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DETECTION OF BRUCELLA ABORTUS FROM RETAIL RAW MILK SAMPLES FROM QUETTA, PAKISTAN

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Abstract

Brucellosis is the most significant zoonotic bacterial disease with huge economic impacts in the livestock production sector, and is a public health menace worldwide, especially in developing countries such as Pakistan. Brucella abortus, the causative agent of bovine brucellosis, usually infects humans through the ingestion of contaminated raw milk and its products. Consumption of contaminated milk is the source of human infections in those areas where the practice of consuming raw milk is common, and no preventive measures have been taken yet. This work was performed to find out the prevalence rate of B. abortus in retail raw milk from milk shops in Quetta, Pakistan, by serological, microbiological, and molecular techniques. A total of 100 samples of raw milk were tested. Out of them, 24% were found positive by the Milk Ring Test (MRT), and 20% were positive by the Enzyme-Linked Immunosorbent Assay (ELISA). Bacteriological and molecular detection showed the presence of B. abortus in 15% of the samples. Isolated colonies possessed the typical characteristics of B. abortus. The presence of living B. abortus in raw milk indicated the exposure of consumers to the disease by ingesting unprocessed milk. These results highlight the importance of enhanced surveillance and testing of bovine brucellosis, milk quality monitoring, better management practices on farms, and public education on the risks of consuming raw milk to prevent zoonosis and foodborne illness.

Keywords: *Brucellosis, ELISA, Livestock, MRT, PCR, Zoonotic*

INTRODUCTION

Brucellosis is one of the most significant bacterial zoonotic diseases affecting both livestock and people across the world. Brucellosis continues to be a major concern in animal health, public health, and livestock production due to its wide distribution, chronicity, and huge economic importance (1,2). The organisms causing brucellosis are small, aerobic, non-motile Gram-negative coccobacilli capable of surviving and replicating inside host cells, where they act as intracellular pathogens (3). Intracellular survival makes it possible for the bacteria to avoid the host immune response, causing persistent and chronic infections in susceptible hosts.

The capability of *Brucella* species to adapt to diverse conditions promotes their successful transmission. The organism can maintain its viability for several months in water, moist soil, aborted fetuses, manure, and secretions from animals, especially under refrigeration conditions (4). However, *Brucella* is very sensitive to sunlight, various disinfectants, and acidity. Heating of milk to 62.7 °C for 30 minutes or 71.6 °C for 15 seconds results in the death of viable bacteria; hence, pasteurization of milk completely kills the organism (5). Thus, consumption of pasteurized milk is one of the main means of prevention of brucellosis acquired through food.

Brucella abortus is the primary cause of bovine brucellosis and mainly causes infections in cattle and buffaloes (6). The bacteria have a high predilection for the reproductive organs in sexually active animals due to the presence of erythritol, which promotes bacterial growth (7). Once an animal is infected, the bacteria settle in the uterus, placenta, mammary glands, and lymph nodes, causing severe inflammatory damage.

The most prominent symptom of bovine brucellosis is abortion in the final trimester of gestation. Other symptoms of bovine brucellosis include retained placentas, metritis, infertility, repeat breeding, orchitis, epididymitis, and decreased milk production (8,9). Despite the majority of the affected animals recovering clinically from the disease, most of them become lifelong carriers of the disease and shed bacteria through milk, uterine discharges, the placenta, and aborted fetuses.

It is known that raw milk serves as one of the primary sources of transmission of *Brucella* species to humans. In most developing countries, especially in Pakistan, the consumption of fresh and raw milk is an established custom, which makes zoonotic infections more prevalent (17). Routine screening of raw milk is essential, not only to protect people from the disease, but also to determine the infection status of dairy herds and evaluate the effectiveness of disease prevention programs.

The accurate detection of brucellosis is very important for epidemiology and control of the disease. Serological techniques, such as the Milk Ring Test (MRT), Rose Bengal Plate Test (RBPT), Complement Fixation Test (CFT), and Enzyme-Linked Immunosorbent Assay (ELISA), are often used due to their speed, cost-efficiency, and applicability for mass screening (18). Nevertheless, these tests identify antibodies, and not the pathogen, and thus sometimes give false positive or negative results. The use of molecular methods like Polymerase Chain Reaction (PCR) allows for increased sensitivity and specificity of the tests and detects *Brucella* DNA directly (19).

While there are many studies on the incidence of bovine brucellosis in Pakistan, little is known about the prevalence of *Brucella abortus* in raw milk supplied by retail milk stores in Quetta, Balochistan. Comparative evaluation of conventional serological assays using PCR as the confirmatory method has also not been carried out in the region. Current epidemiological data are required for disease surveillance, livestock improvement, enhancement of milk safety, and prevention of zoonotic transmission.

Hence, this study was undertaken to assess the prevalence of *Brucella abortus* in raw milk samples taken from retail milk stores in Quetta, Balochistan, Pakistan. In addition, this research aims to compare the diagnostic effectiveness of the Milk Ring Test (MRT), Enzyme-Linked Immunosorbent Assay (ELISA), and Polymerase Chain Reaction (PCR) in the diagnosis of bovine brucellosis. It is hoped that the results of the study will contribute to baseline epidemiological information useful for surveillance, control measures, milk safety, and prevention of brucellosis transmission to humans and livestock.

MATERIALS AND METHODS

STUDY DESIGN

The current research work was a laboratory-based cross-sectional study conducted to detect and identify *B. abortus* using raw milk samples obtained from retail milk shops located in Quetta, Pakistan.
This study was conducted in the Department of Microbiology, Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta, Pakistan.

SAMPLE COLLECTION

A total of 100 raw milk samples were collected from different retail milk shops in Quetta city. Sterile screw-capped plastic containers were used to collect 40 mL of milk from each retail shop (14). The specimens were tagged with the sample number, place, and date of collection. Insulated ice boxes containing ice packs were used to ship the milk samples to the Microbiology Laboratory of CASVAB.

MILK RING TEST (MRT)

Milk Ring Test (MRT), a preliminary serological screening technique, was performed to identify *B. abortus* antibodies in milk samples (15). One milliliter of the milk samples was put into a sterile test tube. ilk samples were stored in a refrigerator at 4 ℃ overnight to separate the cream layer, and 100 µL of stained *Brucella* antigen prepared by the Veterinary Research Institute was added. The samples were mixed gently to enable the antibodies and antigen interaction. The tubes were incubated at 37 ˚C for 1 hour. After incubation, the tubes were visually examined for the formation of a blue colored ring at the cream layer.

A blue ring was formed at the cream layer at the top of the milk column, thus showing the formation of antibodies against *B. abortus* (Positive). The milk column showed uniform coloration without the formation of a ring (Negative).

ISOLATION OF *BRUCELLA ABORTUS*

The *B. abortus* isolation was done using standard bacteriological culture methods. Brucellosis is said to be confirmed by culture using the gold standard method of confirmation (16). 5 mL of each milk sample was transferred to sterile centrifuge tubes. The samples were centrifuged at 10,000 rpm for 15 mins. The sediment and cream layer were collected very carefully. These fractions were cultured on *Brucella* selective agar obtained from BD Difco, Michigan, USA (211086), to which we added selective antibiotics including vancomycin 20 mg/L and bacitracin 2 mg/L for the inhibition of Gram-positive bacteria, and for the inhibition of Gram-negative bacteria, except *Brucella*; nalidixic acid 5mg/L was added. The plates were incubated in a 5-10% CO_2 atmosphere at 37 °C for 72 hours.

Suspect colonies were further put through biochemical tests including Gram staining (Coccobacilli, negative Gram), Catalase test (positive) (17).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

All positive Milk Ring Test samples were confirmed with Enzyme-Linked Immunosorbent Assay (ELISA) in order to confirm *Brucella* antibodies. ELISA kit was obtained from M/S Svanova, Sweden

ELISA microplates that were coated with *Brucella* antigen were utilized. Milk samples were centrifuged at 5000 rpm for 5 minutes for removal of the cream layer. 100 µL of sample dilution buffer was added to each control well, and 4 µL of positive control serum was added to the 1st well, and 4 µl of negative control serum was added to the 2nd well of the microplate as a negative control. 100 µL of milk sample was added to the remaining wells. The plate was gently shaken, covered, and incubated at 37°C for one hour to enable antigen-antibody interaction. The wells were washed using washing buffer (PBS-Tween buffer) three times to eliminate unbound components, then 100 µL of horseradish peroxidase (HRP) conjugate was added to each well and incubated at 37℃ for 1 hour. After washing 3 times again, the plate was poured with 100 µL of tetramethylbenzidine and incubated at room temperature for 15 minutes. Then, 50 µL of stop solution was added to all wells (18)

Measurement of the optical density (OD) values was done with the ELISA microplate reader at 450 nm wavelength. Samples that had a higher OD in comparison to the cut-off value were regarded as positive, whereas those whose OD was less than the cut-off were regarded as negative.

MOLECULAR DETECTION BY POLYMERASE CHAIN REACTION (PCR)

B. abortus molecular detection was done by Polymerase Chain Reaction (PCR) to determine the presence of bacterial DNA (19). Following primers used for PCR (20).

Forward Primer	GACGAACGGAATTTTTCCAATCCC	498 bp
Reverse Primer	TGCCGATCACTTAAGGGCCTTCAT	498 bp

PCR AMPLIFICATION AND AGAROSE GEL ELECTROPHORESIS FOR DETECTION OF *B. ABORTUS* DNA

The polymerase chain reaction (PCR) cycling conditions consisted of an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55–60 °C for 30 seconds, and extension at 72 °C for 1 minute, with a final extension at 72 °C for 7 minutes. Under these amplification conditions, the *B. abortus*-specific gene fragments are detectable (21). The PCR products were then separated using 1.5% agarose gel electrophoresis, with the agarose gel stocked and impregnated with ethidium bromide, and the PCR products and a DNA ladder loaded into the gel wells. Electrophoresis was conducted at 90–100 volts for 30–40 minutes, and the DNA bands were visualized under UV transillumination. A positive result, indicated by a DNA band at the correct position, verified the presence of *B. abortus* DNA in the samples examined.

RESULTS

A total of 100 raw milk samples were collected from retail milk shops in Quetta and tested for the identification of *B. abortus* using the Milk Ring Test (MRT), ELISA, bacteriological culture, biochemical characterization, and PCR. The findings were documented through visual analysis, biochemical tests, and molecular confirmation to provide a critical assessment of *B. abortus* prevalence in the study region.

For the MRT, milk samples were refrigerated overnight after the addition of stained antigen and analyzed for the formation of a colored ring at the cream layer. Twenty-four samples (24%) formed a clear blue-colored ring at the cream layer on top of the milk column and were considered positive, while the remaining 76 samples (76%) were homogeneous in color without ring formation and were considered negative. When a blue ring is formed, *Brucella*-specific antibodies attach to the stained antigen and migrate with the cream layer (Fig, 1a).

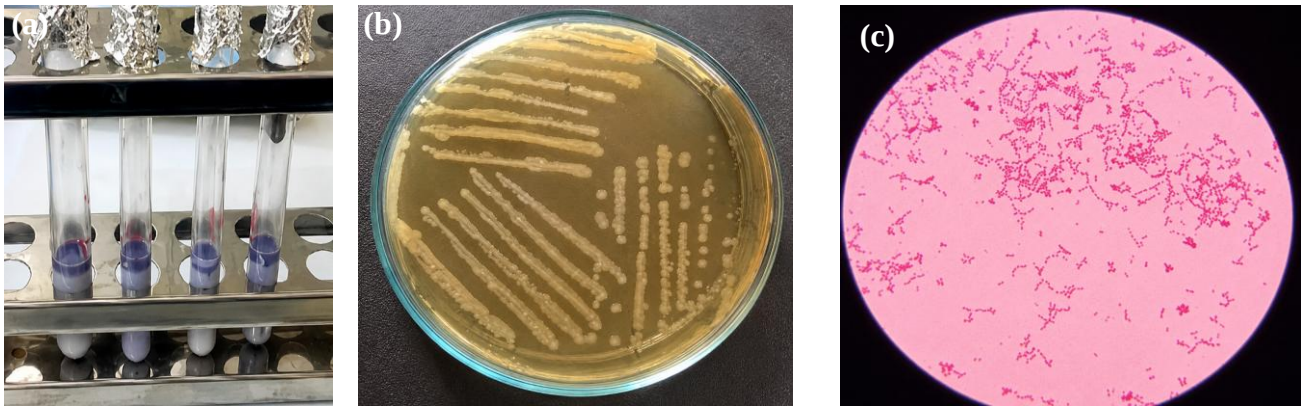

Fig. 1 (a). Milk sample containing stained antigen forming a blue-colored ring at a cream layer on the top of milk; **(b).** *B. abortus* colonies on *Brucella* agar; **(c).** *B. abortus* appears pink under the microscope after Gram staining, showing Gram-negative coccobacilli

The 24 MRT-positive samples were subsequently tested by ELISA, which produced a colorimetric change following the addition of substrate (TMB). After the addition of stop solution, 20 of the 24 MRT-positive samples (83.3%) were positive by ELISA, while 4 samples (16.7%) were negative. Optical density was measured at 450 nm, and samples exceeding the cut-off value were considered positive. The yellow color developed was proportional to the concentration of antibodies. The lower number of positive samples relative to MRT indicates that MRT produces false positives, which is consistent with previous research conducted in Pakistan.

Bacteriological isolation was performed by incubating samples on selective *Brucella* agar and closely monitoring colony morphology. Colonies on positive plates were small (1–2 mm diameter), smooth, glistening, convex, transparent or slightly whitish, with entire margins. Out of the 20 ELISA-positive samples, *Brucella* colonies were isolated from 15 samples (75%), while 5 samples (25%) showed no growth or contamination. The observed colony morphology agreed with regional and international literature, confirming successful isolation of *B. abortus* (Fig. 1b).

Biochemical testing was performed on all culture-positive isolates. Gram staining revealed pink-colored Gram-negative coccobacilli in 100% of isolates (Fig. 1c), and the catalase test was positive in 100% of isolates, as indicated by bubble formation. Phenotypic identification of all isolates was therefore possible based on the biochemical features of *B. abortus* (Fig. 2).

PCR amplification was carried out using *B. abortus*-specific primers. Agarose gel electrophoresis revealed a clear DNA band of approximately 498 bp in all

Fig. 2. Positive catalase, showing bubble formation

15 culture-positive samples (15%). The positive control produced a single band at 498 bp, while the negative control produced no band. A complete correlation was observed between culture and PCR results, with both methods yielding a prevalence of 15%.

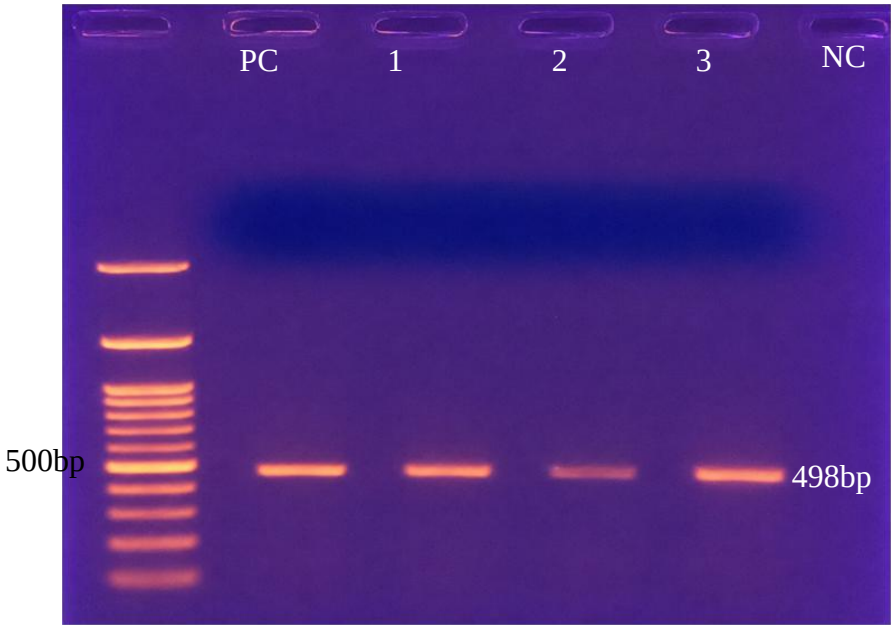

Fig. 3. Gel containing 4-well M containing DNA leader of 100bp lane 1 to 3 containing PCR product of *Brucella abortus* showing a band at 498 bp. PC as positive control, NC as negative control

The detailed observations and results of all diagnostic methods are comprehensively summarized in Table I, while the biochemical characterization of the culture-positive isolates is presented in Table II.

Table I. Comprehensive summary of MRT, ELISA, culture, and PCR results for the detection of *B. abortus* in raw milk samples (n = 100)

Method	Observation	Result	Number of samples	Percentage (%)
MRT	Blue ring formation at cream layer	Positive	24	24%
MRT	No ring, uniform milk color	Negative	76	76%
ELISA (of 24 MRT-positive samples)	Yellow color, OD at 450 nm above cut-off	Positive	20	83.3%
ELISA (of 24 MRT-positive samples)	Low OD	Negative	4	16.7%
Culture (of 20 ELISA-positive samples)	Small, smooth, translucent colonies	Positive	15	75%
Culture (of 20 ELISA-positive samples)	No growth or contamination	Negative	5	25%
PCR (n = 100)	Band at ~498 bp	Positive	15	15%
PCR positive control	Band at ~498 bp	Positive	—	—
PCR negative control	No band	Negative	—	—

Table II. Biochemical characterization of culture-positive *B. abortus* isolates (n = 15)

Test	Observation	Result	Percentage (%)
Gram staining	Pink-colored Gram-negative coccobacilli	Positive	100%
Catalase	Bubble formation	Positive	100%

DISCUSSION

In the present research, the prevalence of *Brucella abortus* was determined in retail raw milk samples purchased from milk shops in Quetta, Pakistan, through serology (Milk Ring Test and ELISA), bacterial culture, and PCR-based diagnosis. Detection of *B. abortus* in 15% of milk samples by culture and PCR demonstrates that contaminated raw milk still persists as a potential agent in the transmission of this zoonotic disease in the study area. This indicates that brucellosis infection among dairy cattle supplying milk to the retail sector remains prevalent and poses a great threat to public health, especially where consumption of unprocessed milk is practiced.

A positivity rate of 24% was observed for the MRT, while 20% of these samples were confirmed positive by ELISA. The higher positivity rate for MRT compared to ELISA indicates that MRT is a good screening tool but lacks specificity due to false-positive reactions caused by mastitis, late lactation period, presence of colostrum, or a higher percentage of fat in the milk. The same observations have also been made by Shafee et al. in Quetta, in which milk ELISA was found to be more specific compared to other screening tests for brucellosis in cattle (18). Similarly, Abnaroodheleh et al. indicated that serologic testing methods are beneficial in herd-level monitoring, but they cannot distinguish between an active infection and prior exposure or vaccination (22).

In this research, 15 out of 20 samples (75%) tested positive by ELISA were found to contain *Brucella* by bacteriologic culture. Even though isolation of *Brucella* bacteria through cultivation has been regarded as the gold standard method, isolation of *Brucella* from milk is usually challenging due to the intermittent shedding of the bacteria, their low number, and contamination by environmental microbes. Thus, the five samples that did not grow *Brucella* in this research might indicate low bacterial counts, previous use of antibiotics, intermittent shedding, and contamination during culturing. De Miguel *et al*. had experienced similar difficulties in isolating *Brucella* bacteria from milk samples (16).

B. abortus DNA was detected using PCR in all the positive cultures, giving a prevalence rate of 15%. Complete correlation between the culture method and PCR in the current study indicates the high level of specificity of the molecular test. PCR results can be obtained much faster without needing the long incubation time required for the culture test, along with a low risk for occupational exposure to live *Brucella* strains. Similar results were obtained by Mishra et al., where it was shown that PCR is a very sensitive confirmation test for bovine brucellosis and is a very effective addition to traditional diagnostic tests (20). Daugaliyeva *et al*. found that PCR tests for *B. abortus* are very accurate when detecting contaminated milk and food products (18). The prevalence recorded in the current study seems to be similar to several previous studies in Pakistan and other nearby countries; however, differences among these studies are also noted. The prevalence of bovine brucellosis was recorded to be almost similar in organized dairy farms in Quetta in a study carried out by Shafee *et al*., using milk ELISA (17). Babaoglu *et al*. also isolated *Brucella* species from raw milk in Turkey, which proved contamination of retail milk to be an important food safety issue in endemic areas (13). Similarly, studies carried out in Iran by Alamian *et al.* showed that *Brucella abortus* continued to circulate in dairy cattle, despite the ongoing control programs (19). Differences in prevalence among various studies can be due to geographical differences, herd management, vaccination status, movement of animals, sample size, diagnostic techniques, and biosecurity.

Isolation of live *B. abortus* from retail milk has important health implications. Ingestion of raw or unprocessed milk continues to be an important source of human brucellosis, especially in developing countries where pasteurization is not usually practiced. Human brucellosis is characterized by prolonged fever, arthritis, endocarditis, neurobrucellosis, reproductive diseases, and chronic illness.

The first limitation of the present study relates to its limited sample size as well as the sampling of samples from retail milk shops located in Quetta city. Besides, the study only focused on *B. abortus* without any investigation regarding other *Brucella* species, including *B. melitensis*, another causative agent of brucellosis. In the future, larger sample sizes, molecular typing, and possible animal- and farm-based risk factors related to the transmission of disease will be considered.

CONCLUSION

This study established the occurrence and prevalence of *B. abortus* in retail milk samples from Quetta using serological, bacteriological, and molecular methods. *B. abortus* was confirmed in 15% of samples by both culture and PCR, demonstrating that brucellosis remains a significant zoonosis in the study area and poses a potential threat to public health. The presence of viable organisms in commercially sold raw milk highlights the urgent need for dairy herd monitoring, routine milk testing, public awareness, and proper pasteurization practices. These findings underscore the importance of effective surveillance programs, improved dairy management, laboratory diagnosis, and public health measures.

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Conflict of interest:

Authors declared no conflict of interest.

Authors' contribution:

RB Experimental work and designed the study; SAK Conceived and designed the study, supervised the research work YH; Performed sample collection and serological testing; SI Carried out bacteriological culture and isolation; IS Conducted PCR amplification and gel electrophoresis; MS Performed biochemical identification and data interpretation; ZR Assisted in sample processing and data compilation; AZ Performed sample collection and laboratory experiments; HN Maintained laboratory records and literature review; MAK Assisted in PCR optimization and statistical analysis; Gd Provided laboratory support and data recording.

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