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Research Article

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Cloning, expression, purification and characterization of two diagnostically important proteins of *Brucella abortus*

Arvind Kumar Tiwari[1*], Vijai Pal[1], Subodh Kumar[1], Bhupendra Bharadwaj[3], G. B. K. S. Prasad[2] and G. P. Rai[1]

[1]*Division of Microbiology, Defence Research and Development Establishment, Jhansi Road, Gwalior, India-474002*
[2]*School of Studies in Biochemistry, Jiwaji University, Gwalior, India-474011*
[3]*Regional Disease Diagnostic Centre, Udaipur, India- 313001*

ABSTRACT

Brucellosis, a bacterial zoonotic disease, primarily of cattle and is prevalent worldwide. The disease results in abortion and/or infertility in affected animals and undulant fever in human. It is an important cause of economical losses and human suffering. This disease is spread worldwide with areas of high endemicity in the Mediterranean, India, Middle East, South and Central America and Asia. The disease is caused by organisms belong to the genus Brucella. The true incidence of human brucellosis however, is unknown in most of the countries including India. In animals, the disease is usually asymptomatic in nonpregnant females, while pregnant females develop a necrotic placentitis and ulcerative endometritis, usually resulting in abortion. Many serological tests have been developed for the diagnosis of brucellosis. The most commonly used tests are standard tube agglutination test (STAT), the coombs anti-brucella test, the rose bengal plate agglutination test (RBPT), complement fixation test (CFT) and indirect haemolysis test (IHLT). The antigenic similarity of Brucella with other gram-negative organisms gives the false positive reactions in serodiagnosis, which reduces the specificity for diagnosis. Keeping in view the importance of brucellosis as an emerging infection and its prevalence in our country, there is a definite need to develop a specific diagnostic assay for Brucella infection. In this study, two diagnostically important recombinant proteins were cloned, expressed, purified and characterized, which can be used as a diagnostic antigen.

Key words: Brucellosis, *Brucella abortus*, cloning, expression, purification

INTRODUCTION

Brucella infection causes contagious abortions in cattle and "undulant fever' in humans. The infection is wide spread globally. It is an important cause of heavy economic losses due to storms of abortion, infertility and loss of milk production. It also has zoonotic importance in terms of its transmissibility to human beings attending infected animals or consuming contaminated products. The disease clinically characterized by undulant fever night sweating, early fatigue, joint pain (spondylitis) and orchitis. *Brucella* can be utilized as bio-weapon.

The disease is caused by very small, non motile, aerobic, Gram-Negative, facultative, intracellular coccobacillus or short rod shaped bacteria of the family Brucellacae. Six main species are distinguished: *B. abortus, B. melitensis, B. ovis B. suis, B. canis and B. neotomae. B. abortus* is normally associated with cattle, *B. melitensis* with sheep and goats, *B.suis* with swine. *B. ovis* causes an infection specific for sheep. *B. abortus B. melitensis and* occasionally *B. canis* cause Brucellosis in human beings. This disease is spread worldwide with areas of high endemicity in the Mediterranean, India, Middle East, South and Central America and Asia [1, 2]. More than 500,000 new human cases are reported world over each year, but according to the World Health Organization (WHO), these numbers greatly underestimate the true incidence of human disease [3]. Brucellosis is widely prevalent throughout India among the

bovine population both in farm and in the village animals. Brucellosis is still an uncontrolled serious health problem in many developing countries including India.

A large number of tests are available presently for the diagnosis of the disease ie RBPT, STAT, ELISA however no single detection test is found to be satisfactory because of their specificity/ sensitivity. Isolation of organism requires expensive laboratory set up and expert lab workers. The molecular diagnosis of brucellosis is costly and not suitable for developing country like India. Therefore, serological identification of antibodies against *Brucella* antigens is a suitable tool for rapid diagnosis of the disease. But serological tests which are currently in use are based on anti-lipopolysacharide (LPS) antibodies detection and are highly cross reactive. These tests can not differentiate between vaccinated and infected animals. The development of LPS-free protein based diagnostic system may be the useful tool for diagnosis of brucellosis. The most commonly used tests are standard tube agglutination test (STAT), the coombs anti-brucella test, the rose bengal plate agglutination test (RBPT), complement fixation test (CFT) and indirect haemolysis test (IHLT). Each of the serological tests has some limitations. In general, their sensitivity is poor in the early stage of the disease and their specificity is also not good [4, 5]. Therefore, there is definitely need to develop a specific non LPS based detection system for specific diagnosis of brucellosis.

In this study we cloned, expressed, purified and characterized two diagnostically useful rrecombinant proteins of *Brucella abortus*. Chirhart-Gilleland *et. al.*, [6] made an attempt to identify *Brucella* proteins capable of inducing protective immune responses, a collection of recombinant *Escherichia coli* clones expressing *Brucella* proteins reactive in immunoassays with sera from a variety of experimentally and naturally infected hosts. One of these clones produced a recombinant *Brucella* protein with an apparent molecular mass of 14 kDa. For ease of communication, the recombinant *Brucella* protein produced by clone was designated BA14K. Expressed recombinant protein was designated as BA14K protein. It appears likely that this protein is associated with the bacterial cell envelope; however, further biochemical characterization will be required to confirm this subcellular localization . Joseph P. Connoly analyzed the cell envelope proteins of *B. abortus* and identified some immunodoninant proteins using 2-DE with MALDI-TOF MS and LC-MS/MS which can be useful for bovine as well as human brucellosis [7]. They identified one immunogenic hypothetical protein (BAB2_0731). The function and sub cellular location of this protein was unknown. These two recombinant proteins can be used as antigen for *Brucella* diagnosis.

EXPERIMENTAL SECTION

B. abortus S99 strain, RBPT and STAT reagents were obtained from Indian Veterinary Research Institute, Izatnagar, India. *Escherichia coli* M15 and BL 21(DE3) for recombinant protein expression were obtained from Qiagen, Germany. The plasmids pQE-30 UA (amp^r), Ni-NTA agarose resin, anti-His monoclonal antibody, DNA purification kit, PCR product purification kit were also procured from Qiagen, Germany. PCR was performed using i-cycler thermal cycler (Biorad, CA, USA). Anti-cow IgG-horseradish peroxidase (HRP) was procured from Dakocytomation, Denmark. Agarose, isopropyl-thio-β-D-galactopyranoside (IPTG), 3-3'-diaminobenzidine tetra hydrochloride (DAB), O-Phenyldiamine (OPD) and other chemicals were purchased from Sigma Chemicals Co., USA. Media components for bacterial growth were purchased from Difco, USA and Hi-Media Laboratories, India.
The nucleotide sequences of genes encoding BA14K and hypothetical (we named as 20 kDa), proteins of *B. abortus* were retrieved from GenBank (www.ncbi.in). The accession numbers of GenBank and primers sequences used for amplifications of desired genes were synthesized commercially. The DNA from *B. abortus* S99 strain was prepared by Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instructions. PCRs were optimized for amplification of genes encoding these two proteins.

The BA14K protein encoding gene was cloned in pET 28a(+) vector using XhoI and NcoI restriction sites, while hypothetical (20 kDa) protein encoding gene was cloned in pQE-30 UA expression vector as per the manufacturer's instructions. *E. coli* (M15) was taken as a expression host for amplicons cloned in pQE-30 UA vector and *E.coli* BL 21(DE3) for the same in pET-28a(+) vector. Fresh competent cells were prepared using $CaCl_2$ procedure described by Cohen and associates [8].

E. coli (M15) was taken as a expression host for amplicons cloned in pQE-30 UA vector and *E.coli* BL 21(DE3) for the same in pET-28a(+) vector. Transformation was carried out by heat shock method described by Mandel and Higa [9]. Following heat shock treatment, competent cells were spread plated on LB-agar plates containing appropriate antibiotics {Kanamycin (25 µg/ml) & ampicillin (100 µg/ml) for M15 host cells and Kanamycin (50 µg/ml) for BL 21(DE3) host cells} and incubated overnight at 37°C. The presence of insert was confirmed by colony PCR. Expression of recombinant proteins was induced with IPTG as per instructions of manufacturer and checked by SDS-PAGE [10], uninduced cells were used as control.

The solubility of the recombinant proteins was determined according to the QIA expressionist protocol (Qiagen, Germany). Various fractions were analyzed on 10 or 15% SDS-PAGE, and the fractions containing purified protein were pooled. The purified protein was quantified by the method of Lowry et al. with bovine serum albumin (BSA) as a standard. The purified recombinant proteins were resolved on 10 or 15% SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. Blocking of the membrane was done with 5% defatted milk powder in phosphate buffered saline (PBS) (pH 7.2; 136 mM NaCl, 2.5 mM KH_2PO_4, 6.9 mM K_2HPO_4) overnight at 4°C. After washing the membrane with PBS containing 0.05% Tween 20 (PBS-T), the blot was probed with a 1:2,000 dilution of anti-His antibody-HRP conjugate for 1 h at 37°C. The membrane was washed with PBS-T, and the reaction was developed using 3-3-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 as a substrate.

RESULTS AND DISCUSSION

Brucella infection requires laboratory confirmation. *Brucella* isolation has so many limitations. Isolation results various due to so many factors. As we know *Brucella* species is slow growing and very risky for handling. Therefore, laboratory confirmation of brucellosis is required. The Rose Bengal plate agglutination test (RBPT), the Standard Tube Agglutination Test (STAT) and the complement fixation test (CFT) are the most commnaly used tests. The tests are associated with false-positive results due to serological cross-reactions with some Gram-negative bacteria having smooth lipopolysaccharides (S-LPS) and are unable to differentiating vaccinated and naturally infected animals.

Chirhart-gilleland, *et. al.*, reported a *Brucella* protein which was reactive in immunoassays with sera from a variety of experimentally and naturally infected hosts with an apparent molecular mass of 14 kDa and designated as BA14K. This protein could be associated with the bacterial cell envelope and has biological significance. The cell envelope proteins of *B. abortus* were analyzed by Connoly *et. al.*, and they identified some immunodoninant proteins using 2-DE with MALDI-TOF MS and LC-MS/MS which could be useful for vaccination and diagnosis of brucellosis. They identified a hypothetical protein (BAB2_0731). The function and sub cellular location of this protein is still unknown. This protein has not been utilized for the diagnosis of brucellosis. Therefore, in this study a primary attempt has been made to express these two recombinant proteins.

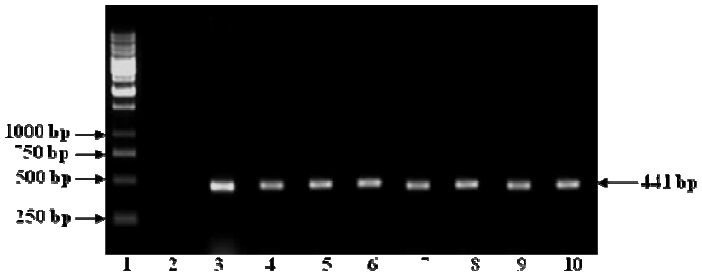

Fig. 1 – Agarose gel of temp. gradient PCR of 441 bp *BA14K* gene

Lane 1- 1 kb DNA Ladder
Lane 2 - Negative control
Lane 3 - 55°C
Lane 4 - 54.5°C
Lane 5 - 53.3°C
Lane 6 - 51.4°C
Lane 7 - 48.9°C
Lane 8 - 47.1°C
Lane 9 - 45.8°C
Lane 10 - 45°C

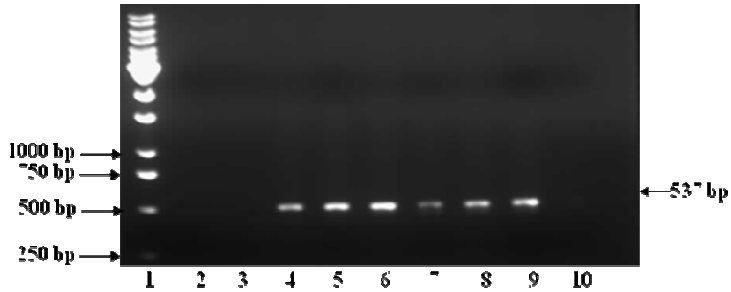

Fig. 2 – Agarose gel of temp. gradient PCR of 537 bp 20 kDa protein encoding gene

Lane 1- 1 kb DNA Ladder
Lane 2 - Negative control
Lane 3 - 55°C
Lane 4 - 54.5°C
Lane 5 - 53.3°C
Lane 6 - 51.4°C
Lane 7 - 48.9°C
Lane 8 - 47.1°C
Lane 9 - 45.8°C
Lane 10 - 45°C

The optimum annealing temperatures for PCR amplification of genes were found to be 55°C for BA14K and 52°C for 20 kDa protein encoding genes (Fig- 1 &2). Genomic DNA of *B. abortus* S99 was used as template for amplification of these genes in PCR. The amplicons size for BA14K and 20 kDa protein encoding genes were 441 and 537 bp, respectively. The amplicon of BA14K protein encoding gene was cloned in pET 28a(+) vector using XhoI and NcoI restriction sites, while 20 kDa protein encoding gene amplicon was cloned in pQE-30 UA expression vector as per the manufacturer's instructions. The 20 kDa protein encoding amplicon was transformed in M15 host cells while BA14K protein encoding amplicon was transformed in BL 21(DE3) host cells (as per materials and methods).

Expression of recombinant proteins was induced with IPTG. Optimum concentration of IPTG for maximum expression of the recombinant proteins was found to be 1 mM. Maximum expression of recombinant proteins was observed after 4 h of induction with 1 mM of IPTG. (Fig- 3&4).

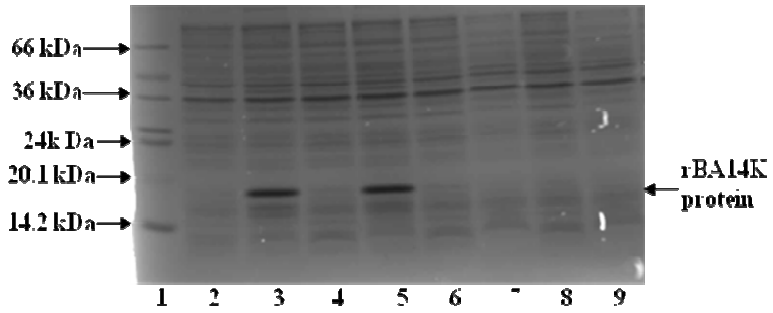

Fig. 3 – Expression of rBA14K protein

Lane 1- Molecular weight markers
Lane 2- Uninduced clones
Lane 3-9 Induced Clones

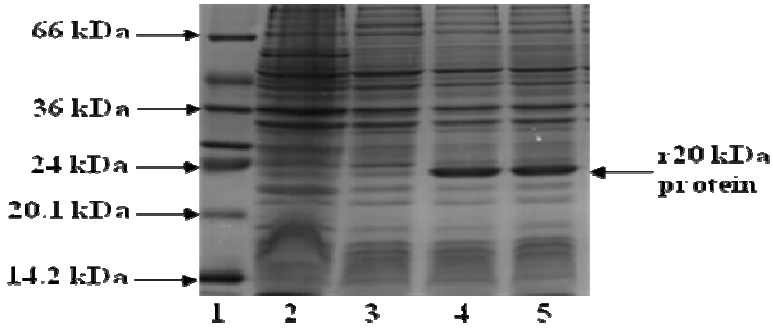

Fig. 4 - Expression positive clones of r20 kDa protein

Lane 1- Molecular weight markers
Lane 2- Uninduced clone
Lane 3-5 Induced clones

In protein localization study, both BA14K, and 20 kDa proteins were found in pellet fraction (inclusion bodies). Both the proteins were purified under denaturing condition, using Ni-NTA affinity chromatography. Elutes containing purified recombinant protein were pooled separately for individual recombinant protein. The pooled recombinant proteins reacted with anti-Histidine-HRP conjugated antibodies in Western blot and confirmed the expression of desired (6x-His-tagged) recombinant protein (Fig- 5&6). The final yields of purified BA14K, and 20 kDa recombinant proteins were approximately 06 and 09 mg per litre of the culture, respectively, using folin-phenol method [11]. The purified recombinant proteins can be utilized for diagnosis of brucellosis.

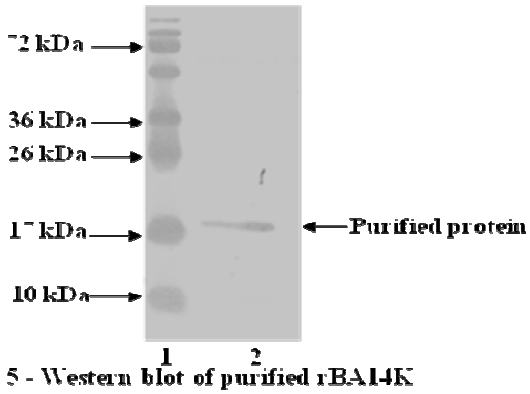

Fig. 5 - Western blot of purified rBA14K protein with anti His HRP

Lane 1 – Molecular weight markers
Lane 2 – Purified rBA14K protein

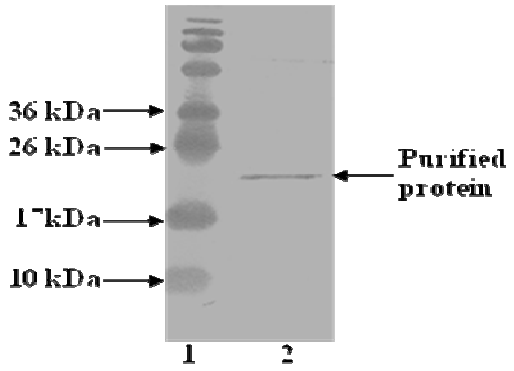

Fig. 6- Western blot of r20 kDa protein with anti His HRP

Lane 1 – Molecular weight markers
Lane 2 – Purified r20 kda protein

CONCLUSION

Brucellosis, a contagious disease primarily affecting animals, is caused by members of the genus *Brucella*. The disease has zoonotic potential in terms of its transmissibility to human beings attending infected livestock. Diagnosis of brucellosis always requires laboratory confirmation, either by demonstration of specific antibodies or by isolation of the pathogen. Isolation of organism is risky and also have poor sensitivity. The most commonly used serological tests are associated with false-positive results. The diagnostic tests based on LPS or whole cell antigens are not specific as chances of cross-reactions with antibodies to other similar bacterial infections increases. On the other hand, use of recombinant antigen increases the specificity of diagnostic assay and makes them more attractive. Moreover, good yield is obtained in one step procedure. Keeping in view, in this study, efforts have been made to generate and characterize two recombinant immunodominant proteins of *B. abortus* for diagnosis of brucellosis. The BA14K and 20 kDa proteins are specific peoteins and can be used as diagnostic antigen for diagnosis of brucellosis.

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