



THE EMERGING TREND OF *BRUCELLA ABORTUS* ZONOTIC INCIDENCE IN BOTH HUMAN AND ANIMAL POPULATION OF DISTRICT SWAT KHYBER-PAKHTUNKHWA

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ABSTRACT

Brucellosis is a common zoonotic disease caused by a member of the genus *Brucella*, which affects both animals and humans. The aim of the current study is to record the incidence of brucellosis among livestock engaged human and the comparative study of two *Brucella* species, *Brucella abortus*, and *Brucella melitensis* in animal and human population in District Swat. A total of two hundred blood samples (100 each from human and animals) were collected from 3 tehsils i.e. Kabal, Khwza Khela and Matta of District Swat. For the analysis of these samples, Standard Plate Agglutination Test (SPAT) and molecular detection were carried out. Out of the total samples, 23% for humans and 18% for animals were found positive on the SPAT test. For species identification, two sets of species-specific primers were used for *Brucella abortus* and *Brucella melitensis* and amplicons having a size of 498 bp and 731 bp were obtained respectively. Out of 200 samples, 11% were confirmed through PCR by detecting IS711 gene in the *Brucella* genome. The presence of *Brucella abortus* and *Brucella melitensis* was identified and confirmed through PCR in the studied area. This study demonstrates that brucellosis is the most prevalent zoonotic disease in district Swat. Our results showed that the zoonotic incidence of *Brucella abortus* infection is increasing in both animals and Human population of the studied area as compared to the *Brucella melitensis* infection.

KEYWORDS: *Brucella*, *Brucella abortus*, *Brucella melitensis*, IS711, Human, Animals District Swat.

INTRODUCTION

Brucellosis is the most common and important zoonotic disease, which affect both humans and domesticated animals at the global level.^[1] After rabies, it is ranked as second amongst the most common zoonotic diseases according to the office of the international Dis epizootic (OIE) and is also known by other common names like undulant fever or Mediterranean fever and Malta fever.^[2,3] This infectious disease is endemic to various countries from Europe, America, Africa and Asia.^[4] The most affected regions are Mediterranean, Africa, the Middle East and also some regions of Latin America and Asia. Worldwide human brucellosis is predicted to be greater than 500,000 infections annually, but the actual incidence rate is 25 times more than the predicted value, and this may be due to the fact that in many countries there is no proper statistical system for reporting and notification of the disease.^[5]

The causative agents for brucellosis are the members of genus *Brucella*. These are gram-negative, small, non-

capsulated, non-spore forming bacteria, and having no flagella. Their size varies from 0.5 µm to 0.7 µm in length and 0.5 µm to 1.5 µm in width. Different animals are affected by this disease such as cattle, swine, sheep, goats, camels and some marine animals.^[6,7] Humans are infected by different species of *Brucella* including *Brucella abortus*, *Brucella suis* biovars, *Brucella melitensis* and in rare cases by *Brucella canis* as well.^[8] About eight species are reported which effect terrestrial animals such as *B. abortus*, *B. neotomae*, *B. suis*, *B. canis*, *B. ovis*, *B. melitensis*, *B. microti* and *B. inopinata*.^[9] Different *Brucella* species have different host i.e. the *B. abortus* is found mostly in cattle, *B. melitensis* is present in goats, *B. suis* in swine and the *B. ovis* in sheep.^[10] The incidence of brucellosis increases with age, as it occurs mostly in sexually mature animals.^[11] Gender wise studies have shown that in comparison with mature males, females are mostly affected by brucellosis.^[12] Brucellosis can occur in any season but the peak duration is from February to July as these months are mostly concerned with the delivery as

well as abortion of animals.^[13] In humans, the infection mostly occurs in the summer season.^[14] In animals, it can cause untreatable infections, abortion, infertility, and a reduction in milk production.^[15]

The causative agent of Brucellosis mostly enters to the host through the digestive tract, mucosal layers or lungs, abraded the skin and circulates in the whole body within circulatory system and cause infection.^[16] Brucellosis is a long-lasting infection in humans with symptoms like intermediate fever, joints pain/arthritis and fatigue. The other signs including retained placenta, inflammation of testicle, epididymitis and the organism mostly excreted in uterine discharge and in milk secretion.^[17] Human is affected directly through contact with infected animals or by using their raw or contaminated material.^[18, 19]

Currently, three kinds of testing techniques are used for the diagnosis of brucellosis; these are microbiological, serological, and molecular-based techniques.^[20] The classical, microbiological (Culturing of concern bacteria from a blood sample) technique is time-consuming and also poses a high risk of causing infection to the lab personnel as *Brucella* requires biosafety level III for its culture and isolation.^[21] Different kinds of serological testing techniques are also implemented for the detection of this disease.^[20] The common serological techniques including Rose Bengal precipitation test (RBPT), standard plate agglutination test (SPAT), Coombs test, immune capture test and Enzyme-linked immunosorbent assay (ELISA).^[22] Currently, these conventional techniques are replaced by a molecular technique, Polymerase chain reaction (PCR) which is used for the detection of *Brucella* and their species.^[23] PCR is used for the accurate clinical diagnosis of brucellosis due to its high sensitivity and specificity.^[24] This molecular technique is used to identify the *Brucella* DNA in blood samples that were considered false negative through SPAT. In addition, the PCR is a sensitive, simple, specific, quick, and relatively inexpensive method for clinical diagnosis of this disease.

In Pakistan brucellosis has been ignored, where limited studies have been done to estimate its prevalence. A full description of the epidemiology of the disease is needed for planning interventional strategies for its prevention and control.

The present study was performed with the aim to identify the *Brucella* genus by targeting BCSP31 gene and to detect the *B.abortus* and *B.melitensis* in animals and in human samples by targeting the IS711 gene through Polymerase Chain Reaction (PCR). This study also focused on Serological technique along with molecular detection of *B. abortus* and *B. melitensis* from blood samples of household animal keepers and livestock populations from various areas of the District Swat.

MATERIALS AND METHODS

Study Area

The current study was performed with the aim to identify the incidence of Brucellosis in the animal keepers and their livestock population in three tehsils of district Swat. A detail information regarding brucellosis was collected on a pre-designed questionnaire. The samples were collected from three tehsils including Kabal, Khwza Khela, and Matta. The samples were brought to the Veterinary Research and Disease Investigation Center (VRI) Balogram, Swat for further analysis.

Sample Collection

Total 200 blood samples (100 human samples and 100 animal samples) were collected from different animals and animal keepers using sterile syringes. Three to five milliliter (ml) of duplicate blood samples were collected aseptically from the jugular vein of each animal and 3ml from the cephalic vein of each human. The samples were brought to VRI and stored through refrigeration for onward processing.

Serological Test

Different kinds of serological based testing techniques were used in the present study for diagnosis of brucellosis.

Standard Plate Agglutination Test (SPAT)

SPAT test was performed accordingly to the OIE protocols as described in their manufacturer's instructions for the presence of *Brucella*-specific antibodies. The serum from all blood samples was isolated through centrifugation and stored at room temperature. Then, 80 µl of each serum sample was placed on a glass plate. After proper shaking 30 µl of antigen, was applied to each of the samples and mixed them thoroughly with the help of a clean glass or plastic rod to form a circular zone of about 3cm in diameter. The samples showing agglutination were considered positive while the samples showing no agglutination were considered negative.

Polymerase Chain Reaction (PCR)

Brucella isolates were identified and confirmed by PCR using the protocol described by.^[25]

DNA Extraction from Blood Samples

DNA was extracted by Nucleospin[®] DNA extraction kit following the manufacturer's protocol.

Primer

Two sets of primers were used for targeting the Bcsp31 and IS711genes in *Brucella* DNA. The Bcsp31gene primers were used for the genus identification of *Brucella* while the IS711gene primers were used for specific *Brucella* species identification.^[25]

Table 1.1; Sets of Primer.

3'to 5' direction	
Bcsp31 G:	Forward : '3TGGCTCGGTTGCCAATATCAA 5' Reverse : '5CGCGCTTGCCCTTTCAGGTCTG3' Primer for Bcsp31 : (223 bp)
Is711 AB:	Forward: '3GACGAACGGAATTTTCCAATCCC5' Reverse: '5TGCCGATCACTTAAGGGCCTTCAT3' Primer for <i>Brucella abortus</i> : (498 bp)
1S711M:	Forward : '3AAATCGCGTCCTTGCTGGTCTGA5' Reverse: '5TGCCGATCACTTAAGGGCCTTCAT3' Primer for <i>Brucella melitensis</i> : (731 bp)

Detection and amplification of *Brucella* DNA through PCR.

For detection and amplification of *Brucella* DNA two assays were used in the current study, first one was used to detect the *Brucella* genus by detecting the BCSP31 gene (BCSP31-PCR) and the second one was used to detect *Brucella* species by detecting the IS711 locus (IS711-PCR).^[26]

The PCR reaction Mixture

The final volume of the PCR reaction was adjusted to 25 µl by adding 3 µl of ready to load Master Mix with 0.5 µl of primer (Forward) and 0.5 µl of primer (Reverse), 18.9 µl of double distilled water, 0.1 µl of enzyme Taq polymerase and 2 µl of template DNA. The primers used in the reaction were diluted to a concentration of 10 Picomoles.^[27]

The PCR Program

The thermal cycler software was edited for all the four steps i.e. initial denaturation step was set at 94 °C for 5 min, then cycle denaturation step by 40 cycles of 94 °C for 1 min, then annealing step with a temperature of 60 °C for 30 second, and then with elongation step with a temperature at 72 °C for 1 min and finally with another elongation/extension step with a temperature at 72 °C for 10 min was performed to polymerize all remaining single strand DNA fragments.

Gel Electrophoresis

Amplified PCR products were then run on the gel for further analysis as described by.^[28] A 1 % Agarose gel was prepared by taking 1 gram of Agarose and then added to the flask containing 100 ml of 1X TBE buffer. The flask was boiled using a microwave oven for at least

1 to 2 minutes. Then 1-2 µl of the prepared gel staining dye i.e. Ethidium bromide (Sigma Aldrich Germany) was added to gel when its temperature is lowered to 56 °C or below.

Statistical analysis

The data collected were statistically analyzed through SPSS (version 16.0.).

RESULTS

Initially, all the blood samples collected from different tehsils of district Swat were screened for the presence of *Brucella*'s antibodies using SPAT test. About 23% of human and 18% of animal's samples were found positive for *Brucella* antibodies using SPAT test.

Area wise human sample

Out of total 33 collected samples from tehsil Kabal, 3 samples were found positive for *B. abortus* and 3 samples were found positive for *B. melitensis* on SPAT test. Similar numbers of samples were also collected from tehsil Matta, out of which 6 and 7 samples were found positive for *B. abortus* and *B. melitensis* respectively. Out of 34 collected samples from tehsil Khwaza Khela, only 4 samples were found positive for *B. abortus* while no sample was detected positive for *B. melitensis*. The results revealed that out of total 100 analyzed samples collected from the three tehsils of district Swat, 13 for *B. abortus* and 10 for *B. melitensis* were found positive through SPAT test (Table 3.1). The total percentage of brucellosis was found 23% in human on SPAT test for *Brucella* in district Swat. A significant difference ($p < 0.05$) was found in area wise incidence of the disease.

Table 3.1; Area Wise Human samples.

Area	Results			Total	Chi-Square
	Brucella abortus	Brucella Melitensis	Negative		
Kabal	3	3	27	33	0.033
Matta	6	7	20	33	
Khwazakhela	4	0	30	34	
Total	13	10	77	100	

Sex-wise Prevalence of Brucellosis in District Swat

Out of the total 34 collected samples from males from the targeted area, 4 samples were found positive for *B.*

abortus and similarly, 4 samples were detected as positive for *B. melitensis*. Out of 66 samples collected from females, 9 samples were found positive for *B.*

abortus and 6 samples were found positive for *B. melitensis* (Table 3.2). No significant difference ($p < 0.05$)

was found in data regarding Sex wise prevalence of brucellosis in three tehsils of district Swat.

Table 3.2: Sex-wise Samples.

Sex	Results			Total	Chi-Square
	Brucella abortus	Brucella Melitensis	Negative		
Kabal	4	4	26	34	0.895
Matta	9	6	51	66	
Total	13	10	77	100	

Age Wise Prevalence of Brucellosis

In the current study three age groups were studied in human but as the sampling method was a convenient one; therefore, no equal number of samples were collected from each group. Only 1 sample was collected from the teenage group (5 to 20 years) which was found positive for *B. abortus*. From a young group (20 to 40 years) a total of 78 samples were collected in which 11 and 10 samples were found positive for *B. abortus* and

melitensis respectively. From an adult group, only 21 samples were collected and only one sample was found positive for *B. abortus* (Table 3.3). On analysis on chi-square test the p-value was found less than 0.05 which show the significant difference among the age group but, as the sampling method was a convenient one, therefore, different numbers of samples were collected from different age groups on the basis of which we cannot estimate the significant difference in the sage group.

Table 3.3; Age Wise Human samples.

Age group	Results			Total	Chi-Square
	Brucella abortus	Brucella Melitensis	Negative		
Teenage	1	0	0	1	.020
Young	11	10	57	78	
Adult	1	0	20	21	
Total	13	10	77	100	

Area Wise Animal Samples

A total of 25 samples from animals were collected from Kabal tehsil out of which only 1 sample was found positive for *B. abortus* and 1 for *B. melitensis* on SPAT test. From Matta tehsil, a total of 38 samples were collected out of which 6 samples were detected positive for *B. abortus* and 2 samples were found positive for *B. melitensis*. Out of the total 37 samples collected from

Khwa Khela tehsil, 4 samples were positive for *B. abortus* and 4 were detected positive for *B. melitensis* (Table 3.4). Chi square analysis of the data showed that there is no significant difference in area wise prevalence of brucellosis in under studied tehsils of District Swat. This might be also due to the sampling method which was convenient one in the whole study.

Table 3.4; Area Wise Animal samples.

Area	Results			Total	Chi-Square
	Brucella abortus	Brucella Melitensis	Negative		
Kabal	1	1	23	25	0.468
Matta	6	2	30	38	
Khawazkhela	4	4	29	37	
Total	11	7	82	100	

Specie Wise Brucella incidence

In the current study, samples were collected from three different species including cow, buffalos, and goat. A total of 33 samples were collected from cows in which only 4 samples were found positive for *B. abortus* and no single sample was positive for *B. melitensis* on SPAT test. From buffalo's total of 19 samples were collected out of which one sample were detected positive for *B. abortus* and 2 samples for *B. melitensis*. Data of the present finding further stated that a total of 48 samples

were collected from goats, out of which 6 and 5 samples were found positive for *B. abortus* and *melitensis* respectively (Table 3.5). From the current study, it was observed that 18% of the animal population was found positive for brucellosis. No significant difference was observed among the species as the p-value is greater than the 0.05 and also the method of sampling was a convenient one.

Table 3.5; Specie Wise Animal samples.

Area	Results			Total	Chi-Square
	Brucella abortus	Brucella Melitensis	Negative		
Kabal	4	0	29	33	0.349
Matta	1	2	16	19	
Khawazakhela	6	5	37	48	
Total	11	7	82	100	

Molecular Identification of the *Brucella* Species

Positive samples identified in the initial screening on SPAT were subjected to molecular identification. Two sets of primes (Genus specific and species-specific) were used. Firstly, all 41 samples (n=23 human, n= 18 animal) were tested for *Brucella* genus, 22 out of 41 samples were detected as positive for *Brucella* genus with an amplicon size of 223bp, which was observed on gel documentation. For confirmation of the *Brucella* species, species-specific primers were used for the species identification. Out of 22 positive samples for *Brucella* genus, only 11 samples were identified as positive for *B. abortus* with an amplicon size of 498 bp, while 8 out of 22 samples were detected positive for *B. melitensis* with amplicon size of 731 bp. The remaining 3 samples were found negative for both the species; maybe they were some other species of the *Brucella* genus. In

human, 9 out of 22 samples were found positive on PCR, 4 of which were positive for *B. melitensis* and 5 for *B. abortus*. A total of 8 out of 9 samples from female were found positive in which 3 were for *B. melitensis* and 5 were found positive for *B. abortus*. Interestingly only one sample from a human male was found positive for *B. melitensis*. Total of 10 samples was found positive on PCR in animals including 4 samples for *B. melitensis* while 6 samples for *B. abortus*. In goats' total, 6 samples were found positive out of which 4 samples were found positive for *B. abortus* and only two samples were found positive for *B. melitensis*. In the cow, only one sample was found positive for *B. melitensis*. In Buffalo, only 3 samples were found positive on PCR, out of which 2 samples were found positive for *B. abortus* while only one sample was found positive for *B. melitensis*.

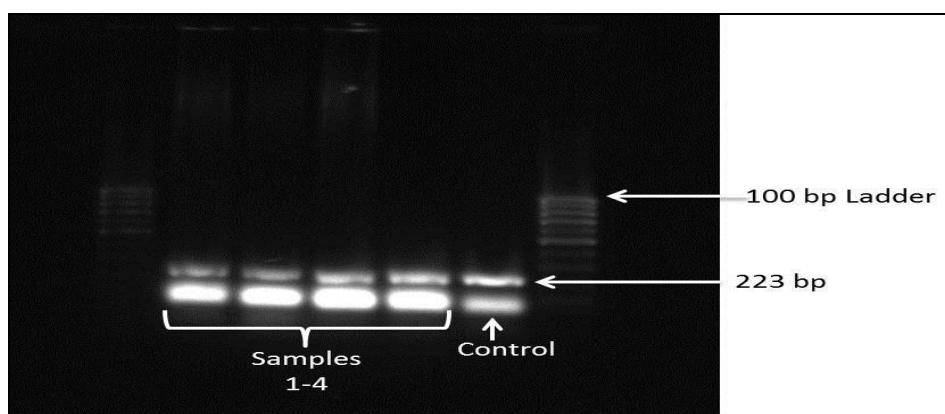


Fig.- 3.6: PCR product of gene BSCP31 in *Brucella* genus having an amplicon size of 223bp of samples collected from different animals and their owners at District Swat.

Ladder 100bp, Positive control, samples 1- 4

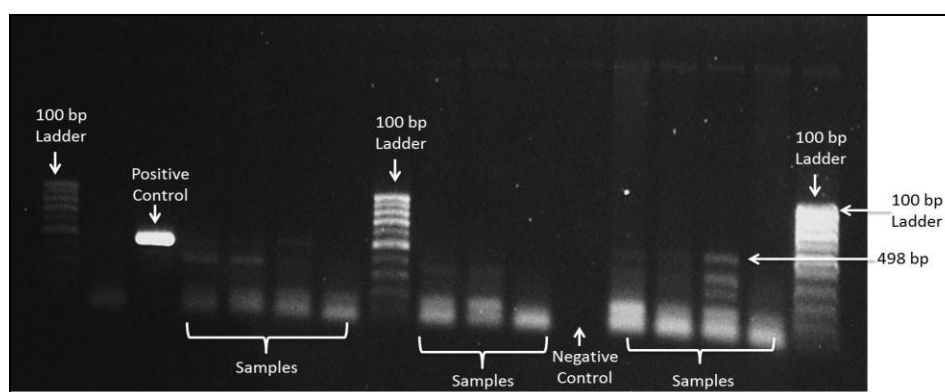


Fig.- 3.7: PCR product of gene IS711 in *Brucella abortus* having an amplicon size of 498bp of samples collected from different animals and their owners at District Swat.

Ladder 100bp, Positive control, samples, and negative control

DISCUSSION

Pakistan is an agricultural country but still, the exact prevalence of brucellosis in bovine is unknown. The author^[29] reported that the prevalence varies from 3.25 to 24.96% in various regions of Pakistan. The incidence of *B. abortus* is particularly increasing in large herds and in organized dairy farms as compared to small holdings in Pakistan.^[30, 31] The overall incidence of *B. abortus* is much higher in cattle and buffalos at various government and private livestock farms. Private livestock farms, showed a higher percentage of seropositive cattle and buffalo.^[32] The prevalence (8.5%) of *B. abortus* in Quetta Baluchistan is found much higher than previously reported (3.97%) positive cases in both cattle and buffalos. The prevalence was reported to be much higher in cattle as compared to buffalos.^[33] In Kohat, 17.58% and 32.5% seroprevalence of brucellosis was recorded at various government and private farms in cattle and sheep/goat, respectively.^[34] In the current study, both human and animal serum samples were tested initially on SPAT test, and about 23% (23/100) and 18% (18/100) samples were identified as seropositive for the *Brucella* antibodies respectively. It was also reported that 11% (11/100) of the samples were identified as positive for *B. abortus* and 7% (7/100) samples were detected as positive for *B. melitensis* on SPAT test in different animal population in district Swat. While in human 13% (13/100) samples were found positive for *B. abortus* and 10% (10/100) samples were found positive for *B. melitensis*. Our results are in line with the findings of the author^[31] who reported that the incidence of *B. abortus* is increasing particularly in large herds and in organized dairy farms as compared to small holdings in Pakistan. Similarly another author^[35] reported the seroprevalence of brucellosis in humans and goat population using various serological techniques like Rose Bengal Plate Test (RBPT), and Serum Plate Agglutination Test (SPAT). The total prevalence of brucellosis in goats was recorded 11.33% while sex wise prevalence in male and female was 8.0% and 14.66% respectively. In human samples, 7.33% of total prevalence was noted while in human male and female 5.33% and 9.33% prevalence was observed. Similar findings were also obtained in the current study. By SPAT test a higher seroprevalence of 11 % (11/100) was found in goats followed by 4% (4/100) and 3% (3/100) in cows and buffalos respectively. Similarly, a higher sero-prevalence of 15% (15/100) in female and 8% (8/100) in male was also recorded in the human population in the studied area. Higher seroprevalence is recorded in females of both animal and human. As the sampling method was a convenient one, therefore a different number of samples were collected from both sexes in human and animals.

Other diagnostic tools are required to overcome all the limitations facing by using conventional ways in order to detect the *Brucella* infection. The introduction of novel techniques like molecular techniques for the diagnosis of *Brucella* including genetic base technology was used as described by two authors.^[36, 37] The polymerase chain

reaction (PCR) is most attractive and best means for the detection and confirmation of brucellosis as referred by.^[26, 38, and 39] The using of gene probes method has been restricted to national reference laboratories for *Brucella*, but the introduction of molecular-based techniques like PCR into clinical diagnostic section is a useful addition to the techniques used conventionally. Different studies have validated the effectiveness of PCR based method for rapid and accurate diagnosis of brucellosis in humans, as described by.^[40, 41, 41, and 43]

This molecular technique hijacked almost all conventional diagnostic methods used for *Brucella* diagnosis in peripheral blood in human cases^[43], where it proved to be the more sensitive and specific technique. Likewise, other investigators.^[41, 44] have also suggested the diagnostic value of PCR-ELISA for the identification of the acute form of brucellosis in blood or serum specimens in humans. Recently the PCR method is considered more advantageous as compared to isolation and conventional serological techniques in order to diagnose the brucellosis. It is because the PCR is able to detect the DNA of *Brucella* in those false negative samples which are detected previously by conventional serological methods.

Another investigator^[45] reported the use of PCR for identification of *Brucella* DNA in sera samples. In his results, he declared the presence of an amplicon having a size of 223 bp in 96% (24/25) of tested sera samples through using primers derived from the nucleotide sequence of a gene that encodes the 31-kDa *B. abortus* antigen. However, when another PCR was performed for the DNA templates of serum, the presence of an amplicon having a size of 731 bp was declared in 60% (15/25) of the tested samples by using *B. melitensis* specific primers which were derived from a polymorphic locus adjacent to the 3V end of IS711. The specificity of each PCR was demonstrated by inclusion of reference *Brucella* DNA strains and by DNA sequence analysis of PCR products. The above-explained results suggested that using PCR for serum analysis is the most convenient and safe technique in order to diagnose the brucellosis more accurately and timely.

In the current study, all the seropositive samples were subjected to PCR and 11% (22/200) samples were identified to contain *Brucella* DNA after subjecting them to the genus-specific primers having an amplicon size of 223bp. For species identification of the *Brucella*, all of the samples were subjected to the species-specific PCR 11% (11/100s) samples were identified, belonging to the *B. abortus* species targeting the IS711 locus in its genome obtaining an amplicon size of 497bp. In the remaining samples, about 8% (8/100) of samples were confirmed positive for *B. melitensis* with an amplicon size of 731bp. Similar primers sets were used by the author^[25] in his research and similar amplicon size was obtained in the different species of *Brucella*, thus our study supports the previous findings.

CONCLUSIONS

In the current study, two techniques (SPAT and PCR) were used to identify the *Brucella* infection in animals and humans from different tehsils of District Swat. About 18% and 23% of the samples were found positive of having *Brucella* Antibodies on the SPAT test in animals and in human respectively. About 11% of samples were confirmed through PCR by detecting the IS711 locus in the DNA of the all collected samples. The Molecular-based technique (PCR) was first time used for the confirmation of both prevalent *Brucella* Species in District Swat. Our results showed that incidence of *Brucella abortus* infection is increasing in both animals and Human as compared to the *Brucella melitensis* infection.

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