considering Milk i-ELISA as gold standard test for diagnosis of *B. abortus* and *B. melitensis* infection. DSe and DSp of MRT for buffalo milk samples was found as 78.9% and 100% respectively. While for goat samples DSe and DSp of MRT was found as 51.7% and 100% respectively. It is concluded that, MRT is less effective for diagnosis of Brucella antibodies in goat milk as compared to buffalo. Adult age, self-reared animals and larger herd size were found as potential risk factors for the spread of brucellosis in both goat and buffalo. Whereas, use of natural insemination method was found as a risk factor in goat population only and artificial insemination in buffalo only. With the advent of corporate dairy sector in the Pakistan, it is highly recommended that routine Brucella screening should be done using Milk i-ELISA on pooled milk samples.

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INTRODUCTION

Brucellosis is the most important zoonotic and contagious disease, found in large and small animals. *Brucella* infection is considered to be the second most important zoonotic problem in the world after rabies (OIE, 2009). It causes major reproductive problems like retained fetal membranes, abortion and weak offspring in sexually developed animals (Wadood *et al.*, 2009; Shahzad *et al.*, 2017). Consumption of unpasteurized milk is an important public health hazard (Kang'ethe *et al.*, 2000; Gul *et al.*, 2015). Milk ring test (MRT) is simple and frequently used test for screening and monitoring of *Brucella* antibodies in dairy cows milk (Alton *et al*., 1988). It is a good screening test for cows but it may yield false-positive response in cattle vaccinated less than 4 months before testing, in mastitic animals and immediately after parturition (Ali *et al*., 2013; Kumar *et al.*, 2016). MRT is considered non-reliable technique for diagnosis of Brucella antibodies from sheep and goat milk (Chand *et al*., 2004; Tittarelli *et al*., 2006; OIE, 2009). MRT is also not reliable to be performed on pooled milk samples because it is unable to detect low level of antibodies efficiently (Kolar, 1984). Also, the smaller fat globuli of goat and sheep milk cream absorb agglutinated stained Brucella in positive milk sample less efficiently and do not rise to form typical ring at top (Kolar, 1984). Whereas, Milk i-ELISA can be performed on up to 250 pooled milk samples (Cadmus *et al.*, 2008). A high prevalence of brucellosis was reported among cattle (4.6%) as compared to buffaloes (1.7%) using MRT in Quetta, Pakistan (Shafee *et al*., 2011). Previously, Milk i-ELISA and serum ELISA were

compared to check the level of antibody in ewe's milk and serum, where milk ELISA was confirmed as superior than serum ELISA (Chand *et al.*, 2005). Milk i-ELISA detects IgG antibodies (Rahman *et al.*, 2013) and the primary advantage of Milk i-ELISA is screening from bulk milk samples, but the individual samples can also be tested (Funk *et al*., 2005). Several studies have been conducted in dairy cattle to look for potential risk factors for spreading brucellosis in animal. Different risk factors like age, herd size, self-reared animals and insemination methods have been previously confirmed linked with the spread of brucellosis in dairy cattle as reported by Aulakh *et al. (*2008), Abubakar *et al.* (2010) and Ali *et al.* (2017). However, no such study has been conducted to look into the situation in goats and buffalo population of Pakistan. Keeping the scenario, the present study was designed to check the prevalence of Brucella antibodies and to determine the risk factors of brucellosis in goat and buffalo. This paper describes application of Milk i-ELISA for screening of *B. abortus* and *B. melitensis* antibodies in goat, buffalo and bulk tank milk (BTM) samples collected from two Districts of Punjab (Jhang and Okara), Pakistan. The present study also describes risk factors associated with the spread of brucellosis in goat and buffalo population in the study areas.

MATERIALS AND METHODS

Study area: Milk samples were collected randomly from goats, buffaloes and milk shops as bulk tank milk (BTM) samples from two Districts of Punjab (Jhang and Okara) for screening of Brucella antibodies.

Samples and data collection: Milk samples were collected directly from all the quarters of animals in a 10ml sterile container (OIE, 2016). Total (n=300) milk samples were collected. An equal No. of milk samples (n=50) were collected from goats, buffaloes and milk shops as BTM respectively from each of the two Districts Jhang and Okara, Punjab. Animals were sampled randomly from the herds having goat breeds (Teddy & Beetle) and buffalo breeds (Nili-Ravi & Kundi). For this purpose total 30 buffalo herds and 27 goat flocks were sampled. Mastitis and colostrum milk samples were excluded in the present study which may give false positive results in MRT.

After collection, samples were transported in an icebox immediately to Microbiology Laboratory, College of Veterinary and Animal Sciences, Jhang. During sample collection, data related to animal species, breed, age, insemination method used, stock replacement, lactation stage, lactation number and herd size were recorded by circulating a pre-structured questionnaire to determine the potential risk factors associated with spread of brucellosis in goat and buffalo.

Milk samples processing: A total of 9ml whole milk sample was used for screening of Brucella antibodies through MRT. Before performing Milk i-ELISA, milk samples were centrifuged in a refrigerated centrifuge machine (HERMILE, Z 216 MK) at 2000g for 15 minutes to separate cream layer from lactoserum (OIE, 2016).

Milk ring test: Commercially available MRT antigen was procured from Veterinary Research Institute (VRI) Lahore, Pakistan. Antigen was used for initial screening of the milk samples for presence of Brucella antibodies. The MRT antigen is a stained suspension of *Brucella abortus* strain 99, which is used for Abortus Bang Ring (ABR) Test for diagnosis of brucellosis. It is dark blue colored (hematoxylin-stained) antigen to be stored at 4ºC. The MRT antigen was kept at room temperature before use, as per manufacturer's recommendation. One ml of whole milk sample was added in a test tube. Then 40µL of antigen was added, mixed and incubated at 37ºC for 1 hour. A sample having change in color at the top of milk was considered as positive (OIE, 2009; Ali *et al*., 2017).

Milk indirect ELISA (i-ELISA): The milk samples were also analyzed by Milk i-ELISA using commercially available kit (ID Screen Brucellosis Milk Indirect®, BRUMILK, IDvet, Grabels, France). The kit was used to screen the *Brucella* antibodies present in samples of goat, buffalo and bulk tank milk (BTM). Milk samples were tested undiluted after the removal of the cream layer as per manufacturer's recommendation. Briefly, samples and controls (negative and positive) were added to antigen coated plate and incubated for 45 minutes at 21°C. The plate was emptied and washed three times using wash solution. Conjugate (1x) was added to wells and incubated again. After that, 1x substrate solution was added. The plate was incubated for 15 minutes at 21°C in dark. Optical density (O.D) was recorded at 450 nm using Bio-Rad ELISA plate reader (PR-4100, USA). The sample producing S/P ratio of <45% was considered negative and with value >50% were considered positive. OD values of samples were analyzed with help of Magellan software, (Bio-Rad, USA). Diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of MRT was evaluated considering Milk i-ELISA as gold standard test for diagnosis of *B. abortus* and *B. melitensis* infection. Lactation number (1^{st}, 2^{nd}–4^{th} and $\geq 5^{th}$ lactation) wise presence of anti-Brucella antibodies in buffalo and goat milk samples was also checked through MRT and Milk i-ELISA. Results were analyzed statistically through Chi Square (Maadi *et al.*, 2011).

RESULTS

In this study 18 milk samples out of total 300 samples were found positive through MRT. This represents overall 6% prevalence of Brucella antibodies in samples collected from goat, buffalo and bulk tank milk (BTM) collected from District Jhang and Okara. Out of 300 samples, 133 (44.3%) samples were found positive through Milk i-ELISA as shown in Table 1. Higher prevalence was found in District Okara (44.6%) as compared to District Jhang (44%). In District Jhang 22.6% of goats, 4% buffaloes and 17.33% of BTM samples were found positive, while in District Okara 28.6% of goat, 6% of buffaloes and 10.6% of BTM samples from milk shops were found positive as confirmed through Milk i-ELISA.

Higher prevalence was found in goats (76%) followed by BTM (42%) and buffalo milk samples (15%). Considering Milk i-ELISA as gold standard test, the DSe and DSp of MRT for detecting Brucella antibodies in

buffalo milk samples was recorded as 78.9% and 100% respectively. While for goat samples diagnostic sensitivity and specificity of MRT was recorded as 51.7% and 100% respectively. Lactation number (1st, 2nd–4th and ≥5th) wise presence of anti-Brucella antibodies in buffalo and goat milk samples as checked through MRT and Milk i-ELISA is shown in Table 2 & 3 respectively. Data represent that, most of the animals (including buffalo and goat) in 2nd-4th lactation were found positive having Brucella antibodies. In the present study, different risk factors were studied. Adult age, self-reared animals and larger herd size were found as potential risk factors for brucellosis in both goat and buffalo. Whereas, use of natural insemination method was found as a risk factor associated in goat only and, use of artificial insemination in buffalo only. The results were statistically significant at 95% CI except for self-reared animals. Data are shown in Table 4.

Table 1: Comparison of positive samples detected through MRT and Milk i-ELISA in buffaloes, goats and milk shops (as bulk tank milk-BTM samples)

Animal Species (n=300)	MRT (No.)	Milk i-ELISA (No.)
Buffalo (n=100)	11	15
Goat (n=100)	06	76
BTM (n=100)	01	42
Total	18	133

Table 2: Lactation number wise presence of anti-Brucella antibodies in buffalo milk samples checked through MRT and Milk i-ELISA

Lactation number (Buffalo)	Positive (through MRT)		Positive (through Milk i-ELISA)	
	No.	Percentage %	No.	Percentage %
1st	0	0	1	1
2nd-4th	8	8	11	11
≥ 5th	3	3	3	3
Total	11	11	15	15

Table 3: Lactation number wise presence of anti-Brucella antibodies in goat milk samples checked through MRT and Milk i-ELISA

Lactation number (Goat)	Positive (through MRT)		Positive (through Milk i-ELISA)	
	No.	Percentage %	No.	Percentage %
1st	0	0	12	12
2nd–4th	6	6	62	62
≥5th	0	0	2	2
Total	6	6	76	76

DISCUSSION

In this study, we evaluated two screening techniques (MRT and Milk i-ELISA) for detection of antibodies against *B. abortus* and *B. melitensis* from milk of goats, buffaloes and bulk tank milk (BTM) samples. According to Guarino *et al*. (2001), milk i-ELISA is a good screening test due to its ability to detect lower level of antibodies especially in an early infection. Several studies have been conducted in dairy cattle to look for prevalence and potential risk factors for brucellosis in Pakistan as reported by Abubakar *et al*. (2012) and Ali *et al*. (2017). However, no such study has been conducted to look into the situation in goats and buffalo population of Pakistan. For this purpose, the above study was designed to determine the prevalence of Brucella antibodies and risk factors associated with the spread of brucellosis in goat and buffalo. In present study, higher prevalence was recorded in goats (76%) followed by BTM (42%) and buffalo milk samples (15%). Similar higher prevalence in goats is being reported by Hamidullah *et al*. (2009) while performing serum-based diagnosis of brucellosis in goats. In present study, the reason for high positivity of Brucella cases detected in goats is because of ability of Milk i-ELISA, to detect antibodies both against *B. abortus* and *B. melitensis*. Whereas, the MRT is less sensitive to detect *B. melitensis* infection as previously reported by Chand *et al*. (2004) and Tittarelli *et al*. (2006). Mikolon *et al*. (1998) has reported that, because of the difference between the physiologic properties of goat and cow milk, the MRT does not perform well with goat samples. Also, the low concentrations of antibodies in goat milk have been cited as a reason for the lack of sensitivity of the MRT in pooled milk samples. The milk ring test, which has been very useful in cattle, is ineffective in small ruminants. However, in large herds (>100 lactating cows), the sensitivity of the test becomes less reliable (OIE, 2009). MRT can efficiently detect antibodies against *B. abortus*; however, the major causative agent of brucellosis in goats is *B. melitensis* (Young, 2006; Blasco *et al*., 2011). Also, MRT might result from false positives, which could be due to many causes, including mastitis, colostrum, milk sample collected at the end of the lactation period, hormonal disorder, pasteurization and homogenization. In present study, the possible reason for high prevalence of Brucella antibodies in BTM is that the milk shops mix the milk from both goats and buffaloes.

In present study, a higher prevalence of anti-Brucella antibodies was found in milk samples of those animals which were in 2nd-4th lactation. Similar findings were also recorded by Mohamand *et al*. (2014). In another study, Sukumar *et al*. (2012) has reported a higher percentage of animals positive for brucellosis at 7 years age group. In present study, the lower prevalence of brucellosis in 1st lactation stage of buffalo could be due to resistance of sexually immature animals or because of passive immunization of calves through colostrum from infected dams as reported by Mohammed *et al*. (2011). Findings of present study suggest that, it is empirical to adopt Milk i-ELISA method instead of MRT in screening caprine brucellosis cases. Another advantage of screening using Milk i-ELISA can detect *Brucella abortus* or *B. melitensis* infection from a pool of up to 250 samples (OIE, 2016). The sensitivity and specificity of commercial IDvet kit (ID

Table 4: Risk factors for spread of brucellosis in goats (n=200) and buffaloes (n=200)

Risk factors		Animal species	Sample positive (No.)	Chi Square value (CI=95%)
Herd size (No.)	1-10	Goat & buffalo	29	10.14 (P=0.006)
	10-30		19	
	>30		43	
Stock replacement	Self-reared	Goat & buffalo	64	0.054 (P=0.816)
	Purchased		27	
Age	Young	Goat & buffalo	40	31.99 (P=0.000)
	Advanced age		51	
Insemination method	Natural	Buffalo	3	8.005 (P=0.018)
	Artificial Insemination + Natural		3	
	Artificial Insemination		9	

Screen Brucellosis Milk Indirect® ELISA) (used in the present study) is claimed as 100% using pool of known positive and negative sera samples as per control sheet of (ID Screen Brucellosis Milk Indirect® ELISA) kit. However, the present study reports diagnostic sensitivity and diagnostic specificity of MRT considering Milk i-ELISA as gold standard test.

In our study, for goat and buffalo, the adult age, self-reared animals and larger herd size were found as potential risk factors for spread of brucellosis. Whereas, the use of natural insemination method was found as a risk factor in goat only and AI in buffalo only. Previously, similar risk factors have been reported in dairy cows responsible for the spread of Brucella infection (Aulakh *et al*., 2008; Abubakar *et al*., 2010; Amanullah, 2015; Ali *et al.*, 2017). Arshad *et al*. (2011) has reported higher prevalence of brucellosis in goat with history of abortion while determining sero-prevalence in goats from public and private livestock farms in Pakistan. In our study, we were unable to get history of abortion because samples were also collected from goats reared by individual farmers.

The present study is the first report on screening of Brucella antibodies using Milk i-ELISA on goat, buffalo and bulk tank milk samples in Pakistan. The study proved that unpasteurized milk from Brucella infected goats, buffaloes and BTM poses serious threat to health of livestock and human population. Especially the people, who reside in District Jhang and Okara are at higher risk of getting the infection. This study added knowledge concerning current prevalence and important risk factors involve in spreading disease in goat and buffalo population in Pakistan. With the advent of corporate dairy sector in the Pakistan, it is highly recommended that routine Brucella antibodies screening of milk should be done using Milk i-ELISA. Because, Milk i-ELISA also exhibit potential to be performed on a pool of up to 250 milk samples whether from caprine or bovine species.

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Authors contribution: SE and IK designed the study and supervised the research. TK collected the samples. TK and SE executed the experiment and analyzed the milk samples. UW, MY and SA analyzed the result and interpreted the data. All authors critically read the manuscript for important intellectual contents and approved the final version.

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