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ASSESSMENT OF BRUCELLOSIS IN MILK OF COWS AND BUFFALOES USING BACTERIOLOGICAL AND SEROLOGICAL METHODS

Original Research

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Acknowledgement: The authors express sincere gratitude to the Department of Microbiology, Government College Madyan, Swat, for providing laboratory facilities and research guidance throughout the study.

Conflict of Interest: None

Grant Support & Financial Support: None

ABSTRACT

Background: Brucellosis is a zoonotic disease caused by *Brucella* species, gram-negative intracellular bacteria that primarily infect food-producing animals such as cattle and buffaloes. The infection poses a serious public health and economic threat in developing countries due to its impact on livestock productivity and its potential for human transmission through unpasteurized milk. The Milk Ring Test (MRT) serves as a rapid, cost-effective, and indirect method for detecting anti-*Brucella* antibodies in milk samples.

Objective: This study aimed to determine the frequency of brucellosis among milk samples of cows and buffaloes in Tehsil Bahrain, Swat, using serological and bacteriological methods.

Methods: The study was conducted in the Department of Microbiology, Government College Madyan, Swat, from July to December 2024 after ethical approval from the institutional research committee. A total of 160 raw milk samples were collected—100 from cows and 60 from buffaloes—from both individual animals and bulk milk tanks. Samples were aseptically collected in sterile containers, refrigerated at 4°C, and analyzed using the MRT. For each test, 1 mL of milk was mixed with 0.03 mL of hematoxylin-stained antigen and incubated at 37°C for one to three hours. The formation of a blue ring indicated a positive result. Biochemical identification of *Brucella* species was performed according to standard protocols, and data were analyzed using SPSS version 16.

Results: Out of 160 milk samples, 20 (12%) tested positive for *Brucella* antibodies. Among these, 14 (14%) were from cows and 6 (10%) from buffaloes. The predominant species isolated were *Brucella melitensis* (64.2% in cows; 66.6% in buffaloes) and *Brucella abortus* (35.7% in cows; 33.3% in buffaloes). Seasonal variation analysis revealed the highest seropositivity in December and the lowest in July, suggesting environmental influence on disease occurrence.

Conclusion: Brucellosis remains a persistent zoonotic and economic concern in Tehsil Bahrain, Swat, with higher seroprevalence observed during the rainy season. MRT proved to be a reliable and practical diagnostic method for rapid surveillance of *Brucella* infection in dairy herds, underscoring the need for routine screening and preventive measures.

Keywords: Brucella abortus, Brucella melitensis, Brucellosis, Buffaloes, Cattle, Milk Ring Test, Seroprevalence.

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INTRODUCTION

Brucellosis remains a globally significant zoonotic disease that continues to challenge both veterinary and public health sectors, particularly in developing regions. It is notably more prevalent in the Middle East, the Indian subcontinent, and countries bordering the Mediterranean Basin, despite the successful eradication efforts in many European nations, the United Kingdom, and Japan. Nonetheless, sporadic epidemics continue to emerge from endemic regions of Asia, Africa, and Central and South America, highlighting the persistent nature of this infection and the challenges in its control (1-3). The disease is caused by gram-negative bacteria of the genus *Brucella*, with four main pathogenic species responsible for human infection—*B. abortus*, *B. melitensis*, *B. suis*, and *B. canis*. Among these, *B. suis* is regarded as the most virulent, followed closely by *B. melitensis*. Remarkably, infection can occur with an inoculum as low as 10–100 organisms, underscoring the organism's high infectivity and zoonotic potential (4). In livestock, particularly in cows and buffaloes, *B. melitensis* has been identified as the predominant causative agent. Although this species was historically considered restricted to the Mediterranean region, recent epidemiological data reveal its wider prevalence across South-East Asia, suggesting shifting patterns of distribution likely linked to trade, animal movement, and inadequate disease surveillance (5). Microbiologically, *Brucella* species are small, aerobic, non-motile, non-spore-forming, gram-negative coccobacilli that exhibit slow growth and often require CO_2-enriched incubation for up to four weeks at 37°C. The addition of serum or blood enhances their in vitro growth, a feature that aids in their laboratory isolation (6). Human transmission primarily occurs through ingestion of unpasteurized milk or dairy products, consumption of undercooked meat, or direct contact with infected animal secretions. Abraded skin and mucous membranes act as common entry points, while inhalation of aerosols or exposure during obstetric procedures are also recognized occupational hazards (7,8).

Once inside the host, *Brucella* invades lymphoid tissues of the intestinal submucosa, where it is phagocytosed by polymorphonuclear leukocytes and macrophages. However, these immune cells often fail to eliminate the pathogen, allowing it to persist intracellularly. The bacteria subsequently disseminate through the lymphatic and circulatory systems to organs such as the liver, spleen, kidneys, lymph nodes, and bone marrow, leading to chronic infection (9,10). In animals, the reproductive system serves as a critical site of bacterial colonization, particularly during breeding seasons. High bacterial loads in placental and foetal fluids contribute significantly to transmission during abortion events. Infected animals can shed the bacteria for prolonged periods through milk, urine, and mucosal secretions, perpetuating the cycle of infection. Beyond its public health implications, brucellosis imposes substantial economic losses by reducing livestock productivity and restricting the trade of milk, meat, and related products on the global market (11,12). Accurate diagnosis is essential for disease surveillance and control. Among the various serological and bacteriological methods available, the Milk Ring Test (MRT) remains a widely used indirect screening technique for detecting anti-*Brucella* antibodies in milk samples, particularly in field settings (13). Despite its utility, limited implementation of effective diagnostic and control strategies continues to impede eradication efforts in several endemic regions. Given the unsatisfactory management of livestock brucellosis in Tehsil Bahrain, Swat, the present study aims to determine the prevalence of brucellosis using both serological and bacteriological methods, thereby contributing to the understanding of its epidemiology and informing future disease control strategies.

METHODS

The present study was conducted in the Department of Microbiology, Government College Madyan, Swat, Pakistan, between July 2024 and December 2024, following approval from the Institutional Research Committee. Ethical principles for animal research and sample collection were strictly followed throughout the study. Prior informed consent was obtained from the livestock owners before sample collection, ensuring voluntary participation and transparency in data handling. A total of 160 milk samples were collected, comprising 60 from buffaloes and 100 from cows. Samples were obtained from both individual animals and bulk milk tanks to ensure a representative selection from the region's dairy population. The inclusion criterion was lactating cows and buffaloes from farms or households actively engaged in milk production, while visibly diseased animals or those undergoing antibiotic treatment were excluded to avoid confounding factors that might interfere with serological results. Each sample was aseptically collected into sterile containers, labeled accurately with animal species and source information, and immediately transported to the microbiology laboratory under a controlled cold chain. The samples were stored at 4°C overnight to maintain their integrity before processing. The Milk Ring Test (MRT) was employed for the serological detection of anti-*Brucella* antibodies. For each test, 1 mL of milk was dispensed into a clean, narrow

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test tube (11 × 100 mm) and mixed with a single drop (0.03 mL) of hematoxylin-stained *Brucella* antigen. The tubes were then incubated at 37°C for one to three hours. The development of a distinct blue ring at the top of the milk column was interpreted as a positive reaction, indicating the presence of *Brucella*-specific antibodies. Biochemical identification of *Brucella* species was performed according to standardized protocols as described by Al-Mashhadany (2018), using conventional diagnostic characteristics such as oxidase, urease, and catalase activity tests, as well as CO_2 dependence and H_2S production for differentiation of species. Data obtained from the study were systematically entered and analyzed using Statistical Package for the Social Sciences (SPSS) version 16. The results were expressed as frequencies and percentages to describe the prevalence and distribution of brucellosis among the tested samples.

RESULTS

A total of 160 raw milk samples were analyzed for *Brucella* antibodies using the Milk Ring Test (MRT). Among these, 20 samples (12%) tested positive for *Brucella* antibodies, while 140 samples (88%) were negative. When compared by species, 14 out of 100 cow milk samples (14%) were seropositive, whereas 6 out of 60 buffalo milk samples (10%) were positive. Although the prevalence was higher in cows than in buffaloes, the difference was statistically insignificant ($p = 0.442$). Biochemical identification revealed that *Brucella melitensis* and *Brucella abortus* were the only species isolated from the milk samples. *B. melitensis* accounted for the majority of isolates, representing 64.2% (9/14) of the positive cow milk samples and 66.6% (4/6) of the positive buffalo milk samples. In contrast, *B. abortus* was detected in 35.7% (5/14) of cow samples and 33.3% (2/6) of buffalo samples. Overall, *B. melitensis* was found to be the dominant species in both animal types. Seasonal variation analysis showed a marked fluctuation in the prevalence of *Brucella* antibodies across the study period. The highest rate of seropositivity was observed in December, while the lowest prevalence was recorded in July, suggesting possible environmental or management-related influences on disease transmission during colder months. A chi-square analysis was performed to assess the association between animal species and *Brucella* seropositivity. Although cow milk samples demonstrated a slightly higher positivity rate (14%) compared to buffalo milk (10%), the difference was not statistically significant ($\chi^2 = 0.59$, $p = 0.442$). The odds ratio (OR) for brucellosis in cows compared to buffaloes was 1.46 (95% CI: 0.53–4.01), indicating no significant increase in risk between the two species. This suggests that both cows and buffaloes in the studied region are comparably susceptible to infection. Additionally, seasonal trends revealed a gradual increase in seropositivity from July to December, with the peak incidence observed during colder months, supporting the hypothesis that environmental factors and herd management practices may influence *Brucella* transmission dynamics.

Table 1: Brucella antibody seroprevalence in raw milk from cows and buffaloes

Milk ring test Samples		Positive N (%)	Negative N (%)	P value
Cow	100	14(14%)	86(86%)	0.442
Buffaloes	60	6(10%)	54(90%)	
Total	160	20(12%)	140(88%)	

Table 2: Isolated Brucella species distribution in studied milk samples

Brucella species	Cow milk	Buffaloes milk	P value
B. melitensis	9(64.2%)	4(66.6%)	0.442
B. abortus	5(35.7%)	2(33.3%)	
Total	14(100%)	6(100%)	

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Table 3: Association of Animal Type with Brucella Seropositivity

Animal Species	Total Samples (n)	Positive (n, %)	Negative (n, %)	χ^2 Value	p-value	Odds Ratio (95% CI)
Cow	100	14 (14.0%)	86 (86.0%)	0.59	0.442	1.46 (0.53–4.01)
Buffalo	60	6 (10.0%)	54 (90.0%)	—	—	Reference
Total	160	20 (12.5%)	140 (87.5%)	—	—	—

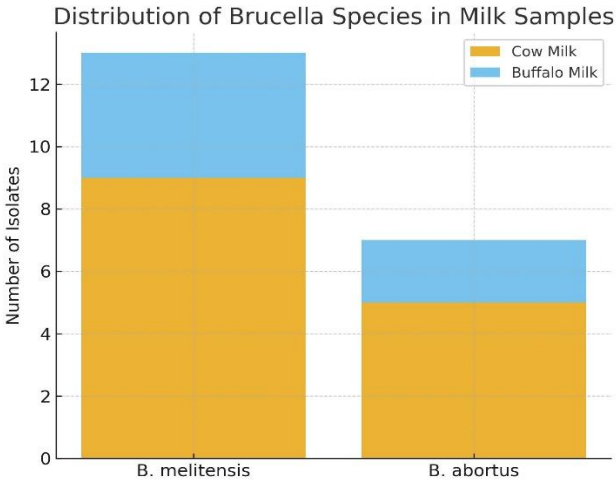

Figure 1 Distribution of Brucella Species in Milk Samples

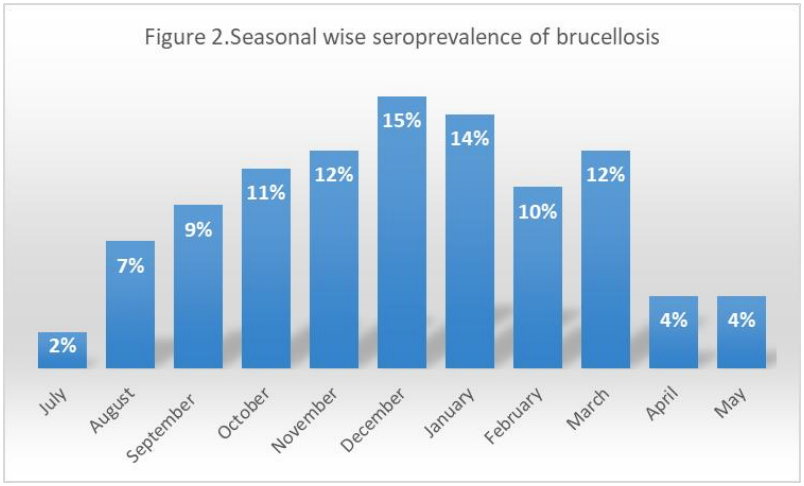

Figure 1 Seasonal Wise Seroprevalence of Brucellosis

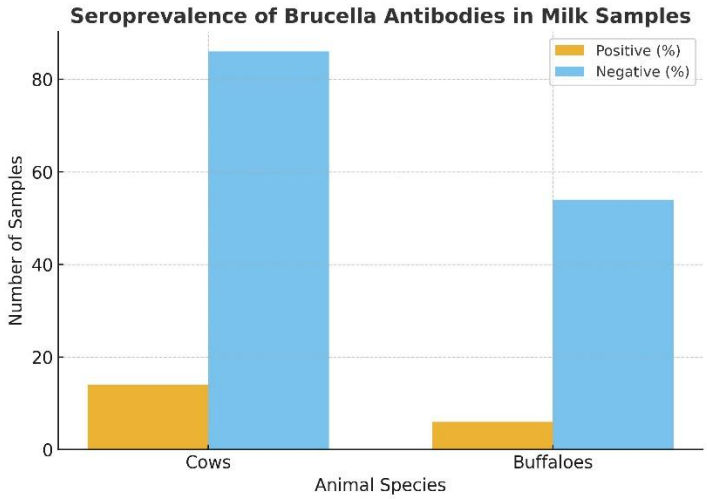

Figure 3 Seroprevalence of Brucella Antibodies in Milk Samples

DISCUSSION

Brucellosis continues to pose a significant global challenge, particularly in regions where livestock rearing constitutes a major component of the agricultural economy. The disease primarily affects food-producing animals such as cattle, buffaloes, camels, and pigs, but transmission to humans occurs frequently through the consumption of contaminated raw milk or undercooked meat (10). The World

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Health Organization recognizes brucellosis as one of the most prevalent zoonotic diseases worldwide, emphasizing its considerable public health and socioeconomic impact (11). The findings of the present study revealed a seroprevalence of 14% in cows and 10% in buffaloes, highlighting an ongoing circulation of *Brucella* infection among dairy herds in the Swat region. The observed prevalence aligns closely with findings reported from several Middle Eastern countries, including Iraq, where recent investigations using diverse serological methods have documented prevalence rates ranging from 10% to 15% (12). Such consistency suggests comparable environmental and husbandry conditions that sustain endemic transmission. In contrast, studies conducted in other regions, including India, have demonstrated substantially higher prevalence levels, reaching up to 38% (13), whereas much lower rates, such as 2.67%, have been reported in Turkey (14). These discrepancies across countries likely stem from variations in epidemiological settings, veterinary infrastructure, diagnostic capacities, vaccination strategies, and animal co-rearing practices (15). The differences underscore the influence of local herd management, biosecurity measures, and livestock–wildlife interactions on the epidemiology of brucellosis.

The present findings reaffirm the diagnostic utility of the Milk Ring Test (MRT) in field-based surveillance programs. MRT remains one of the most widely employed serological screening tools for detecting *Brucella* antibodies in raw milk and has demonstrated high sensitivity and reliability in multiple settings (16,17). The detection of both *B. abortus* and *B. melitensis* in the study further supports global evidence that these two species are the predominant etiological agents of bovine and buffalo brucellosis (18). While *B. abortus* typically infects cattle, *B. melitensis*—classically associated with sheep and goats—has increasingly been isolated from bovine and buffalo populations, particularly in mixed-farming systems where interspecies contact facilitates cross-transmission (19). The predominance of *B. melitensis* observed in the current study may therefore reflect inter-herd interactions and shared pastures between large and small ruminants. A critical epidemiological aspect highlighted in this study is the role of lactating females in disease perpetuation. Over 80% of infected females harbor *Brucella* organisms within the mammary glands and supramammary lymph nodes, leading to persistent excretion of bacteria through milk over their lifetime (20,21). This chronic shedding not only sustains herd-level transmission but also constitutes a major source of human infection through raw milk consumption. Therefore, regular screening of lactating animals and stringent pasteurization practices remain essential components of brucellosis control programs. The study also noted a seasonal trend, with higher seropositivity observed during colder months, particularly in November and December. Seasonal patterns of brucellosis have been inconsistently reported in different species, with some studies identifying the wet or breeding season as a risk factor for seropositivity, particularly among goats and camels (22,23). The observed winter peak may correspond to changes in reproductive activity, environmental stress, or animal congregation patterns during these months. However, due to the limited temporal span of the current study, which covered only six months, the observed seasonal variation should be interpreted cautiously. A more extended study encompassing all seasons would provide a more reliable understanding of temporal dynamics in brucellosis transmission.

The strengths of this study lie in its combined use of serological and biochemical methods, providing both screening and confirmatory evidence of *Brucella* infection in local dairy herds. The inclusion of samples from both cows and buffaloes allowed species-specific comparisons within the same geographic context. However, certain limitations should be acknowledged. The sample size, although adequate for preliminary estimation, limits the statistical power to detect subtle interspecies or seasonal differences. Additionally, the study relied on the MRT and biochemical identification methods, which, though practical, lack the molecular precision of polymerase chain reaction (PCR)–based assays. Absence of data on animal reproductive history, vaccination status, or farm-level management practices also constrained the interpretation of risk factors. Overall, the findings highlight that *Brucella* infection remains endemic in the dairy herds of Swat, posing an ongoing threat to both animal productivity and human health. Expansion of surveillance through year-round monitoring, integration of molecular diagnostics, and implementation of vaccination programs are warranted to reduce disease burden. Strengthening intersectoral collaboration under a One Health framework could further enhance control efforts by addressing the interconnected nature of animal and human brucellosis in endemic settings.

CONCLUSION

The findings of the present study concluded that brucellosis remains a significant zoonotic and economic concern in Tehsil Bahrain, Swat, affecting the productivity and health of local dairy herds. The infection demonstrated a noticeable seasonal trend, showing increased prevalence during the rainy months, which may be linked to changes in environmental conditions and herd management practices that favor bacterial transmission. The use of the Milk Ring Test (MRT) proved to be a rapid, practical, and cost-effective diagnostic approach for the regular screening and surveillance of *Brucella* antibodies in milk from cows and buffaloes. These findings emphasize the need for continuous monitoring, implementation of preventive measures, and awareness among livestock owners to mitigate the impact of brucellosis on both animal health and rural livelihoods.

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AUTHOR CONTRIBUTION

Author	Contribution
Mian Syed Kashif*	Substantial Contribution to study design, analysis, acquisition of Data Manuscript Writing Has given Final Approval of the version to be published
Ihsan Ullah	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published
Nasar Hussain Khan	Substantial Contribution to acquisition and interpretation of Data Has given Final Approval of the version to be published
Izaz Ali	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published

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