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Detection of virulence associated gene in *Brucella abortus* from blood samples of cows in District Swat of Khyber Pakhtunkhwa, Pakistan

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Contribution All authors contributed to the study as follows: **W. Khan** (conceptualization and supervision); **N. Khan** (methodology and original draft preparation); **Nasirullah** (methodology and formal analysis); **F. Khan** (methodology and formal analysis); **D. Ghafoor** (formal analysis and investigation); **M.N. Uddin** (original draft preparation and supervision); **S.A.** Khan (original draft preparation); **I. Uddin** (resources and review and editing); and **N. Qayum** (review and editing).

Brucella abortus is a zoonotic pathogen causing brucellosis in livestocks and human, a disease with signifinat economic and public health concern. The present study investigated the molecular detection of Brucella species, and the frequency of virulent genes (vir B and ure) in the Brucella species circulating in livestock of the district Swat Khyber Pakhtunkhwa, Pakistan. Blood samples were collected from suspected cases of brucellosis in cows of the two tehsils of district Swat. The genomic DNA was extracted from a blood sample and subjected to genus and species-specific primer of Brucella through polymerase chain reaction (PCR) assay. The Brucella species DNA was then screened for the presence of virulent genes. The PCR using specific primers confirmed genus Brucella, species *abortus,* and detected virulence-associated gene *vir*B and ure operon in *B. abortus* in the current location. Out of the total tested samples, 22.8% were positive for the Brucella genus and 68.7% for *B. abortus,* with high percentage in Tehsil Charbagh. The virB virulent gene and ure gene frequency were 72.7% and 40% respectively. The high frequency of virulent genes in *B. abortus* indicated a circulation of virulent strains of *B.abortus* in cows.

Keywords: *Ptyas mucosa*, Lakki Marwat, Morphological diagnosis, Taxonomic differentiation, Habitat degradation, Conservation

INTRODUCTION: Brucellosis is a zoonotic disease caused by the members of the genus *Brucella* and transmitted from the infected animals to humans by aerosol inhalation, ingestion of contaminated food products, and direct contact with an infected animal or persons (Corbel, 2006; Chan and Surendran-Nair, 2025). Taxonomically, *Brucella* is classified as α-Proteobacteria and subdivided into *B. abortus*, *B. melitensis B. canis*, *B. suis, B. ovis* and *B. neotomae* (Alton *et al.*, 1988; Rahimoon *et al.*, 2024). The development of new species and the expansion of host ranges can result from Brucella species' constant genetic and phenotypic adaptation to changing environmental, agricultural, and social factors. This has important ramifications for both human and animal health. (Godfroid *et al.*, 2005; Al Dahouk *et al.*, 2017). Among the reported species of *Brucella*, *B. suis* and *B. abortus* are very important zoonotic agents after *B. melitensis*, the most virulent and the most prevalent worldwide. *B. abortus* is the major cause of late gestation abortion in cows and makes the milk contaminated (Barkallah *et al.*, 2014). The pathogen follows a different strategy to enter into host cell and then overcome the host immune system for propagation (Martirosyan *et al.*, 2011). In livestock, Brucella abortus mainly reproduces in the genital-reproductive organs, such as the mammary glands and supra-mammary lymph nodes resulting in milk contamination. (Refai, 2003; González-Espinoza *et al.*, 2021). Many Brucella species' reported unique genetic traits ehich reflects their ongoing diversification and evolution, which may have an impact on their pathogenicity, host range, and environmental fitness (Poester *et al.*, 2013; Banai, 2025). Therefore, an outbreak of brucellosis may be expected in the future. Different virulent factors of *Brucella* spp. are responsible for the infection. *Vir*B proteins forming the type IV secretion system (T4SS) are one of the most virulence factors of *Brucella* that have a role in intracellular replication (Lapaque *et al.*, 2006; Roop *et al.*, 2021). Another virulence factor of *Brucella is* virulence factor A (bvfA), which is a small 11kDa protein of periplasm and might have a role in establishing an intracellular niche, but its specific virulence role is still unrevealed (Lavigne *et al.*, 2005; Głowacka *et al.*, 2018). Besides *vir*B and *bvf*A, urease is another important virulence factor of *Brucella* encoded by *ure* genes. The number and combination of different virulent factors mediated the pathogenesis of species of *Brucella* (López-Santiago *et al.*, 2019). Thus, the identification and characterization of these virulent factors play a vital role in the management and control of the outbreaks.

OBJECTIVES: The current study investigates the molecular diagnoses and frequency of virulent genes (*vir*B and *ure* gene) of *B. abortus* affecting the local population of cows.

MATERIALS AND METHODS: A standardized procedure was used to collect blood samples from the cows suspected of having brucellosis (Zulfiqar *et al.*, 2012). A total of seventy sampes were collected (n=70) from the two tehsils (Charbagh and Barikot) of district Swat, with 35 samples from each tehsil. About 3-5mL of blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes from the jugular vein of cows via disposable syringes. The blood samples were brought to the laboratory of the Centre for Biotechnology and Microbiology, University of Swat, and properly labeled. The blood samples were processed for serum separation and handled for further analysis following the standardized procedure of Oddoze *et al.* (2012). The Rose Bengal plate test and Standard agglutination test were performed and the methodology used by Ebid *et al.* (2020). All samples tested positive by RBPT and SPAT were further confirmed by PCR. The Genomic DNA extraction was performed via Thermo Scientific Gene JET Genomic DNA Purification Kit (Cat#FSB2147) following the manufacturer procedure with no modification. The concentration and the purity of DNA were measured by a NanoDrop™ spectrophotometer (NanoDrop 2000 Thermo Scientific). The calculated value of ~1.8 from the absorption ratio (A260nm/280nm) is considered the purity of the sample.

Four different PCR assays were used in the current study. The first and second assays identified the genus *Brucella* and species *abortus*, previously described by Garshasbi *et al.* (2014). The third and fourth assays investigated the virulent genes (*vir* B and *ure*) in the *B. abortus* (Lavigne *et al.*, 2005). Briefly, the total volume of the 25µL PCR reaction was prepared by the addition of 3µL 5x FIREPol® Master Max, 2µL template DNA (different for each reaction), 0.5µL forward and 0.5µl of the reverse primer (different for each assay) was added to 19µL of double distal water. The oligonucleotide primers (table 1) used in the current study were supplied by Macrogen (Korea). PCR assay was accomplished in the thermocycler (Bio-Rad T100) using the following parameters: primary denaturation at 94°C for 4 min, 40 cycles of application with denaturation at 94°C for 30 sec, 30 sec of annealing at 64°C, 65°C and 54°C for Bcsp31, Is711 and *vir*B respectively, and primer extension for 1 min., and 10 min. a final extension of incomplete synthesized DNA was performed at 72°C. The amplified PCR products were separated on 1% gel containing 2µL Ethidium bromide, with the help of electrophoresis performed in 1X TBE buffer. The gel was visualized under UV light for the determination of the amplified product. The photos were then saved on the computer.

RESULT AND DISCUSSION: Brucellosis is one of the most widespread zoonotic bacterial diseases, which represents a serious threat to humans and animals and can also cause high economic losses (Maadi *et al.*, 2011). Annually, 500,000 cases of brucellosis occur in humans and animals (Pappas *et al.*, 2006).

Target	Primer sequence(5'-3')	Amplified segment	References
*Bcsp*31	B4: TGGCTCGGTTGCCAATATCAA B5: CGCGCTTGCCTTTCAGGTCTG	223 bp	(Garshasbi *et al*., 2014)
Is711, *B. abortus*	F:TGCCGATCACTTAAGGGCCTTCAT R:GACGAACGGAATTTTTCCAATCC	498 bp	(Garshasbi *et al*., 2014)
virB	F: CGCTGATCTATAATTAAGGCTA R: TGCGACTGCCTCCTATCGTC	881 bp	(Lavigne *et al*., 2015)
ure	F GCTTGCCCTTGAATTCCTTTGTGG R:ATCTGCGAATTTGCCGGACTCTAT	1282 bp	Derakhshandeh *et al*., 2013

Table 1: Oligonucleotide primers used in the PCR assay.

Large, organized dairy farms and large herds are more vulnerable than small holdings because of management procedures and herd size. In Pakistan, organized dairy farms show a substantial prevalence of *B. abortus*, and the incidence is frequently higher in larger herds and disorganized farms (Shafee *et al.*, 2011; Arif *et al.*, 2019). Different molecular and serological methods are used for the detection of *Brucella* spp. However, the preferred diagnostic method for brucellosis detection is PCR assays, which offer quick, sensitive, and specific detection of Brucella spp., surpassing other diagnostic methods such as culture, immunohistochemistry, and traditional serological tests for direct diagnosis of infection in animals and clinical samples (Asif *et al.*, 2009; Pereira *et al.*, 2023). In the current study, the presence of *B. abortus* and the virulence genes were investigated via PCR assay (table 2). The total samples were first tested for the *Brucella* genus through PCR using a specific primer targeted Bcsp31. Out of the total (70) tested samples, 22.8% were positive with high percentage in Charbagh and the remaining 77.2% samples were negative for the *Brucella* genus. The positive sample appeared on the gel with an amplicon size of 223 bp (figure 1).

Target genes	Results	
	Positive (%)	Negative (%)
Bcsp31, Brucella	(22.8%)	(77.2%)
IS711, B. abortus	(68.7%)	(31.3%)
virB, T4SS	(72.7%)	(27.3%)
ure	(40%)	(60%)

Table 2: Detection of *B. abortus* and their virulence-associated genes (*vir*B and *ure*).

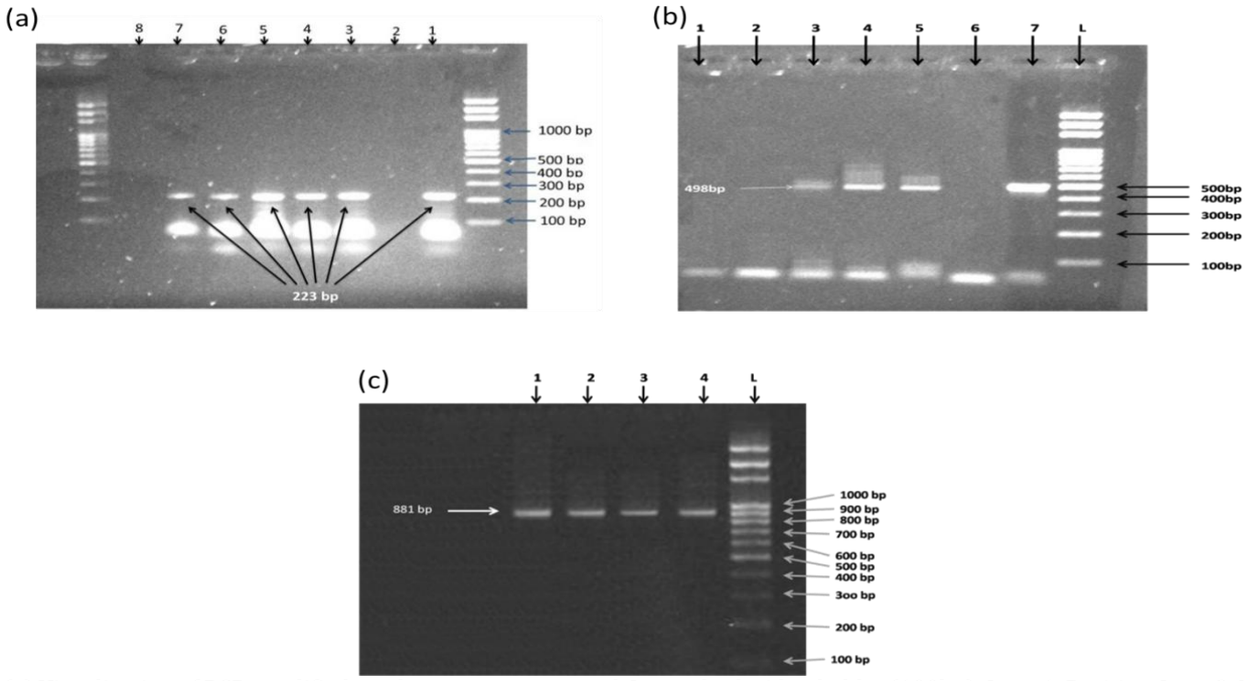

Figure 1: (a) Visualization of PCR amplified product on an agarose gel. Lanes: both sides, ladder (100bp); Lane 1, Positive; Lane 2, Negative control; Lane 3-7, samples. All the samples (3-7) showed positive PCR products for the *Bcsp*31 gene, with an amplicon size of 223 bp, (b) Visualization of DNA amplified fragments by gel electrophoresis. Lanes: L, Ladder (100bp); 7, positive control; 6, negative control; 1-5, samples. 1 and 2 show negative results while the remaining 3, 4 and 5 show a positive result for IS711 with an amplicon size of 498 bp, (c) Visualization of PCR amplified product on an agarose gel. Lanes: L, ladder (100bp); 1-4, samples. All the samples (1-4) showed positive results for the virB gene with an amplicon size.

Furthermore, 68.7% of the tested samples were identified as positive for *B. abortus*, with an amplicon size of 498 bp (figure 1b). (Garshasbi *et al.*, 2014) reported 73.8% for *B. abortus* showing results near to the current study. The virulence and pathogenicity of *Brucella*e species are mostly because of their astonishing ability to survive in unfavorable environmental situations encountered within the cell. *Brucella* has many virulent associated factors (Gándara *et al.*, 2001). These virulent factors are involved in pathogenesis and other important activities. Among the reported virulent factors, T4SS is one of the central virulence traits essential for the intracellular replication of the bacteria (Delrue *et al.*, 2004) . In addition to T4SS, Brucella's lipopolysaccharide (LPS) is a virulence factor that avoids substantial host immune response due to its distinct structure. The T4SS gene is one of several virulence genes that are regulated by the BvrR/BvrS two-component regulatory system. In order to infect and survive inside host cells, Brucella depends on these specialized systems rather than traditional virulence factors like exotoxins, capsules, or fimbriae (Głowacka *et al.*, 2018). The findings of the study revealed that 72.7% of the *B. abortus* positive samples were positive for *vir*B (figure 1c). A different percentage of *vir*B gene has been reported in the genome of *Brucella* spp, according to the previous studies (Ramadan *et al.*, 2019). The other important virulence factor involved in the pathogenicity of *Brucella* spp. is the urease enzyme coded by *ure* operon (Sangari *et al.*, 2007; Lin *et al.*, 2022). The urease hydrolyzes the urea and results from the formation of ammonium which helps the bacteria to survive in an acidic environment (Bandara *et al.*, 2007; Kornspan *et al.*, 2020).

The findings of the study revealed the frequency of *ure* gene of 40% in *B. abortus* which showed disagreement with the finding of Mirnejad *et al.* (2017). This difference in the results might be attributed to the number of tested samples, variation in the source, and prevalent strains in the under-investigated areas.

CONCLUSION: A high percentage of *B. abortus* (68.7%) was detected in the tested samples of cows for brucellosis. The prevalence of *vir*B virulent gene and *ure* genes were 72.7% and 40% respectively highlighting the potential virulence of the bacterium. Furthermore, it is recommended that public health authorities and veterinary officials set a national campaign for awareness and control of this hazard.

CONFLICT OF INTEREST: All the authors declared no conflict of interest.

LIFE SCIENCE REPORTING: In current research article no life science threat was reported

ETHICAL RESPONSIBILITY: This is original research, and it is not submitted in whole or in parts to another journal for publication purpose.

INFORMED CONSENT: The author(s) have reviewed the entire manuscript and approved the final version before submission.

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