



Expression of immunogenic genes of *Mycobacterium avium* subsp. *paratuberculosis*

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Johne's disease (JD), caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic inflammatory bowel syndrome in wild and domestic ruminants. JD is of economic importance worldwide and because of its chronic nature, early diagnosis and control has always been challenging. Currently available vaccines do not confer good protection. Similarly, the present day diagnostic tools are unable to detect early, subclinical infections. Moreover, these assays will not be useful in differentiating vaccinated animals from infected animals. To resolve the above issues, recombinant antigen based vaccines and assays could be an alternative in rapid diagnosis and control of JD. MAP codes for various immunogenic proteins, and a 38 kb locus region of MAP considered as the pathogenicity island consisting of *mpt* operon that is associated with virulence of the organism (Stratmann *et al.* 2004, Li *et al.* 2018). The *mpt* operon codes for *mptA*, *mptB*, *mptC*, *mptD*, *mptE* and *mptF* genes and among these, *mptD* codes for proteins exposed on the surface of MAP (Heinzmann *et al.* 2008). Similarly, 35-kDa antigen gene codes for a strongly immunoreactive major membrane protein (MMP) and is associated with its virulence (Banasure *et al.* 2001, Bannantine *et al.* 2003). Since these genes could be candidates for developing newer diagnostics and vaccines, we generated recombinant *mptD* and 35 kDa antigens.

A cattle isolate of MAP recovered in 2007 from Tamil Nadu, India (GenBank number: NZ_CP015495.1) was used. Genomic DNA was isolated by cetyl trimethyl ammonium bromide (CTAB) method. The 35 kDa antigen and *mptD* gene were amplified by PCR using the following newly designed primer pairs (MptD FP 5' CAT ATG ACC GCG ACC AGC AGC A 3'; MptD RP 3' GAA TTC CGC CAG GCC CGC GCG CT 5'; 35kDa Ag FP 5' CAT ATG GCG AAC CCG TTT GTG 3'; 35kDa Ag RP 3' GAA TTC CTG GCC CAG CGG TTTT 5').

mptD gene was amplified with an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 45 sec), annealing (52°C for 45 sec), extension (72°C for 1 min) and a final extension of 72°C for 7 min. Likewise, 35 kDa antigen gene was also amplified with the same

conditions except that annealing was at 50°C for 45 sec. Amplified PCR products of both the genes were cloned into pTZ57R/T cloning vector. Recombinant plasmids that were found positive by colony PCR were subjected to restriction enzyme (RE) digestion using *EcoRI* and *NdeI* to release the insert in order to confirm as recombinant clones for subsequent transformation into expression host.

Recombinant vector was subjected to RE digestion with *EcoRI* and *NdeI*, and the released insert was purified and subsequently used for cloning. pET22b vector was digested with *EcoRI* and *NdeI*, and the digested vector was gel purified. Further, the *mptD* and 35 kDa antigen genes were cloned into pET22b vector and transformed into DH5α cells. Recombinant plasmids identified by RE digestion were again digested using *EcoRI* and *NdeI* to confirm the recombinants. The rpET-22b/*mptD* and rpET-22b/35kDa vectors containing the *mptD* and 35 kDa genes respectively were isolated from *E. coli* DH5α host cells and transformed into BL21 (DE3) host system. BL21 (DE3) cells containing recombinant rpET-22b/*mptD* and rpET-22b/35kDa were induced with 1 mM IPTG (Isopropyl β-d-1 thiogalactopyranoside) and expression was checked by SDS-PAGE.

Good recombinant antigens can be used as a double edged weapon both as diagnostic and protective antigen, and the search for such antigens continues (Li *et al.* 2018). These recombinant proteins can be of use in DIVA (Differentiating Infected from Vaccinated Animals) strategies. In this context, we cloned and expressed two important proteins of MAP namely, *mptD* and 35 kDa antigen which have been reported to be associated with virulence and hence could be of diagnostic and or immunogenic importance (Banasure *et al.* 2001, Bannantine *et al.* 2003, Stratmann *et al.* 2004). The A260/A280 ratio of isolated MAP genomic DNA was found to be between 1.7 and 1.8 indicating the purity of the DNA as expected. *mptD* and 35 kDa antigen genes were amplified with the designed primers resulting in the amplification of 630 bp and 830 bp products, respectively. PCR amplified *mptD* and 35 kDa antigen genes were cloned separately into pTZ57R/T and then transformed into *E. coli* DH5α cells. Plasmid DNA extracted from the transformed colonies were subjected to restriction enzyme digestion which resulted in 630bp and 830bp inserts of

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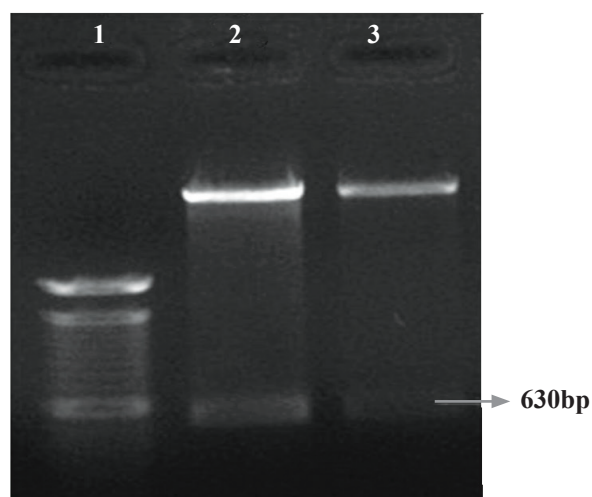


Fig. 1. Agarose gel electrophoresis of cloned *mptD* gene in pET-22b. Lane 1: DNA marker (100 bp); Lanes 2, 3: Released insert of 630 bp after *EcoRI* and *NdeI* digestion of rpET-22b/*mptD*.

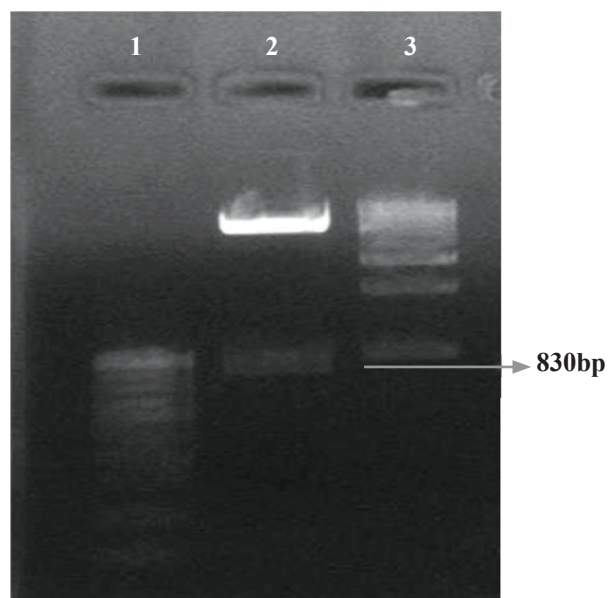


Fig. 2. Agarose gel electrophoresis of cloned 35 kDa gene in pET-22b. Lane 1: DNA marker (100 bp); Lane 2: Released insert of 830 bp after *EcoRI* and *NdeI* digestion of rpET-22b/35 kDa; Lane 3: DNA marker (1 kb).

mptD and 35 kDa antigen genes. Recombinant plasmids rpET-22b/35kDa and rpET-22b/*mptD* were subjected to RE digestion with *EcoRI* and *NdeI*. RE digestion released inserts of about 630 bp and 830 bp from rpET-22b/*mptD* and rpET-22b/35kDa, respectively (Figs 1, 2).

Expression of recombinant proteins was maximum at 4 h after IPTG induction (Figs 3 and 4). Recombinant proteins with immunogenic potential can be used as candidates for developing different diagnostic assays or vaccines against JD. *mpt* proteins are expressed on the mycobacterial cell wall surface and reported to be associated with the virulence of the organism. Moreover, *mptD* codes for

proteins exposed on the surface of MAP during infection (Heinzmann *et al.* 2008). *Mpt* operon clones of *M. bovis* BCG were found to reduce the amplification of MAP significantly in mice. Similarly, 35 kDa antigen gene codes for a strongly immunoreactive MMP that plays a role in invasion of epithelial cells and associated with the virulence of the organism (Banasure *et al.* 2001, Bannantine *et al.* 2003). Earlier work on the 35 kDa antigen protein had indicated that p35 recombinant protein has the potential for use in the serodiagnosis of animals with JD (El-Zaatari *et al.* 1996).

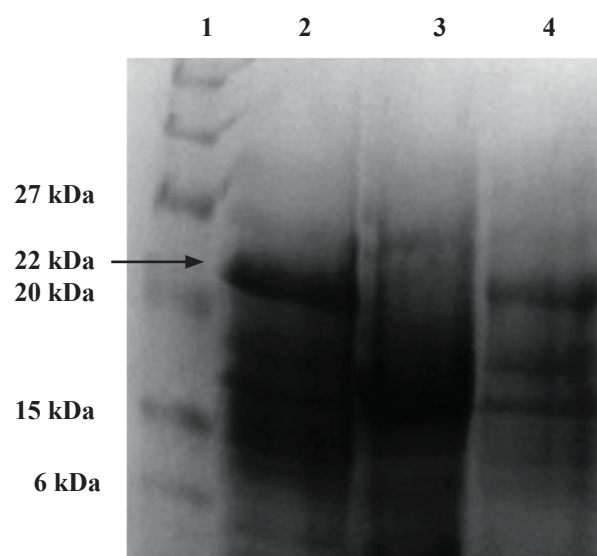


Fig. 3. SDS-PAGE analysis of recombinant *mptD* protein expressed in *E. coli* BL21. Lane 1: Protein molecular weight marker; Lane 3: Whole cell extract of *E. coli* BL21 harbouring rpET-22b/*mptD* (uninduced); Lanes 2, 4: Whole cell extract of *E. coli* BL21 harbouring 22 kDa *mptD* protein (IPTG induced).

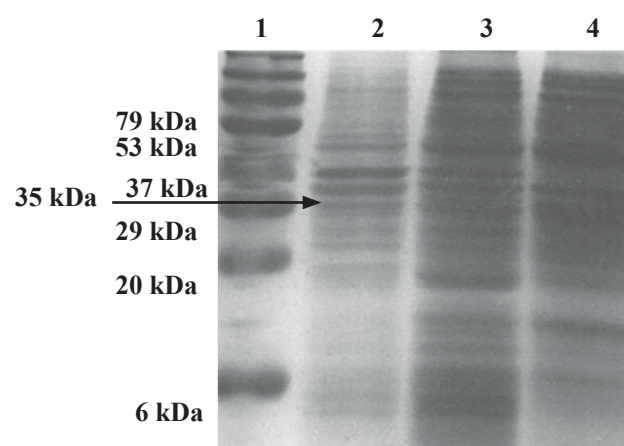


Fig. 4. SDS-PAGE analysis of recombinant 35 kDa protein expressed in *E. coli* BL21. Lane 1: Protein molecular weight marker; Lanes 2, 3: Whole cell extract of *E. coli* BL21 harbouring 35 kDa protein (IPTG induced); Lane 4: Whole cell extract of *E. coli* BL21 harbouring rpET-22b/35 kDa (uninduced).

As these genes are reported to possess immunogenic properties, surface expressed, and associated with virulence of the organism, they could be of use in generating recombinant antigens of diagnostic value (Stratmann *et al.* 2006). The major issue in the prevention and control of JD is the early diagnosis of sub-clinically infected animals. MAP is a slow growing pathogen and subclinically infected animals do not show the symptoms until several months of infection, but still will be excreting the pathogen and contaminating the environment. In this scenario, the need of the hour is a good antigen which can detect early infections. Furthermore, immunogenic recombinant antigens could be of use in developing a vaccine which apart from being protective, could also aid in differentiating vaccinated from infected animals. In this context, this study has generated recombinant *mptD* and 35 kDa antigens which might open up the avenue for the development of newer diagnostics and vaccine candidates for MAP. Having expressed these two important proteins, it is now possible for us to explore the possibilities of testing these recombinant antigens either as diagnostic or immunogenic antigens which could be of use in the early diagnosis and control of MAP infections.

SUMMARY

Johne's disease is an economically important disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Continuous efforts are being made to identify MAP genes with antigenic and immunogenic capabilities which may serve as vaccine candidates and diagnostic antigens. *mptD* and 35 kDa antigen genes of MAP amplified by polymerase chain reaction resulted in 630 bp and 830 bp products respectively. Both *mptD* and 35 kDa antigen genes were cloned into pTZ57R/T cloning vector, and subsequently subcloned into pET22b and expressed in BL21 (DE3) host system. The expressed recombinant antigens could be tested in diagnostic assays and might open up the avenue for the development of newer diagnostics and vaccine candidates for Johne's disease.

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