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In Vivo Evaluation of *purD* Gene Deleted *Brucella abortus* in Mice as Potential Vaccine Candidate for Control of Brucellosis

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Brucella abortus, *purD* gene deletion, Mice evaluation, Attenuation, Histopathology

ABSTRACT

Brucella abortus (*B. abortus*) RB51 is a globally recognized and widely practiced vaccinal strain for the control of bovine brucellosis but has several reported drawbacks including pathogenic potential for humans and excreted in body fluids of vaccinated animals. In the present study, we constructed a more attenuated *B. abortus* Δ*purD* mutant by deleting the *purD* gene from the RB51 strain through site-directed mutagenesis. For virulence attenuation comparison with the parent RB51 strain, the constructed mutant was evaluated for attenuation estimation and clearance in BALB/c mice. The *B. abortus* Δ*purD* mutant exhibited significant attenuation of virulence and cleared from the mice spleen after 20th DPI when inoculated at the dose of 10^{8} CFU per mice. In contrast to this, the parent RB51 strain induces splenomegaly and showed higher persistence with significantly higher splenic CFUs in the mice group at 10th and 20th DPI as compared to *B. abortus* Δ*purD* mutant. The histopathological comparison of spleens from both groups also revealed much infiltration of giant macrophages in the *B. abortus* Δ*purD* mice group as compared to the parent RB51 mice group indicating superior immune response generation. The findings of this study revealed that the highly attenuated *B. abortus* Δ*purD* mutant can be used effectively as a potential live attenuated vaccine candidate for the control of bovine brucellosis both in local and international settings.

INTRODUCTION

Brucellosis is one of the prime and globally recognized infections of public health significance, disseminated to humans through direct contact with the diseased animal and by consumption of contaminated dairy and meat products of affected animals (Saeedinia *et al*., 2015). According to World Health Organization (WHO) annually more than 50,000 new cases of human brucellosis are reported all over the world (Ezama *et al*., 2018). Bovine brucellosis is caused by *Brucella abortus* and is characterized as a chronic infection targeting the reproductive system which leads to reproductive losses in the form of abortion in cattle and buffalo, reduced fertility in bulls, and, dreadful illness in humans (Ficht, 2003).

Previously, several vaccines have been developed and these vaccines are considered a key factor for the mitigation and control of bovine and ovine brucellosis in different disease control programs. Among these vaccines, live attenuated rough vaccine strain RB51 has been widely used in different geographical regions including Pakistan, both in the field and commercial dairy sector for control of bovine brucellosis (Schurig *et al*., 2002). Vaccination with RB51 strain does not generate immunoglobulins against the O-side chain of LPS in vaccinated animals, eliminating the serological issue of disease detection associated with smooth *B. abortus* strain S19 immunization (Olsen, 2000). In the past, the safety and stability of the RB51 strain have been investigated and published in different studies (Lord *et al*., 1998; Palmer *et al*., 1997).

However, the RB51 strain has several disadvantages including residual virulence, secreted in different body secretions, and could be isolated from vaccinated cows and buffalo fetuses, showing the possibility of enormous bacterial multiplication in the vaccinated animals (Galiero *et al*., 2006; Longo *et al*., 2009). The immunization with

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the RB51 strain also reported abortion in heifers, pregnant cows, and buffalo in many studies (Kreeger *et al*., 2000). In humans, the occupational and accidental exposure to the RB51 strain leads to severe local and systemic disorders (Ashford *et al*., 2004; Prevention, 2008). Therefore, the safety of the RB51 strain for human exposure is mainly undermined.

In this study, intending to enhance the safety of the RB51 vaccine, we developed a more attenuated mutant of the RB51 strain by deleting the *purD* (phosphoribosylamine-glycine ligase) gene through site-directed mutagenesis. The *purD* gene is involved in the de novo purine nucleotide biosynthesis pathway of the RB51 strain which has a pivotal role in the intracellular survival and replication inside the host. Further, in comparison to the parent RB51 strain, the Δ*purD* deleted RB51 mutant was characterized by evaluation in the mouse models to comprehend its attenuation, replication, clearance from the body, and splenic effects through histopathological investigations (Truong *et al*., 2015).

MATERIALS AND METHODS

Bacterial strain and media

The RB51 strain of *Brucella abortus* was taken from the Diagnostic Laboratory of Central Lab Complex (CLC), of University of Veterinary and Animal Sciences, Lahore, Pakistan (UVAS) and was used by adopting strict biosafety practices as recommended by OIE (2018) for the culture of *B. abortus*. The culture of BA15 was done in Tryptone Soy Agar (TSA, Oxoid Ltd, Basingstoke, UK) supplemented with rifampicin (250 µg/ mL) and 1% fetal bovine serum (Gibco, Life Technologies Ltd, Paisley, UK) for the period of 4-5 days in CO_2 incubator at 37°C (Saxena and Raj, 2018). Similar conditions were also adopted for culture in Tryptone Soy Broth (TSB, Oxoid Ltd, Basingstoke, UK) in CO_2 shaking incubator for 24 h at 37° C and 150 rpm culturing conditions (Sergueev *et al*., 2017). Initially, the single isolated colonies were identified for characteristic *B. abortus* colony morphology and further processed for Gram staining technique for confirmation of Gram-negative and coccobacilli features of *B. abortus*. Afterwards, the molecular confirmation was done through PCR by using primers listed in Table1 targeting the *IS711* repetitive region of the bacterial genome by adopting conditions published by (O'Leary *et al*., 2006).

Construction of purD *gene deletion cassette*

For the construction of the purD gene deletion plasmid cassette (pUC19-Kana-Up-Down) we choose kanamycin (K) resistant pUC19 (Thermoscientific, Vilnius, Lithuania) cloning vector and transformed it into *Escherichia coli* DH5α competent cells and positive colonies were screened on kanamycin supplemented (30µg/mL) LB media agar plate. For the cloning of the K gene into the pUC19 vector, the K sequence was amplified through PCR by using the pEP- kan DNA Template (Raza *et al*., 2016) and K-specific primers as mentioned in Table I and confirmed on agarose gel electrophoresis and confirmed amplicons were gel purified (Gene JET Gel Extraction and DNA Cleanup Micro Kit, Thermoscientific, USA). The K purified PCR product and pUC19 were subjected to restriction enzyme digestion analysis by using *KpnI* (Thermoscientific, Vilnius, Lithuania) and *BamHI* (Thermoscientific, Vilnius, Lithuania) restriction enzyme and confirmed on agarose gel electrophoresis. Afterward, K and pUC19 confirmed restriction enzyme digested products were gel purified and ligated by using T4 DNA Ligase (Thermoscientific, Vilnius, Lithuania) and transformed into *E. coli* DH5α cells which were further screened on K supplemented LB agar plat and named as pUC19-Kanamycin.

Table I. List of primers used in this study.

Primer	Name	Sequence (5'to 3')	Product size (bp)	References
Br. abortus	B.A (F)	GACGAACGGAATTTTTCCAATCCC	498	(O'Leary *et al.*, 2006)
	B.A (R)	TGCCGATCACTTAAGGGCCTTCAT		
Kanamycin	KM (F)	GC**GGTACC**TAGGGATAACAGGGTAATCGATTT **(Kpn1)**	1004	(Raza *et al.*, 2016)
	KM (R)	CG**GGATCC**GCCAGTGTTACAACCAATTAACC **(BamH1)**		
Upstream	UP (F)	GC**GAATTC**TCCTGATCGACCAGATCATTATAG **(EcoR1)**	492	(Riaz *et al*., 2022)
	UP (R)	CG**GGTACC**CATGCCTTGCTCCCTGCGCTTAAGATC**(Kpn1)**		
Downstream	DN(F)	GC**GGATCC**TGATCGGTTTATGTTTCAGGTTACATG **((BamH1)**	482	(Riaz *et al*., 2022)
	DN(R)	CG**CTGCAG**TCGCCGTGGCTTCGACCGTCACGT(**PstI)**		
purD	PD(F)	AACTGCAGGATGAAAGTTCTGTTGATC	1280	(Truong *et al.*, 2015)
	PD(R)	GCTCTAGAGTCAGCGATTAGCCTTCTCA		

Bold letter indicates sequence of restriction sites inserted.

Cloning of upstream and downstream sequences into purD *gene deletion cassette*

For deleting the purD gene we designed two sets of primers to amplify DNA fragments in the upstream (Up) and downstream (Down) regions of the *purD* gene of *B. abortus* for cloning into the *purD* gene deletion cassette. The primer sequences of both upstream and downstream are shown in Table I. The purified DNA fragments of Up sequence and pUC19-Kanamycin were subjected to *KpnI, EcoRI* (Thermoscientific, Vilnius, Lithuania) restriction enzyme digestion and cloned into *E. coli* DH5α cells, and the resulting plasmid was isolated from transformed *E. coli* and confirmed through restriction enzyme digestion and named pUC19-Kanamycin-Up. Similarly, the purified DNA fragment of Down sequence and pUC19-Kanamycin-Up plasmid were subjected to *BamHI*, and *PstI* (Thermoscientific, Vilnius, Lithuania) restriction enzyme digestion and cloned into *E. coli DH5α* cells, and the resulting plasmid was isolated from transformed *E. coli* and confirmed through restriction enzyme digestion and named pUC19-Kanamycin-Up-Down.

Construction and confirmation of B. abortus ΔpurD *mutant*

The confirmed pUC19-Kanamycin-Up-Down gene deletion plasmid cassette was introduced into electrocompetent *B. abortus* cells prepared by adopting the method of (McQuiston *et al.*, 1995) and electroporation conditions as per the protocol described previously (Lalsiamthara *et al*., 2020). The *B. abortus* Δ*purD* mutant was screened on kanamycin supplemented TSA agar plate and final confirmation was done through PCR by detecting *B. abortus*, Kanamycin, and *purD* sequence by using primers listed in Table I.

Evaluation of B. abortus ΔpurD *mutant in bagg and albio (BALB/c) mice*

For the evaluation of *B. abortus* Δ*purD* mutant seven-week old female BALB/c mice were purchased from the Department of Theriogenology, UVAS, and kept in three groups in individually ventilated cages in Animal House of Institute of Microbiology, UVAS. The two mice groups including (n= 10 per group) RB51 and *B. abortus* Δ*purD* were intraperitoneally (i.p.) inoculated with standardized $3X10^8$ CFU dose of the vaccinal RB51strain and *B. abortus* Δ*purD* mutant in 0.2 mL of PBS (OIE, 2018) by adopting the method of (Truong *et al*., 2016). As a control, one mice group (n=10) was intraperitoneally inoculated with sterile PBS.

Virulence of B. abortus ΔpurD *mutant in BALB/c mice*

The clearance of the RB51 and *B. abortus* Δ*purD* mutant was estimated by calculating the splenic bacterial colony-forming units (CFUs) in both groups as per the protocol described previously (Truong *et al*., 2015). On the 10^{th} and 20^{th} day post-inoculation (DPI), four mice from each group were sacrificed by following with the Institutional Guidelines of Ethical Review Committee, University of Veterinary and Animal Sciences and their spleens were removed and processed aseptically. The splenic weights were recorded and homogenized in sterile PBS (1 X) solution because of its isotonic and nontoxic nature and processed for ten-fold serial dilutions. The splenic homogenates were plated on TSA plates supplemented with kanamycin and purine bases including 1mM adenine, 1mM guanine, 1mM hypoxanthine, and 0.05 mM thiamine (Sigma-Aldrich, Merck, Darmstadt, Germany) by adopting the method of (Truong *et al*., 2015). The TSA plates were incubated at 37°C for 5 days and splenic CFUs were estimated to determine virulence and colonization efficiency of both parent RB51 and *B. abortus* Δ*purD* mutant. The CFU results are expressed as the mean ± standard deviation of the $\log_{10}$ (log) for each group of mice.

Investigations of histopathological changes in BALB/c mice spleens

For the analysis of histopathological changes, spleens from each group were taken and processed in the Histopathology Lab, Department of Pathology, UVAS. The cutting of splenic tissues was done into suitable segments. After this, the tissues were fixed in a 10% neutral buffered formalin solution and subjected to multiple steps involving dehydration, clearing, and infiltration. After these steps blocks were prepared and 0.4 µm thick tissue slices were produced. In the final stage, these tissue sections were subjected to Hematoxylin and Eosin staining by adopting the previously described method (Bancroft *et al*., 1994). The slides were examined by using software DP20 under 40X objective lens of Microscope (Olympus CX31).

Statistical analysis and software used

The graphical maps of the pUC19-Kanamycin up down gene deletion cassette were constructed by using the software SnapGene 6.0.2. The data of the splenic weight and CFUs were analyzed by using the software GraphPad Prism 5. Analysis of variance (ANOVA) test was applied for comparison of *B. abortus* Δ*purD* group with RB51 group. The *P*-value < 0.05 was considered statistically significant.

RESULTS

Colony morphology and molecular confirmation of Brucella abortus

After 4-5 days of incubation, *B. abortus* showed

characteristics small, circular colonies with smooth margins and yellow honey color morphology on TSA media agar. The single colonies were further processed for Gram staining and under (100 X) oil immersion lens showing pink color coccobacilli appearance. The final confirmation was done through PCR by targeting the *IS711* repetitive region of the bacterial genome indicating 498 bp band size on agarose gel electrophoresis.

Confirmation of pUC19-kanamycin-up-down gene deletion plasmid cassette

The pUC19-Kanamycin-Up-Down gene deletion plasmid cassette was constructed through the schematic manner (Fig. 1). The pUC19 cloned *E. coli* DH5α colonies plasmid confirmed pUC19 insertion by showing 2686 bp band size on agarose gel (Fig. 1B1). The Kanamycin sequence amplified DNA fragment showed 1004 bp band size (Fig. 1C1) and Kanamycin cloning into pUC19 transformed DH5α was confirmed through pUC19-Kanamycin transformed DH5α colonies plasmid restriction enzyme digestion showing two bands of 2686 bp of pUC19 and 986 bp of Kanamycin on agarose gel (Fig. 1C2). The UP fragment sequence amplified product showed 492 bp size (Fig. 1D1) and Up sequence insertion into pUC19-Kanamycin plasmid was confirmed via restriction enzyme digestion of pUC19-Kanamycin-up transformed *E. coli DH5α* colonies exhibiting two bands 2686 bp of pUC19 and 1455 bp of Kanamycin-Up fragments on agarose gel (Fig. 1D2).The down fragment sequence amplified product showed 482 bp size (Fig. 1E1) and down sequence cloning into pUC19-Kanamycin-Up plasmid was confirmed via restriction enzyme digest analysis of pUC19-Kanamycin Up–Down transformed *E. coli* DH5α colonies indicating two fragments of 2686 bp of pUC19 and 1909 bp size (Fig. 1E2) of Kana-Up-Down on agarose gel electrophoresis.

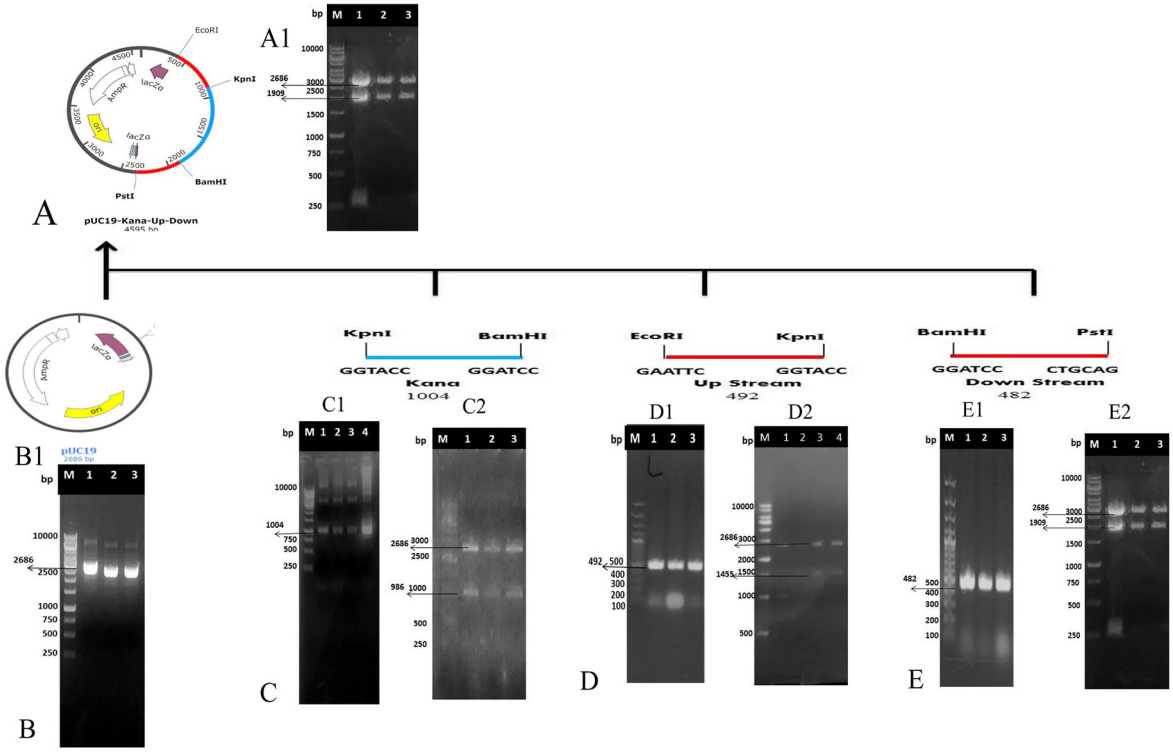

Fig. 1. Construction and confirmation of purD gene deletion plasmid cassette "pUC19-Kanamycin-Up-Down". (A) Illustration of map of purD gene deletion plasmid cassette pUC19-Kanamycin-Up-Down (A1) confirmation of pUC19-Kanamycin-up-down gene deletion plasmid cassette (B) illustration of map of pUC19 plasmid (B1) confirmation of pUC19 cloning in DH5α cells (C) illustration of Kanamycin fragment with restriction sites KpnI (GGTACC) and BamHI (GGATCC) (C1) amplification of Kanamycin fragment. (C2) Confirmation of Kanamycin cloning in pUC19 (D) Illustration of Up sequence fragment with restriction sites EcoRI (GAATTC) and KpnI (GGTACC) (D1) amplification of upstream fragment (D2) confirmation of up sequence cloning into pUC19-Kanamycin plasmid (E) Illustration of downstream fragment with restriction sites BamHI (GGATCC) and PstI (CTGCAG) (E1) amplification of downstream fragment (E2) confirmation of Downstream fragment into pUC19-Kanamycin-up.

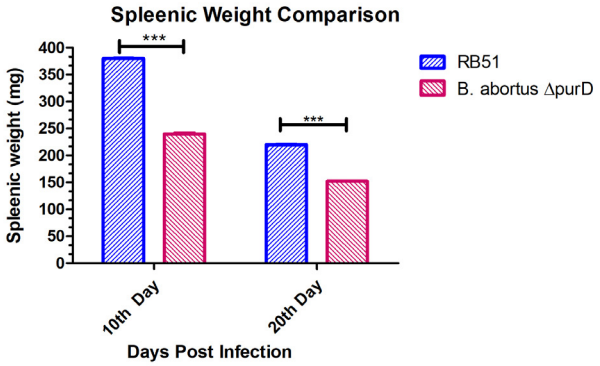

Fig. 2. Comparison of splenic weight between RB51 and B. abortus ΔpurD mutant mice groups. Splenic weight comparison of the RB51 and B. abortus ΔpurD mutant mice groups infected with 3 X108 CFU/ dose of the RB51 and B. abortus ΔpurD mutant at 10th and 20th DPI. Error bars represent average values ± the standard deviations. The asterisks denote values that are significantly different (*P <0.05) between groups with the parent RB51 and the B. abortus ΔpurD mutant at each time point, as determined by two away ANOVA.

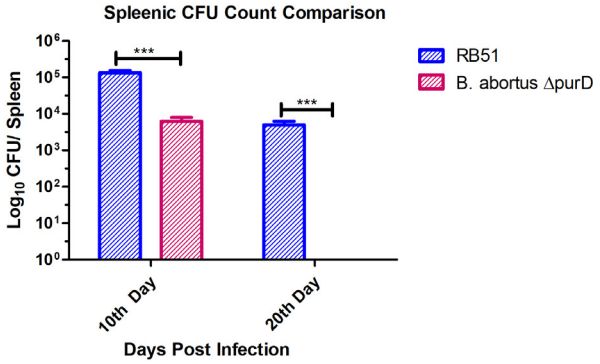

Fig. 3. Comparison of Splenic CFUs between RB51 and B. abortus ΔpurD mutant mice groups. Clearance of the RB51 and B. abortus ΔpurD mutant in mice. Recovery of viable bacterial CFUs count from spleens of mice at 10th and 20th DPI. Results are expressed as total CFU per spleen. Error bars represent average values ± the standard deviations. The asterisks denote values that are significantly different (*P <0.05) between groups with the parent RB51 and the B. abortus ΔpurD mutant at each time point, as determined by two way ANOVA.

BALB/c mice splenic weight estimation

The splenic weights of both groups were also recorded for comparison and estimation of splenomegaly at 10th and 20th DPI (Fig. 2). The weighted analysis revealed that the mice group inoculated with *B. abortus* Δ*purD* mutant exhibited significantly lower splenic weight at both time intervals in comparison to the other group inoculated with RB51, as was apparent by the lack of splenomegaly due to its less persistence and rapid clearance from spleen in comparison to parent RB51 strain. These findings indicate that the *B. abortus* Δ*purD* mutant is more attenuated than the RB51 parent strain and also support our presumption that the *purD* gene of the purine biosynthesis pathway played a crucial role in the survival of RB15 in mice host.

Virulence of B. abortus ΔpurD *mutant in (BALB/c) mice*

The splenic CFUs count in both RB51 and *B. abortus* Δ*purD* showed significant differences at 10th and 20th DPI. The *B. abortus* Δ*purD* mutant enormously affected bacterial persistence in the spleen as compared to the parent RB51 strain by showing significantly lower CFUs count in the spleen (3.90-log CFU; P <0.001) in comparison to the parent RB51 strain (5.12-log CFUs) at 10th DPI. At 20th DPI, splenic CFUs decreased distinctly as no CFUs were observed in the *B. abortus* Δ*purD* mutant mice group. However, 3.85 log CFUs counts were recorded from the spleens of the mice group inoculated with RB51strain as shown in (Fig. 3).

B. abortus ΔpurD *mutant induced splenic histopathological changes*

The splenic tissue histopathological investigation of the RB51 mice group revealed atrophy of follicular area and dead tissue masses with few giant cells in the focused area in the figure (Fig. 4A). However, in the *B. abortus* Δ*purD* mutant mice group, the spleen showed numerous phagocytic cells with phagocytized bacterial cells inside with much infiltration of lymphocytes observed in the area focused in the figure (Fig. 4B). In the spleen of the control group, no tissue changes were observed and normal tissue parenchyma was noticed when observed (Fig. 4C).

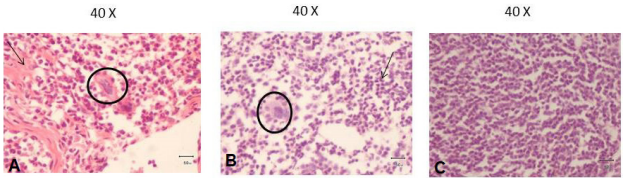

Fig. 4. Representative images of histopathology of the RB51 and B. abortus ΔpurD mutant in mice. Six out of eight mice from RB51 mice group spleen showed atrophy of follicular area and dead tissue mass is also seen in the (arrow) with few giant cells are also present (circle) (A). Six mice out of eight from B. abortus ΔpurD mice group spleen showed giant phagocytic cells with phagocytized bacteria were seen (circle) and higher infiltration of lymphocytes (arrow) (B). Control mice group spleen showed no tissue changes and almost normal tissue parenchyma was seen (C).

DISCUSSION

Even though *B. abortus* RB15 live attenuated strain is widely used and recommended vaccinal strain for the mitigation and control of bovine brucellosis all over the globe. However, this strain has several documented side effects and limitations reported in both animals and human beings (Arellano-Reynoso *et al*., 2004; Yazdi *et al*., 2009). Therefore, the prime objective of this study was to construct and evaluate a gene-deleted *B. abortus* mutant by targeting a crucial gene responsible for intracellular replication and survival inside the host by improving the safety and enhancing the attention of the RB51 strain. To our knowledge, this is the first study in Pakistan reporting the *in vivo* evaluation of gene deleted *B. abortus* in mice model in comparison to the parent RB51 strain.

In our study, we targeted the *purD* (phosphor ribosylamine-glycine ligase) gene which played a key role in bacterial *de novo* purine biosynthesis during its replication in the targeted host. In several bacterial species, the mutations in *de novo* purine biosynthesis pathways have been studied, and established that these pathways played a critical role in bacterial virulence and survival inside host cells. The search for different virulence factors in *Brucella* has recognized several purine biosynthesis genes contributing to replication and survival within the host (Drazek *et al*., 1995; Kim *et al*., 2003). One of the studies reported that mutation in the *purE* gene outstandingly attenuate the *B. melitensis* in goat and mice and, protection against brucellosis with this mutant's vaccine recommended that purine auxotrophy could be a prime choice to construct and develop more attenuated and much safer vaccines for brucellosis mitigation (Drazek *et al*., 1995).

In this study, kanamycin gene sequence insertion in the *purD* gene of *B. abortus* showed auxotrophy for *de novo* purine bases, which are the prime source of DNA and RNA nucleotide synthesis and imperative for Brucella proliferation and survival inside the host system (Köhler *et al*., 2002). We developed a *B. abortus* Δ*purD* mutant by constructing a pUC19-Kanamycin-Up-Down gene deletion plasmid cassette and transfecting it into electrocompetent *B. abortus* cells through electroporation. Before the construction of the plasmid cassette, the RB51 strain was cultured and its final confirmation was done through PCR against the *IS711* repetitive region similar to the study published on biocontrol of *B. abortus* (Shaheen *et al*., 2021).

The pUC19-Kanamycin-Up-Down gene deletion plasmid cassette was constructed by cloning the pUC19 vector into *E. coli DH5α* cells and transformed *DH5α* colonies plasmid size 2686 bp confirming transformation similar to the results reported by (Schweizer, 1991). The kanamycin gene amplification was done through PCR on gel electrophoresis and the amplicon size 1004 bp was similar to a previous study published by (Raza *et al*., 2016). The *purD* gene upstream and downstream sequences were amplified, cutted, and inserted into the previously transformed pUC19-Kanamycin vector and confirmed on gel electrophoresis allied to mentioned (Saeedinia *et al*., 2015). The *B. abortus* Δ*purD* mutant was constructed successfully by transfecting constructed *purD* gene deletion plasmid cassette into *B. abortus* electrocompetent cells and mutant colonies characteristics on TSA agar were quite similar to an earlier study published by (Lalsiamthara *et al*., 2020). The final confirmation of constructed *B. abortus* Δ*purD* mutant was done through PCR with no band of *purD* gene in the deleted mutant observed which was similar to the preceding study reported by (Truong *et al*., 2015).

In the present study, we also aimed to evaluate *B. abortus* Δ*purD* mutant persistence and attenuation status in the mice model in comparison to the parent RB51 strain. The constructed *B. abortus* Δ*purD* mutant showed remarkably reduced splenomegaly at both 10th and 20th DPI as compared to the mice group inoculated with RB51 strain. Furthermore, distinctly reduced splenomegaly linked with mice inoculation recommended that *B. abortus* Δ*purD* mutant have higher attenuation for virulence as compared to the parent RB51 strain, which instigates splenomegaly in the RB51 mice group and these findings are similar to one of the studies published by (Truong *et al*., 2016).

Moreover, the splenic persistence evaluation results of both groups revealed that *B. abortus* Δ*purD* mutant showed minimal persistence in spleen and was cleared from mice spleen at 20th DPI as compared to RB51 mice group which showed higher splenic persistence even at 20th DPI. Additionally, the splenic CFUs count was quite lower in the *B. abortus* Δ*purD* group at 10th DPI with no CFUs count at 20th DPI, while high splenic CFUs count was recorded in the RB51 mice group at both 10th and 20th DPI indicating lower attenuation status of RB51 strain as compared to *B. abortus* Δ*purD* mutant which coincides with the findings of (Truong *et al*., 2015).

In this study, we also investigated histopathological changes within the spleen of all three mice groups. In the RB51 mice group, splenic changes were characterized by a lower degree of aggregates of macrophage giant cells with some degree of the atrophic area also noticed during microscopic analysis. In the *B. abortus* Δ*purD* mutant group a higher degree of aggregates of giant macrophage infiltration and tissue, spaces are well occupied by these infiltrating giant bacterial engulfed cells which

closely resembled the findings published by (Stranahan *et al*., 2019). In the control group without any bacterial inoculation, no cellular changes were noticed and normal parenchyma was observed.

CONCLUSION

These findings collectively stipulated that higher attenuation and lower splenic persistence inside the host make *B. abortus* Δ*purD* mutant an attractive and suitable live attenuated vaccinal candidate from a safety and virulence attenuation point of view for control of bovine brucellosis all over the globe.

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IRB approval

The study was approved by Advanced Studies and Research Board (ASRB), UVAS (DAS/1072/250518).

Ethical statement

The housing and handling of lab animals during animal experimentation was performed in accordance with the University of Veterinary and Animal Sciences, Lahore guidelines for the care and use of lab animals. All procedures were approved by the Ethical Review Committee of University of Veterinary and Animal Sciences, Lahore (Permit Number 442-291021).

Statement of conflict of interest

The authors have declared no conflict of interest.

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