

Original Research Article

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Expression of *Brucella* Lumazine Synthase Gene in *E. coli* System

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ABSTRACT

Brucellosis is an important zoonosis of serious economic and public health consequences affecting livestock and human beings caused by *Brucella* spp. The available whole cell antigen and smooth lipopolysaccharide (sLPS) based diagnostics show false positive reactions due to cross reactivity. Therefore, in this study, *Brucella* lumazine synthase (BLS), an immunogenic protein of *Brucella* spp. coding 477 bp *bls* gene was amplified from DNA extracted from *Brucella abortus* S99 strain and the amplified product was cloned in pET directional TOPO expression vector and transformed in *E. coli* TOP 10F' cells. After verifying sequence, *bls* recombinant clones were subsequently transformed in *E. coli* BL21 (DE3)/pLysS cells for expression. BLS protein expression was induced with 1mM IPTG and optimized, six hours induction yielded maximum expression of BLS protein. The expressed protein was purified by His-tag affinity purification method using Ni-NTA and characterized by SDS-PAGE and Western blot. Recombinant BLS protein expressed in *E. coli* has potential use as diagnostic antigen or immunogen for development of diagnostics and vaccine for brucellosis.

Keywords

Brucellosis, *Brucella* Lumazine Synthase (BLS), Recombinant protein, TOPO vector.

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Introduction

Brucellosis is an important zoonosis of serious economic and public health consequences affecting livestock and human beings, caused by a bacteria belonging to alpha proteobacteria. *Brucella* comprises of six classical species *Brucella abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* (Moreno, 2001) and they cause diseases in cattle, sheep and goats, pigs, sheep, dogs and desert wood rats respectively. Most of the times, specific *Brucella* species infects specific host, but they can also infect

non-natural hosts such as *B. abortus* infection in swine, *B. melitensis* in cattle and swine, and *B. suis* in cattle (Cook and Noble, 1984; Verger, 1985). The species that may infect man are *B. melitensis*, *B. suis*, *B. abortus*, and *B. canis* and species of main concern in India are *B. melitensis*, and *B. abortus* (Mantur and Amarnath, 2008). Globally, the annual occurrence of brucellosis cases were more than 500,000 cases (Pappas *et al.*, 2006) and the disease is transmitted from animals to human by contact with infected animals,

consuming milk and milk products from infected animals. In India, prevalence of brucellosis in animals reportedly varied from as low as 0.13 % (Chatterjee *et al.*, 1986) to as high as 44 % (Zaki *et al.*, 1975) In India, brucellosis costs Rs 350 million in the form of food animals and man days of labour (Tiwari *et al.*, 2013).

Brucellosis can be treated in human beings by prolonged treatment whereas not recommended in animals due to the high cost of treatment and favorable outcomes happen too infrequently. Vaccines are available for cattle and sheep and goat whereas no vaccines are available for human beings. The vaccines for cattle include *B. abortus* S19 and RB51 and for sheep and goat *B. melitensis* Rev1 strain (Olsen and Palmer, 2014). The vaccines used are live vaccines and risk exists for human beings while handling them at production levels and at vaccination points since these vaccine strains can cause infection in human beings. The disease is diagnosed by isolation of the organism, serological and molecular tests. Isolation of the organism is tedious and risky. Serological tests are the most widely used tests in the field for diagnosis of brucellosis whereas molecular tests are carried out in institutions and high end diagnostic laboratories (Nagalingam *et al.*, 2012). The serological test include Rose Bengal Plate test (RBPT), Enzyme Linked Immuno Sorbent Assay (ELISA), Complement Fixation Test (CFT), Serum Tube Agglutination Test (SAT), Fluorescence Polarization Assay (FPA) and Milk Ring Test (MRT). Most of the serological tests are based either on whole antigen or smooth lipopolysaccharide (sLPS) (OIE, 2009). False positive results may occur due to antibodies against closely cross reacting organisms (Al Dahouk *et al.*, 2003). As *Brucella* is a biosafety level 3 (BSL 3) organism, handling of *Brucella* in the laboratory for the production of vaccines and diagnostics is risky. Hence recombinant protein based

diagnostics and vaccines are being developed to address the aforesaid problems. One such novel protein is *Brucella* lumazine synthase (BLS) which has shown promising results to use in the diagnosis (Goldbaum *et al.*, 1993) and as vaccine and adjuvant due to its high immunogenicity. BLS is an ~17 KDa cytoplasmic protein of *Brucella* spp. and this protein has low but significant homology (30%) with lumazine synthases involved in bacterial riboflavin biosynthesis. In this study an attempt has been made to express BLS protein of *B. abortus* S99 in *E. coli* system using TOPO vector which can be further used either in vaccine and diagnostics development.

Materials and Methods

***B. abortus* S99 strain**

B. abortus S99 strain was procured from Indian Council of Agricultural Research – Indian Research Veterinary Institute (ICAR-IVRI) and maintained at ICAR-NIVEDI. The culture was grown in Tryptose soya agar at 37°C for 48 hrs. The organisms were identified for their morphology by Gram's staining.

DNA extraction

The DNA was extracted from *B. abortus* S99 culture using QIAamp DNA mini Kit (Qiagen, Germany) following the kit procedures. The purity and concentration of the DNA were estimated by spectrophotometer and the extracted DNA was stored at -20°C for further use.

PCR reaction

The *bls* gene is of 474 bp in length coding for protein of molecular weight of ~18 kDa. The primers were designed (F-5'-CACCGGATC CCACCATCACCATCACCATATGAACCA AAGCTGTCCGAACAAGAC-3'; R-5'- GC

ATGCGGCCGCATGGTGATGGTGATGGT GGACAAGCGCGGCGATGCG -3') for TOPO as well as other vectors for producing recombinant protein from *bls* gene sequences. PCR was carried out for amplifying *bls* gene from DNA extracted from *B. abortus* S99 culture. PCR reaction mixture comprised of 10 X PCR buffer- 5 µl; Forward and reverse primers 10 µM each 1 µl; dNTP (10 mM)-1 µl; DNA 2 µl (94 ng); High fidelity *Taq* polymerase 0.5 µl (Fermentas, USA). and nuclease free water was added to make up 50 µl. PCR reaction was performed in the thermal cycler (Eppendorf, Germany) with initial denaturation of 94°C for 3min, followed by denaturation, annealing, extension with 94°C, 50-60°C and 72°C respectively with each step for 1min for 35 cycles and final extension was done with 72°C for 10 min. The amplified product was run on 1.5 % agarose gel containing ethidium bromide (0.5 µg/ ml) and visualized at 300 nm wavelength and documented in UV trans-illuminator.

Cloning in TOPO vector

Cloning of *bls* gene of *B. abortus* S99 in pET directional TOPO expression vector (pET100/D-TOPO) was carried out by setting up the reaction with fresh PCR product 2 µl; salt solution 1 µl; TOPO vector 1 µl and made up the volume to 6 µl with sterile water, mixed gently and incubated at room temperature for 5 min. Then 3 µl of cloning reaction mixture was added to TOP10F^c chemically competent *E. coli* cells, mixed gently and incubated on ice for 30 min. Then the cells were heat-shocked for 30 s at 42°C followed by transfer of the tube to ice. After two min, 250 µl of S.O.C. medium was added at room temperature and kept at shaker incubator at 37°C for 1 hr at 200 rpm. Then 100 µl was spread on a pre-warmed ampicillin containing plate and incubated overnight at 37°C. Next day, each clone was subjected to

colony PCR and from confirmed clones having the insert, two clones were selected further and plasmid was extracted and nucleotide sequencing was carried out commercially.

Expression of BLS protein

After the sequence was confirmed and ensuring the gene sequence was in frame with vector, plasmid DNA was transformed into *E. coli* BL21 (DE3)/pLysS cells, and confirmed by colony PCR for the presence of the insert. Four confirmed clones were grown individually in LB broth containing ampicillin(50µg/ml) at 37°C till the culture reached mid log phase or obtaining an OD_{600nm} of 0.4 – 0.5. The expression was induced at 37°C using 1mM isopropyl-β-D-thiogalactoside (IPTG). Samples were collected at 0, 2, 4 and 6 h post induction and were analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method described by Laemmli (1970), with some modifications. The electrophoresis was carried out with 12 per cent separating gel and five per cent stacking gel in Tris-Glycine buffer on a Vertical slab gel electrophoresis system (Bio Rad, Germany) using a constant voltage of 100 V.

The samples were boiled at 100°C for five min in 5x loading buffer prior to loading. The samples were loaded along with molecular weight marker to determine the molecular size of the loaded protein samples. Once the electrophoresis was over, the gel was stained with Coomassie brilliant blue (R 250) for 30 min and destained with destaining solution. Purification of the recombinant protein was performed with a Nickel-nitrilotriacetic acid (Ni-NTA) affinity column (Qiagen, Germany) as per the manufacturer protocol. The purified BLS protein was further analyzed by SDS-PAGE and Western blot.

Western blot

For Western blot, the SDS-PAGE gel was washed in transfer buffer for three times at five min intervals and placed over the nitrocellulose membrane (NCM) which was placed over three sheets of Whatman No. 3 filter papers, soaked in transfer buffer at the anode plate of the Western blot apparatus. Further the gel was covered with three sheets of filter papers and cathode plate was placed in position and transfer of proteins was done at 10 V for 45 min. At the end of transfer, the NCM was separated out and the gel was stained to check for the efficiency of protein transfer. The NCM was immersed in blocking buffer and incubated overnight at 4°C. Next day, NCM was incubated on rocker at 37°C for one hr. The NCM was washed three times using wash buffer and placed in brucellosis positive cattle serum (Pourquier, IDEXX, USA) which was diluted in 1:100 dilution and kept at 37°C for one hr. Then the membrane after washing was placed in anti-bovine HRP conjugate (1:4000) prepared in blocking buffer and incubated at 37°C for one hr. The NCM was washed three times and transferred to 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution and incubated in dark about 10 min or until the desired band intensity was achieved. The reaction was stopped by washing the NCM with excess of distilled water and then the results were documented.

Results and Discussion

Brucellosis is a complex zoonotic disease with significant epidemiological, economic, and global health impact affecting wide variety of livestock, wildlife and humans. For better surveillance and control of the disease, newer innovative ideas and techniques are being explored and development of recombinant protein based diagnostics and vaccine is one among them. This study

targeted the production of recombinant protein of *bls* gene of *B. abortus* S99 under prokaryotic expression system. The culture of *B. abortus* S99 has grown well and Gram's staining (Fig. 1) showed gram negative cocco bacilli. The DNA extracted was containing 94ng/ µl (100 µl from 5ml culture) and the purity was 1.87. The normal ratio of A260/A280 is 1.8 for pure DNA, the DNA extracted in this study was considerably pure. While designing primers, in addition to the requirements for TOPO cloning, *EcoRI* site in the forward primer and *NotI* RE site in the reverse primer were included. Also His tags (six consecutive Histidine amino acids) were included in both forward and reverse primers so that even His tag from any one of the terminal (N or C) is masked, His tag from other terminal is able to bind to the Ni-NTA resin for purification of protein. In addition, N terminal of vector also contains His tag.

The amplified PCR product matched with the *bls* gene size of 477 bp (Fig. 2). Cloning of amplified product with TOPO vector and transformation in Top 10F' cells yielded many clones and colony PCR identified the positive clones (Fig. 3). The pET directional TOPO expression system utilizes a highly efficient, 5-minute cloning strategy to directionally clone a blunt-end PCR product into a vector for high-level, T7-regulated expression in *E. coli*. In this system, PCR products are directionally cloned by adding four bases to the forward primer (CACC). The overhang in the cloning vector (GTGG) invades the 5'end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation (Invitrogen, 2006). The directional cloning is greater than 90%, with no ligase, post-PCR procedures, or restriction enzymes required. This cuts down our cost of research expenditure in addition to ample reduction in time.

Fig.1 *B. abortus* S99 strain stained with Gram's staining and observed under microscope (100 X)

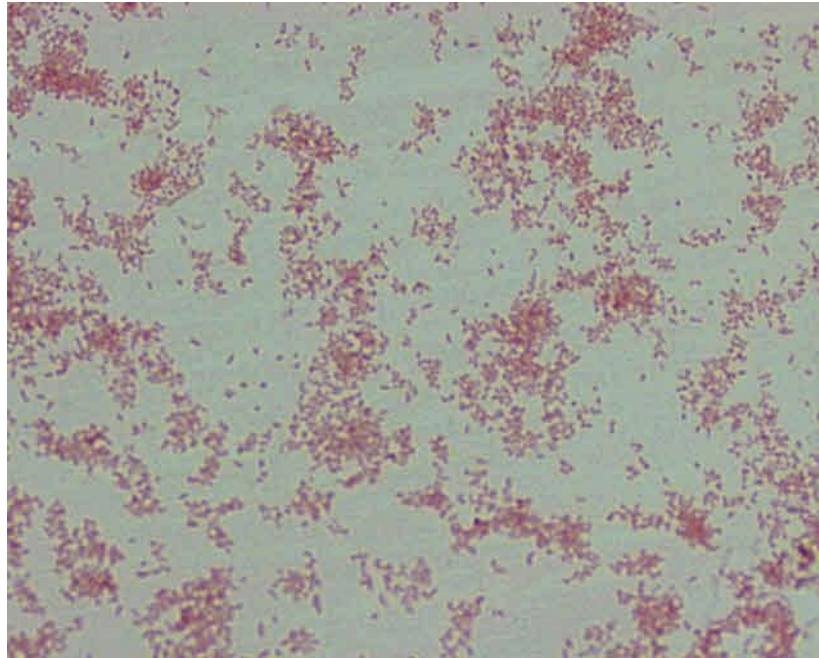
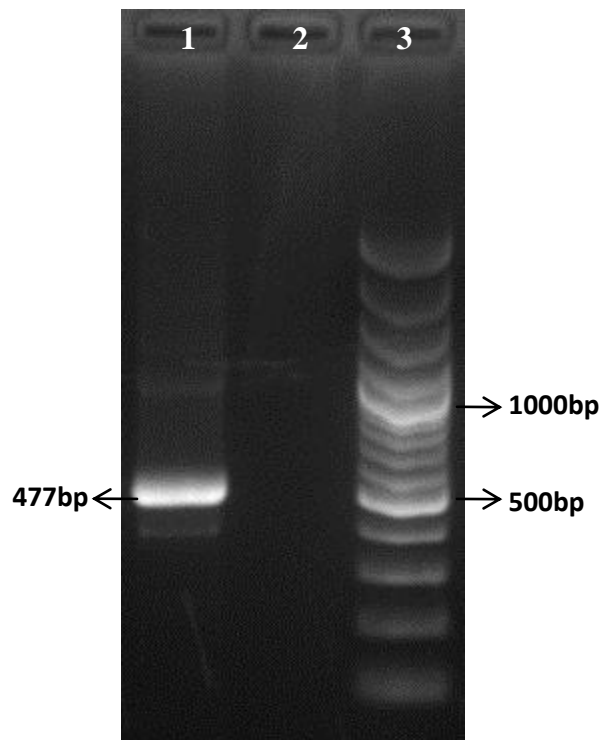
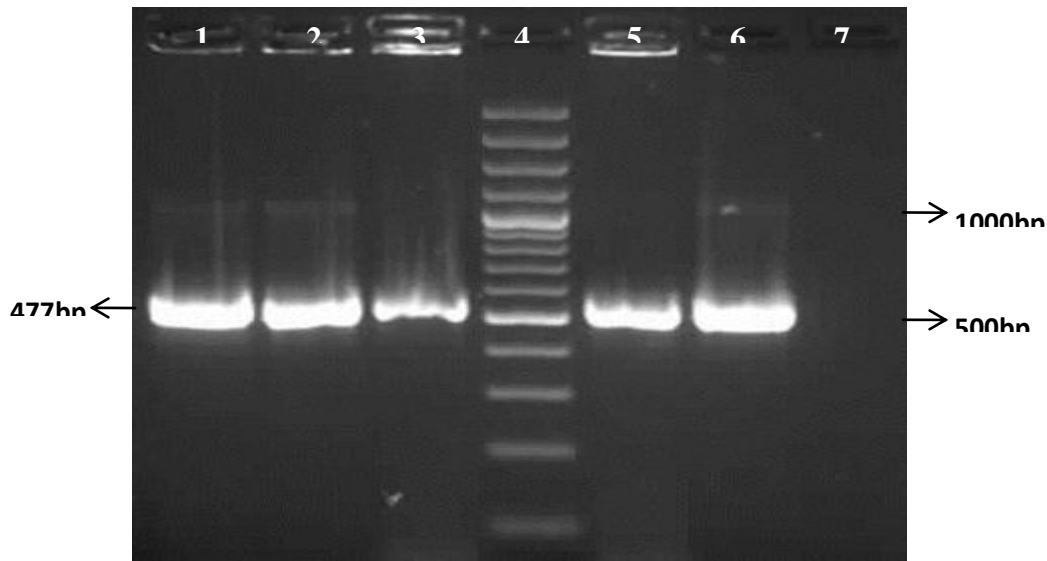


Fig.2 Agarose gel electrophoresis showing PCR amplicon of *bls* gene from *Brucella abortus* S99 DNA



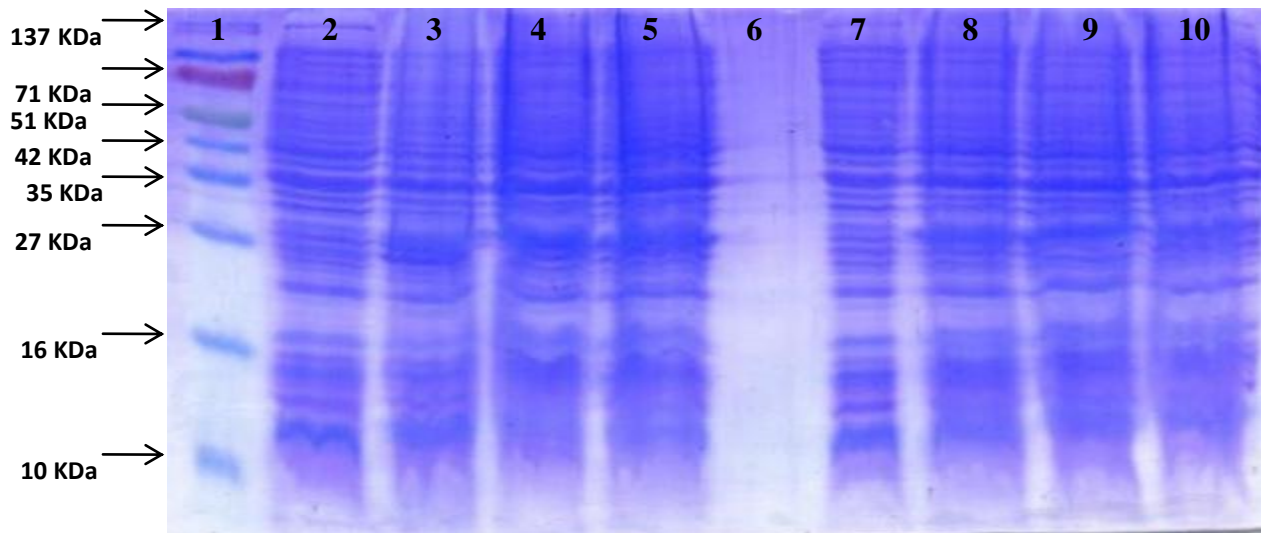
Lane1: PCR amplicon of *bls* gene;
Lane2: Non template control;
Lane3:100bp plus DNA ladder (Fermentas)

Fig.3 Colony PCR profile of *bls* gene cloned in pET100/D-TOPO vector



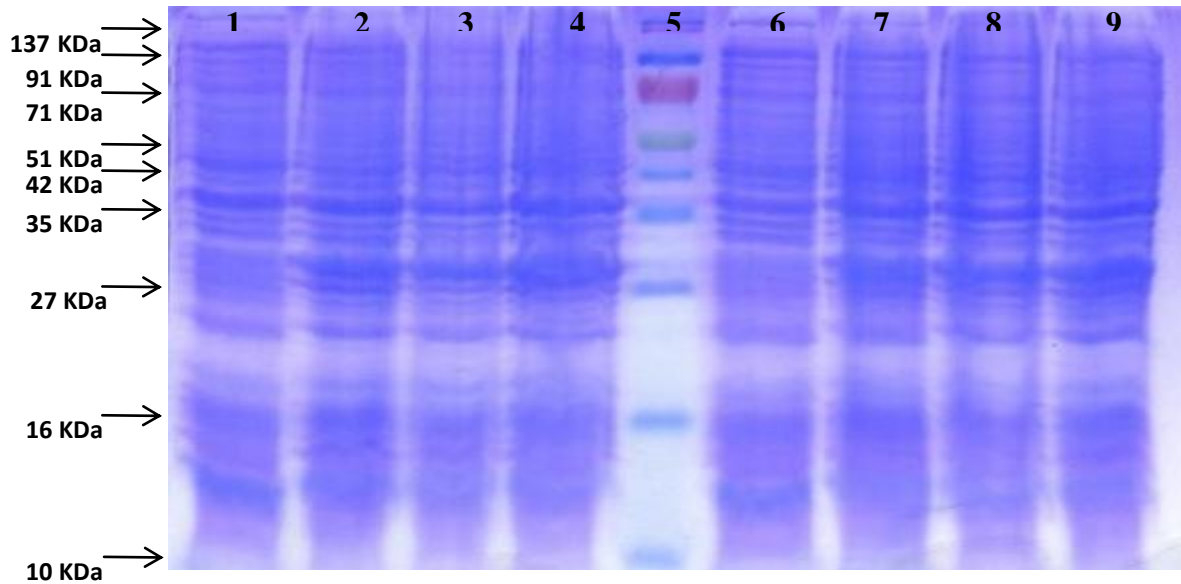
Lane 1, 2, 3, 5 and 6: single colony containing cloned gene;
Lane 4: 100bp plus DNA ladder (Fermentas)
Lane 7: Non template control

Fig.4 BLS Protein expression in BL21 (DE3)/pLysS cells induced at different time intervals using 1mM IPTG



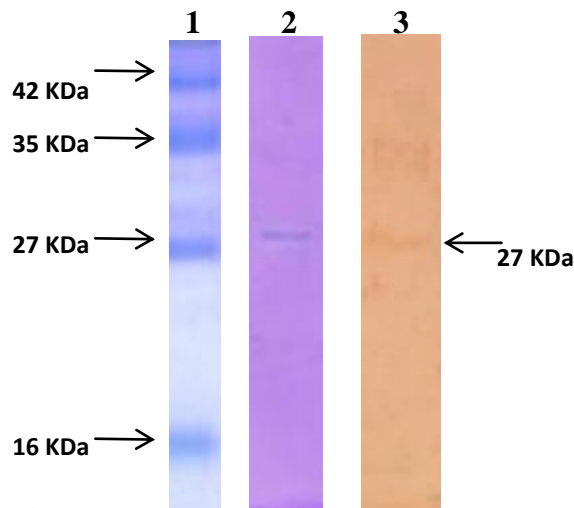
Lane 1: Pre stained protein Marker (Genetix)
Lane 2: Clone1 induced for 0 hr.
Lane 3: Clone1 induced for 2 hrs.
Lane 4: Clone1 induced for 4 hrs.
Lane 5: Clone1 induced for 6 hrs.
Lane 6: Blank.
Lane 7: Clone2 induced for 0 hr.
Lane 8: Clone2 induced for 2 hrs.
Lane 9: Clone2 induced for 4 hrs.
Lane 10: Clone2 induced for 6 hrs.

Fig.5 BLS Protein expression in BL21 (DE3)/pLysS cells induced at different time intervals using 1mM IPTG



Lane 1: Clone3 induced for 0 hr.
Lane 2: Clone3 induced for 2 hrs.
Lane 3: Clone3 induced for 4 hrs.
Lane 4: Clone3 induced for 6 hrs.
Lane 5: Pre stained protein Marker (Genetix)
Lane 6: Clone4 induced for 0 hr.
Lane 7: Clone4 induced for 2 hrs.
Lane 8: Clone4 induced for 4 hrs.
Lane 9: Clone4 induced for 6 hrs.

Fig.6 SDS-PAGE of purified BLS protein and Western blot with rabbit hyper immune serum against *B. abortus* S99 strain



Lane 1: Protein ladder (Genetix);
Lane 2: SDS-PAGE showing purified BLS protein;
Lane 3: Western blot of BLS protein with brucellosis positive cattle serum

The sequence results of two positive clones have shown 100% identity with the *bls* gene of *B. abortus* S99 strain. One clone which was transformed in *E. coli* host BL21 (DE3)/pLysS cells yielded many clones which were again subjected to colony PCR which yielded many positive clones for the presence of *bls* gene. Further, four clones which were subjected to IPTG induction, successful expression was seen in all the clones (Figs. 4 and 5) and the molecular size was around 27 KDa which includes around 17.3 KDa of recombinant protein along with around 10 KDa from vector and additional His tags put together were further purified by the Ni-NTA purification system. It involves affinity chromatography where in the high affinity and selectivity of Ni-NTA agarose for recombinant fusion protein that are tagged with six tandem histidine residues yields purified recombinant protein for further target applications. In Western blot, the purified protein produced a band around 27 KDa with the cattle serum positive for *Brucella* antibodies indicating the expressed protein is *Brucella* specific (Fig. 6).

Reactivity of this BLS protein with cattle, sheep, dog and human serum samples positive for brucellosis shows its potential to be used in the brucellosis diagnostics (Goldbaum *et al.*, 1993; Hemmen *et al.*, 1995; Goldbaum *et al.*, 1999; Cassataro *et al.*, 2002). Plasmid carrying the *bls* gene injected in to Balb C mice induced both humoral and cellular immune responses (Velikovskiy *et al.*, 2003). Bellido *et al.*, 2012 was able to use BLS as an immuno modulator to enhance the antibody response in hens in order to increase egg yolk antibodies. BLS has been reported as a carrier for antigen delivery (Sciutto *et al.*, 2005), based on its physicochemical and immunogenic properties (Zylberman *et al.*, 2004). Rosas *et al.*, (2006) showed that BLS can be used as both an antigen-carrier and as an adjuvant in the design of new oral subunit vaccines. There are other reports of using BLS as an immuno modulator to enhance the antibody response in hens

(Bellido *et al.*, 2012). Hence, the recombinant BLS protein expressed in *E. coli* can be exploited as diagnostic antigen or immunogen for development of immuno assay and vaccine for brucellosis.

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Conflict of interest

No conflicts of interests are declared by authors for the contents in the manuscript.

Authors' contribution

M. Nagalingam designed and carried out the experiment and wrote the draft of manuscript. Thaslim J. Basheer, N. Vijaya Gowri, Rajeswari Shome, G. B. Manjunatha Reddy assisted in carrying out the experiment. RK Gandham and V. Balamurugan provided guidance and edited the manuscript. B. R. Shome, H. Rahman and Parimal Roy provided guidance and support to carry out the experiments.

References

- Al Dahouk, S., Sprague, L. D. and Neubauer, H. 2013. New developments in the diagnostic procedures for zoonotic brucellosis in humans. *Review. Rev. Sci. Tech.* 32:177-88.
- Bellido, D., Chacana, P., Mozgovej, M., Gonzalez, D., Goldbaum, F., Wigdorovitz, A. and Santos, M. J. D., 2012. *Brucella* spp. Lumazine synthase as a novel immunomodulator to produce egg yolk antibodies. *Biomed. Life Sci.*, 3(1): 80-86

- Chatterjee, A., De, B. N., Mondol, P., Bidyant, J., Chakraborty, N. K., Nandy, A. K. and Sen, G. P. 1986. Studies on sero-prevalence of bovine brucellosis in rural areas of West Bengal. *Ind.Vet. J.* 63: 95–100.
- Cook, D., Ri., and Noble, J. W., 1984. Isolation of *B. suis* from cattle, *Aust. Vet. J.*, 61, 263–264
- Goldbaum, F. A., Velikovskiy, C.A., Baldi, P. C., Mortl, S., Bachr, A. and Fossati, C.A., 1999. The 18-kDa cytoplasmic protein of *Brucella* species - an antigen useful for diagnosis-is a lumazine synthase. *J. Med. Microbiol.*, 48: 833-839
- Goldbaum, F. A., Leoni, J. C., Wallach and Fossati, C. A., 1993. Characterization of an 18-kilodalton *Brucella* cytoplasmic protein which appears to be a serological marker of active infection of both human and bovine brucellosis. *J. Clin. Microbiol*, 31: 2141–2145
- Invitrogen 2010. Champion pET Directional TOPO Expression Kits, Manual part no. 25-0400, p.3
- Laemmli, U. K., 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 227: 680-685.
- Manish, K., C. Puran., C. Rajesh., R. Teena. and K. Sunil. 2013. Brucellosis: An updated review of the disease. *Indian Journal of Animal Sciences* 83 (1): 3–16
- Mantur, B. G., and Amarnath. S.K. 2008 Brucellosis in India – a review; *J. Biosci.* 33539–547
- Moreno, E., and Moriyon I. 2001. Genus *Brucella*. The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community.(Ed.) Dworkin M. Springer, New York.
- Nagalingam, M., Shome, R., Balamurugan, V., Shome, B. R., Narayana Rao, K. Vivekananda, R., Isloor, S., and Prabhudas, K., 2012. Molecular typing of *Brucella* species isolates from livestock and human. *Trop Anim Health Prod.* 44(1), 5-9.
- Olsen, S.C., and M. V. Palmer 2014 Advancement of Knowledge of *Brucella* over the Past 50 Years *Vet. Pathol.* 51(6) 1076-1089.
- Pappas, G., 2010. The changing *Brucella* ecology: novel reservoirs, new threats. *Int. J. Antimicrob. Agents.* 36:S8–S11.
- Rosas, G., Fragoso, G., Ainciart, N., Guadarrama, E. F., Santana, A., Bobes, R. J., Pliego, R. O., Toledo, A., Revilla, R. C., Meneses G., Berguer, P., Goldbaum, F. A. and Sciutto, E., 2006. *Brucella* spp. lumazine synthase: a novel adjuvant and antigen delivery system to effectively induce oral immunity. *Microbes and Infect*, 8(5): 1277-1286
- Sciutto, E., Toledo, A., Cruz, C., Rosas, G., Meneses, G., Laplagne, D., Ainciart, N., Cervantes, J. and Fragoso, G., 2005. *Brucella* spp. lumazine synthase: a novel antigen delivery system. *Vaccine*, 23(21): 2784-2790
- Tiwari, R., Sharma, M. C., Mishra, K. K. and Singh, B. P. 2013. Economic impacts of infectious diseases of livestock. *Ind. J. Ani. Sci.* 83: 316–320
- Verger, J.M., 1985. *B. melitensis* infection in cattle. In J. M. Verger and M. Plommet (eds), *Brucella melitensis*. (Martinus Nijhoff Publishers, Dordrecht, the Netherlands). Pp. 197–203.
- World Organisation for Animal Health (OIE) (2009). – Chapter 2.4.3. Bovine brucellosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris.
- Zaki, S., Murty, B. S. K., Rao, S. and Nanjiah, R. D. 1975. Serological incidence of brucellosis in sheep and goats in Karnataka. *Cur.Res.*4: 196–97.
- Zylberman, V., Craig, P. O., Klinke, S., Braden, B. C., Cauerhff, A. and Goldbaum, F. A. (2004) High order quaternary arrangement confers increased structural stability to *Brucella* sp. lumazine synthase. *The Journal of biological chemistry*, 279, 8093-8101.

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