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Cloning and expression of AhpC gene of *Mycobacterium avium* sub sp. *paratuberculosis*

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ABSTRACT

The present study was undertaken to amplify, clone and express AhpC gene from *Mycobacterium avium* sub sp. *paratuberculosis* (MAP). Primers specific for AhpC gene with restriction enzyme sites, viz. NdeI and XhoI were designed. AhpC gene was amplified using DNA from MAP culture with designed primers by polymerase chain reaction (PCR). An amplicon of size 588 bp was obtained. The AhpC gene was first cloned into TOPO vector pCR2.1 and subcloned into prokaryotic expression vector pET22b. Colony PCR was carried out for the selection of the recombinant clones and further confirmed by restriction enzyme digestion. The recombinant clone was induced with 0.3 mM final concentration of isopropyl-ß-D-thiogalactopyranoside (IPTG) for the expression of the recombinant AhpC gene. The expressed protein was analysed by 12% SDS-PAGE. As AhpC gene exist as a homodimer, 2 protein fractions of 24 KDa and 45 KDa were obtained after induction. The specificity of the protein was determined by immunoblot analysis with polyclonal MAP antibodies.

Key words: AhpC gene, *Mycobacterium* avium, Paratuberculosis, Polymerase chain reaction, Prokaryotic expression vector, SDS PAGE, TOPO cloning vector, Western blot

Paratuberculosis (Johne's disease, JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), results in substantial economic losses at farm level due to premature culling and production losses. Cattle become infected early in life but often do not develop clinical signs until 2–5 years of age (Larsen *et al.* 1975). Eradication of the disease is hampered by the difficulty in diagnosis at an early stage and the non-availability of a potent vaccine. Bacterial culture is considered as the gold standard for the diagnosis of paratuberculosis. Constant attempts are in progress for identifying potent antigens for use in diagnostic assays and as a vaccine candidate (Chen *et al.* 2008, Kumanan *et al.* 2008 2009).

Olsen *et al.* (2000) identified 2 antigens in MAP, which are homologous to alkyl hydroperoxide reductases C and D (AhpC and AhpD) of *M. tuberculosis*. The high expression of AhpC and AhpD in *M. avium* sub sp. *paratuberculosis* without the need for peroxide induction is a unique feature of this bacterium. They are the most recently characterized immunogenic proteins of MAP. Goats experimentally infected with *M. avium* subsp. *paratuberculosis* had strong gamma interferon (IFN-γ) responses towards both AhpC and AhpD, and they also had antibodies against AhpC. The ability of AhpC and AhpD to induce IFN-γ production shows that these proteins could potentially be used in future vaccines or in diagnostic assays. The present study describes cloning and expression of AhpC gene of MAP in a prokaryotic expression (pET22b) vector.

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MATERIALS AND METHODS

Bacterial culture: *Mycobacterium avium* subsp. *paratuberculosis* culture available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai was used in this study.

Genomic DNA isolation: Mycobacterium avium subsp. *paratuberculosis* was grown in Middle brook 7H9 broth and the genomic DNA was isolated from the culture by hexacetyl trimethyl ammonium bromide (CTAB) method (Braunstein *et al.* 2002)

PCR amplification of AhpC gene: Primers were designed to have built in restriction enzyme sites (NdeI and XhoI) to enable directional cloning of the PCR product into the expression vector (FP 5' CAT ATG CCT CTG CTG ACC ATC 3' and RP 5' CTC GAG TTA AGC AGA GGC CTT 3'). Oligonucleotide primers were procured commercially. The amplification of AhpC gene (588 bp) including the restriction sites was carried out with an initial denaturation at 94°C for

5 min followed by a 94°C for 90 sec, 50°C for 45 sec and 72°C for 90 sec for 35 cycles and a final extension at 72°C for 7 min. 5 µl of the amplified product was analysed in a 2% agrasoe gel with a 100 bp DNA molecular weight marker.

Cloning of AhpC gene: The PCR amplified, gel purified AhpC gene was cloned into TOPO pCR2.1 vector as per the manufacture's protocol and the recombinant clones were selected based on kanamycin resistance and restriction enzyme digestion. Restriction enzyme digested AhpC gene was obtained from recombinant TOPO clones and used for ligation with *Nde* I and *Xho* I digested pET22b vector. Recombinant clones were selected based on ampicillin resistance, colony PCR and restriction enzyme digestion.

Expression of AhpC gene: The *E. coli* BL21 cells containing recombinant pET22b was induced with 0.3 mM IPTG. Aliquots of cells were collected every 1 h after induction up to 6 h and analyzed for expression by 12% SDS-PAGE at constant voltage of 50 V. The recombinant AhpC of MAP was separated by SDS-PAGE and transferred to 0.45 µm PVDF membrane. The membrane was soaked in blocking buffer for overnight and washed thoroughly in wash buffer. Primary antibody (polyclonal MAP antibody raised in rabbit) was added at a concentration of 1 in 100 and incubated for 5 h at room temperature and washed; secondary antibody (anti rabbit IgG-HRP conjugate) was added at a concentration of 1 in 1000 and incubated at room temperature for 1 h followed by a wash in washing buffer. The membrane was immersed in substrate solution (DAB-5 mg/10 ml) containing fresh hydrogen peroxide (10 µl).

RESULTS AND DISCUSSION

Cloning AhpC gene into TOPO vector: Elsaghier *et al.* (1992) have shown that only MAP infected mice but not *Mycobacterium avium* sub sp. *avium* infected mice had elevated antibodies against a protein that was later identified as AhpC. It is therefore a distinct possibility that AhpC and

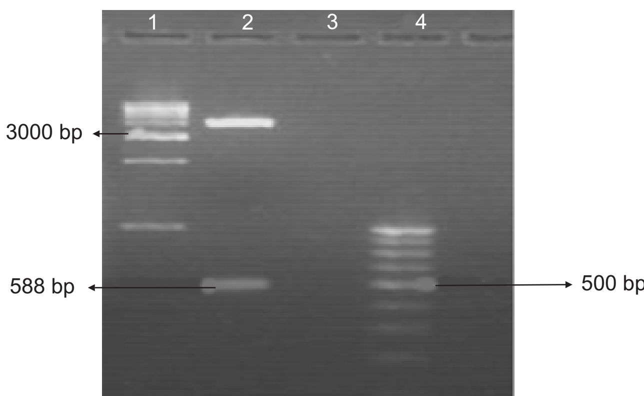

Fig. 1. Agarose gel (1%) electrophoresis of *NdeI* and *XhoI* digested recombinant pCR2.1 plasmid showing 588 bp AhpC gene. Lane 1,1 kb Ladder; lane 2,Recombinant pCR2.1 plasmid digested with Ndel and Xhol; lane 4, 100 bp ladder.

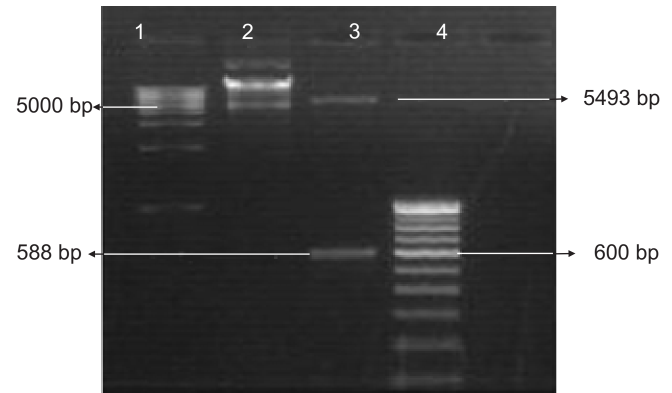

Fig. 2. Agarose gel (1%) electrophoresis of restriction enzyme digestion of recombinant pET22b with *Ndel* and *Xhol* showing insert release at 588 bp. Lane 1, 1 kb ladder; lane 2, uncut pET22b vector; lane 3, recombinant vector DNA digested with *Ndel* and *Xhol*; lane 4, 100 bp ladder.

Fig. 3. SDS-PAGE showing expression of recombinant fusion protein in a 12% gel. Lane 1, protein marker; lane 2, 5, 8, uninduced culture at 4 h, 6 h and overnight respectively; lane 3, 6, 9: Induced culture at 4, 6 h and overnight post induction with 0.2 mM IPTG; lane 4, 7, 10: Induced culture at 4, 6 h and overnight post induction with 0.3 mM IPTG.

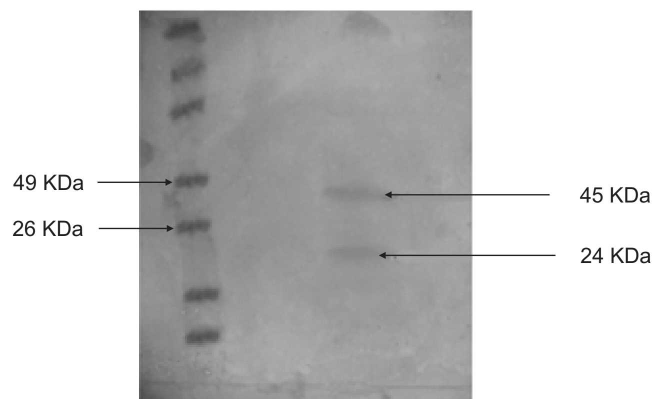

Fig. 4. Western blot analysis of recombinant AhpC gene using hyper immune serum of rabbit against MAP showing bands at 45 KDa and 24 KDa. Lane 1, Prestained protein marker; lane 2, recombinant AhpC protein.

AhpD can be used to distinguish infection with different *Mycobacterium avium* complex organisms. Olsen *et al.* (2000) identified 2 antigens in MAP, which are homologous to AhpC and AhpD from *Mycobacterium tuberculosis*. The present study was undertaken to clone and express the virulence gene AhpC of MAP so that it can be used as a candidate in developing a recombinant vaccine against Johne´s disease and also for developing a diagnostic kit for Johne´s disease. As expected**,** 588 bp long AhpC gene was amplified by PCR using gene specific primers having restriction enzyme sites *Nde* I and *Xho* I. The PCR amplicon of AhpC gene was ligated to TOPO pCR2.1 cloning vector and transformed into *E.coli* DH5α cells. The resultant recombinant colonies were selected by colony PCR and restriction enzyme digestion. 10/12 colonies were found positive for the 588 bp AhpC gene. Digestion of the recombinant plasmid with *Nde*I and *Xho*I, released the insert of approximately 588 bp size (Fig.1).

Subcloning of AhpC gene into the expression vector pET22b: Prokaryotic plasmid expression vector pET22b was used for subcloning because of its high efficiency and universal application (Xia *et al.* 2009). Recombinant plasmids were screened by colony PCR and by restriction enzyme digestion. Out of 30 colonies screened, two colonies were found positive which yielded an expected amplified product of 588 bp in 2% agarose gel, which was again confirmed by digestion with *Nde*I and *Xho*I (Fig.2).

In vitro *expression of the AhpC recombinant protein:* The AhpC gene was in frame in pET22b vector. The recombinant clone designated as pET22b-AhpC showed high level of expression under T_7 promoter of the vector when induced with IPTG. The molecular weights of the expressed protein were approximately 45 KDa and 24 KDa (Fig. 3). The protein kinetics study in SDS-PAGE showed that the maximum level of expression was between the 4 h and overnight incubation of post induction at 0.3 mM concentration of IPTG.

The sixth hour induced lysed cells were transferred to PVDF membrane from polyacrylamide gels. The PVDF was blocked with skim milk powder first and treated with rabbit polyclonal serum raised against MAP culture to check the specificity of the expressed protein. The membrane was treated with anti rabbit goat IgG antibody coated with HRP conjugate and later DAB substrate was added which reacted and gave a band at a molecular weight of 24 KDa, and 45 KDa (Fig. 4). The specificity of the recombinant AhpC protein obtained in this study was assayed using western blot technique which reacted with polyclonal serum (Fig. 4). In this study, the AhpC protein existed as a homodimer and hence 2 bands at the molecular weights of 24 KDa and 45 KDa were obtained for recombinant AhpC from the expression vector. Olsen *et al.* (2000) had already reported that AhpC was seen as broadband at about 45 KDa and another band at about 24 KDa in western blotting indicating that AhpC is linked with disulphide bridges and that it exists as a homodimer in its native form. Olsen *et al.* (2000) reported that AhpC protein produce a strong IFN-γ response and the AhpC protein obtained in the present study after cloning and expression of the AhpC gene in pET22b can be used for the development of a IFN-γ based diagnostic kit for paratuberculosis and can also be tested as a candidate for inclusion in a new vaccine cocktail against paratuberculosis.

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