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Research Article

IMMUNOREACTIVE RECOMBINANT NITRATE REDUCTASE EXPRESSED BY *NIR A* GENE OF *Mycobacterium avium* sub sp *paratuberculosis IS900* ELEMENT IN *E.coli*

PADMAVATHY C.*[1], VIJAYARANI K.[2], KUMANAN K.[3], THANGAVELU A.[4] AND SRITHAR A.[5]

[1]Institute of Veterinary Preventive Medicine (IVPM), Ranipet, 632402, Vellore District, Tamil Nadu, India
[2]Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, 600 007, Tamil Nadu, India
[3]Centre for Animal Health Studies (CAHS), Tamil Nadu Veterinary and Animal Sciences University, Chennai, 600 051, Tamil Nadu, India
[4,5]Department of Veterinary Microbiology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, 600 007, Tamil Nadu, India
*Corresponding Author: Email-kpkumarr@rediffmail.com

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Abstract- *Mycobacterium avium* subsp *paratuberculosis* (MAP) is classified under list B transmissible organisms signifying the public health concern and needs specific early diagnosis. Due to the cross reacting antigens of environmental mycobacterium and the diverse immune responses of individuals, cocktail of antigens capable of inducing strong immune response should be identified to improve serum-based methods of detection. Insertional sequence (*IS900*) was specific to par tuberculosis among the mycobacterium family, and among the *IS900* elements, a *nir A* gene at locus 4 was putative to MAP and absent from the *M. avium* genome. In an attempt to identify the candidate antigens, nitrate reductase gene *nir A* was successfully cloned and expressed in *E. coli*. In Western blot analysis strong immune reactivity was shown by the recombinant protein nitrate reductase with bovine anti MAP antibodies facilitates its use as a diagnostic candidate to improve the specificity of MAP diagnosis.

Key words- Mycobacterium-*IS900*-Recombinant antigen –diagnostic candidate.

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Introduction

Paratuberculosis (pTB) is a fatal inflammatory disease caused by *Mycobacterium avium* subsp. *paratuberculo*sis (MAP) affecting both wild and domestic ruminants including cattle, sheep and goats. *M. avium* subsp. *paratuberculosis*, is difficult to diagnose due to closely related other *M. avium* subspecies. The principal difference between these pathogens and other members of the *M. avium* complex is the presence of 14–18 copies of the insertion element *IS900* within *M. avium* subsp. *paratuberculosis*. Among them *nir A* gene at locus 4 of *IS900* is unique to *M. avium* subsp. *paratuberculosis,* and absent in other *M. avium* species [1]. Any immune response to its derived protein products would be indicative of specific infection. Expression and purification of these proteins will provide material suitable for ELISA and cell-mediated immunoassays [2]. The present study was focused on cloning and expression of recombinant *Nir A* product of *IS900* in *Escherichia coli* and to assess the immunoreactivity with bovine anti MAP antibody.

Materials and Method

Bacterial strains and growth conditions: Mycobactin. J dependent *Mycobacterium avium* sub sp. *paratuberculosis*, K10 strains maintained at Department of Animal Biotechnology, Madras Veterinary college (TANUVAS) was used. Static culture of MAP was done by inoculating 1 ml of glycerol stock of seed culture in 50ml of 7H9 medium (Difco) supplemented with 5g/liter glycerol,1g/liter BactoCasitone (225930-Difco, France), Middle brook enrichment medium oleic acid-albumin-dextrose catalase (OADC- cat no 211885 BD-BBL) at the rate of 10% , and 2 mg/liter mycobactin. J. After 8 weeks of growth, bacterial cells were pelleted by centrifugation and used for genomic DNA isolation by using DNAzol (cat no 10503-027, Invitrogen, USA). *Mycobacterium phlei* freeze dried culture was received from MTCC-cat no 1724 T (ATCC 11758, Chandigar) and cultivated in 7H10 agar (Difco) supplemented with 10%OADC and from which cellular protein extracts were prepared and used to absorb cross-reacting serum antibodies prior to immunoblotting (preabsorption). *E. coli* strains, BL 21 and Nova blue were maintained by Department of Animal Biotechnology, Madras Veterinary college (TANUVAS) cultured in Luria Britani (LB) broth and stored as glycerol stock at-20° C. Plasmid vector pET 21b vector (Novagen, Germany) was used as cloning vector for PCR amplified gene insert, followed by its propagation in *E. coli* (Nova blue) and finally expression in *E. coli* BL21 (DE3). Bovine sera: MAP antibody positive and negative bovine sera samples tested by IDEXX ELISA kit were obtained from Central Referral Laboratory (IVPM, Ranipet). Amplification of *Nir A* gene of *IS900*: PCR amplification of *Nir A* gene from genomic DNA of MAP was done and was used as insert DNA for directional cloning in pET 21b vector. PCR was carried out by using Pfu polymerase (Novagen) with following gene specific primers having shine dalgarno sequence with *Bam HI* and *Hind III* restriction sites. FP: 5' GG GGAT CCA GAA GGA GAT ATA CAT ATG ACC ACC GCA CGT CCC 3' RP:5' ATG AAG CTT AGC TGC AGG TTT TCC CGG 3'. The PCR conditions included initial denaturation at 94°C for 5 mins and 35 cycles of template denaturation at 94°C for 45 sec, primer annealing at 64°C for 45secs and primer extension at 72°C for 1.10 mins, followed by final extension at 72°C for 7mins.The expected product size of 400 bp was gel eluted using Qiagen gel elution kit. The insert nucleotide sequence was determined by DNA sequencing (Eurofins Genomics India Pvt. Ltd) and analysed by NCBI BLAST for similar genes. The

theoretical pI and instability/stability was calculated using ExPASy-ProtParam proteomic tool.

Cloning and expression of Nir A gene of *IS900* was carried out as described by [3]. In brief the gel purified PCR product (Product size: 400bp) was ligated into pET 21b vector (Novagen) and transformed into competent cells of *Escherichia coli* (Nova Blue) prepared by treatment with ice cold CaCl2. Transformed clones were selected by using ampicillin as selectable marker. Plasmid DNA was isolated from the transformed clones using Qiaspin miniprep system (Qiagen) and the recombinants were confirmed by restriction enzyme double digestion using *Bam HI* and *Hind III* for insert release. The recombinant plasmids were transformed to BL21 *E. coli* expression vector. The recombinant plasmid insertion was confirmed by restriction enzyme double digestion. The recombinant *E. coli* carrying histidine tagged fusion gene of pET21b was induced for expression by the addition of 1.5mM IPTG (isopropyl thiogalactoside) when the OD at 600 reaches 0.5 and kept for overnight Incubation at 37°C. The expressed proteins were analysed by 12.5% SDS- PAGE and checked for the solubility [4]. The expressed protein nitrate reductase was purified by Ni-NTA resin (Invitrogen) by column purification. In brief the cells were pelleted from 50 ml of culture and lysed in 6ml of phosphate buffer containing 200mM of lysozyme with 1mM PMSF in ice bath for 30 min. Freezing and thawing done thrice in -80° C deep freezer followed by sonication in 5 cycles at 40 amplitude for 30 sec in ice with 30 sec interval. The cell lysate was clarified by centrifugation and the supernatant was used for Ni-NTA purification under non denaturing native purification condition as per manufacturer's guidelines. Elutes were checked for protein concentration by Bradford assay [5] using Bradford reagent (catlog 500-0205 Bio-rad). The molecular weight was checked by 12% SDS-PAGE with molecular weight marker (cat no: 161-0318 Bio-Rad).The immune-reactivity of the purified *IS900* encoded nitrate reductase protein was determined by Western blot analysis. For blot preparation purified recombinant protein was loaded on to vertical gel electrophoresis using denaturing 12%SDS- PAGE [6] at a concentration of 25 µg per well. The separated proteins were transferred to nitrocellulose membrane by electro blotting [7] using Tris-glycine buffer containing 0.0375% SDS and 20% methanol.

Results

The present study was aimed at cloning and expression of *IS900 Nir A* gene encoding Nitrate reductase in *E. coli*. The targeted *Nir A* of insertion sequence was identified with full length ORF [Seq-1] for primer designing based on the known nucleotide sequence available in the NCBI database [GenBank ID:CAB96057].

Seq-1: *Mycobacterium avium* sub sp. *paratuberculosis* insertion sequence IS900, Locus 4, AJ250018.1, "nirA",
Protein: CAB96057

ATGACCACCGCACGTCCCGTCAAGACCCGCAACGAGGGTCAGTGGGCGCTG
GGAGATCGCGAACCGCTCAACGACACCGAGAAGATCAAGCTGGCCGACGGG
CCGCTGAACAGATCGGCGAGCGCATCATCAACGTCTACGCCAAGCAGGGTT
TCGACAGCATCGACAAGTCCGACCTGCGCGGCCGCTTCCGGTGGATGGGCC
TGTACACCCAACGCGAGCAGGGCTACGACGGCAGCTGGACCGGTGACGACA
ACACCGACAAGATCGAAGCCAAGTACTTCATGATGCGGGTCCGCTCCGACG
GCAAGGCGATGTCGGCGCACACCATGCGCACGCTCGGCCAGATCTCCACCG
AATTCGCCCGCGACACCGCCGATATCAGCGACCGGGAAAACCTGCAGCT

The PCR in the presence of 3% DMSO, with annealing temperature at 64° C for 45sec was optimized. In PCR 400 bp amplicon was obtained as a distinct band representing the full length ORF of Nir A gene of insertional sequence *IS900* revealed by 1.5 % Agarose gel electrophoresis. [Fig-1]. Complete nucleotide sequence of the 400 bp PCR amplified product found to have 100% homology to MAP strains by NCBI blast analysis. Estimation of the theoretical pI: 9.92 and molecular weight: 22602.10 were obtained for Nitrate reductase protein of *IS 900* containing 198 amino acid sequence, by using ExPASy-ProtParam tool. The instability index (II) is computed to be 64.3 classifies the protein as unstable and the estimated half-life is: >10 hours (*Escherichia coli*, *in vivo*).Ligation of the 400 bp gene insert with pET 21 b vector was done in a 20 µl ligation reaction, containing restriction digested (*Bam H*I and *Hind III*) 250 ng of insert and 50ng of plasmid at the ratio of 5:1 for 16 hrs at 16º C, and transformed into 100 µl *E.coli* competent cells with heat shock given at 42º C for 50 sec. One clone was identified as recombinant by insert release after screening of five transformed clones with Restriction enzyme double digestion [Fig-2]. The recombinant protein expression induced by 1.5mM concentration of IPTG with overnight incubation gave high yield which was identified by increased band width in SDS-PAGE analysis. The molecular weight of the expressed protein was approximately 22.5 kDa [Fig-3]. The recombinant protein was found in the soluble fraction. The protein concentration of the Ni NTA purified elute [Fig-4] fraction was 0.168µg/µl by Bradford assay. Western blot analysis [Fig-5] with bovine anti MAP antibody positive serum showed immuno reactivity with the protein nitrate reductase of *IS900* gene.

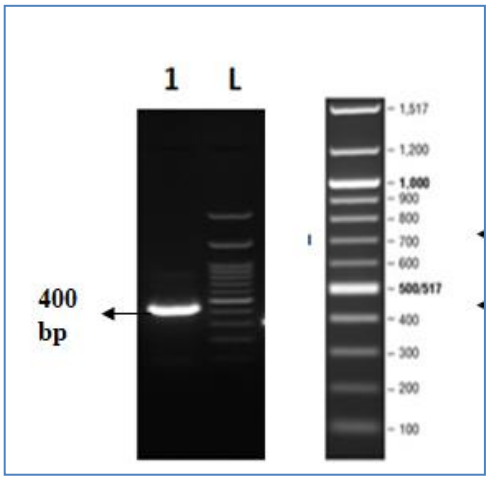

Fig 1- 1.5% Agarose gel Electrophoresis showing PCR amplified *Nir A* gene of *IS 900* Lane L: 100 bp Ladder; Lane 1: PCR product of 400 bp *Nir A* gene of *IS 900*

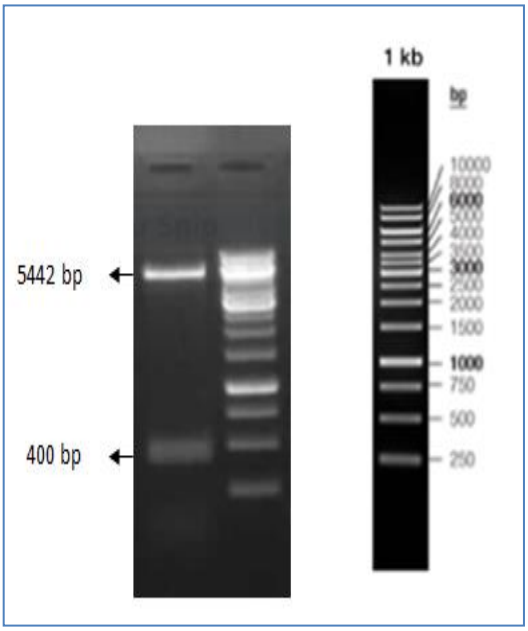

Fig 2- 0.8% Agarose gel electrophoresis of Restriction enzyme digestion of recombinant pET- *Nir A* gene. Lane 1: 1Kb Ladder Lane 2: Restriction enzymes double digestion (*Bam HI* and *Hind III*) of recombinant plasmid

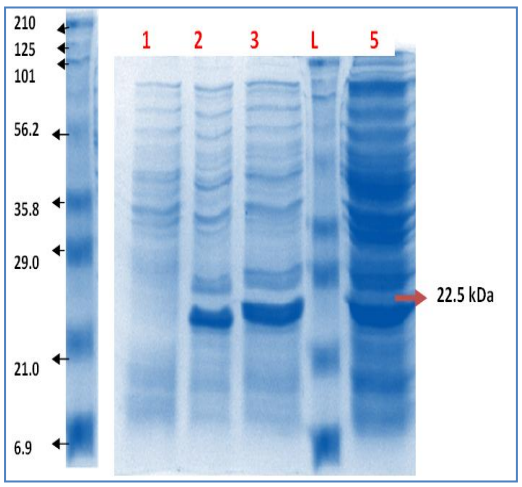

Fig 3- 12% SDS-Polyacrylamide gel electrophoresis of Nitrate Reductase protein of *Nir A* gene of *IS900* at different hours of induction with IPTG. Lane 1: Un induced; Lane 2: 4 hrs of induction; Lane 3: 6 hrs of induction; Lane L: Molecular weight marker; Lane 4; Overnight induction.

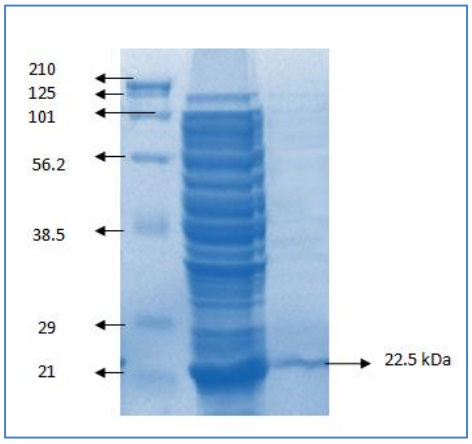

Fig 4- 12% SDS- Polyacrylamide gel electrophoresis analysis of Ni NTA purification of Nitrate Reductase protein of *Nir A* gene of *IS900* Lane 1: Molecular weight Marker; Lane 2: Unpurified; Lane 3: Purified Elute

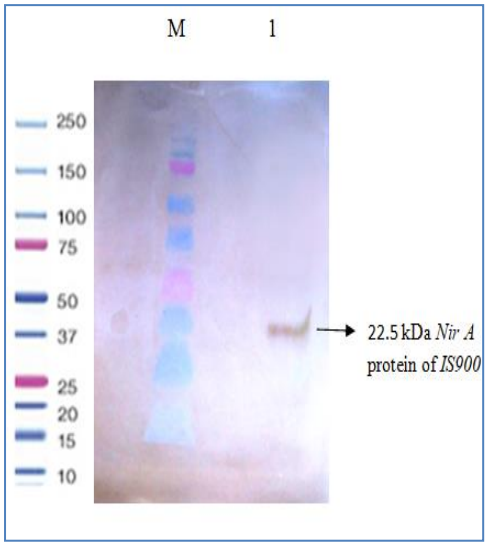

Fig 5- Immunoblotting of Nirate reductase*Nir-A* protein of *IS900* Western blot analysis with Anti MAP antibody Positive bovine serum (Lane M: Molecular weight marker,Lane 1: Purified 22.5 kDa protein).

Discussion

The main focus of the present study was production of recombinant nitrate reductase by cloning *nir A* gene of *Mycobacterium avium* subsp. *paratuberculosis* in *E.coli*. The molecular weight of the expressed recombinant protein was approximately of 22.5 kDa in size by SDS-PAGE which is in agreement with the theoretical value of the expected protein. The strong immune-reactivity with bovine anti MAP antibodies in western blot analysis demonstrated the diagnostic potential of the recombinant protein encoded by *Nir A* gene of *IS 900* elements of MAP. The present study supports the findings [2] that the components of *IS900* elements may facilitate to improve the specificity of MAP diagnosis.

Conclusion

Development of MAP Diagnostics and Vaccine will require a cocktail of antigens due to the range of heterogeneous MHC molecules of out-bred animals [8] and to develope DIVA (Differentiation of Infected from Vaccinated Animals) strategy [9, 10]. Since *IS900* is unique to *M. avium* subsp. *paratuberculosis*, any immune response to its derived protein products would be indicative of specific infection as *IS900* is the major genetic difference between *M. avium* subsp. *paratuberculosis* and *M. avium*. Hence the *Nir A* products can be a valuable component in development of MAP diagnostic with cocktail of antigens

Application of Research: There is a continuous research in identifying immunogenic proteins of MAP. Several antigens were identified and characterized for the development of candidate antigen with vaccine and diagnostic potential. Recombinant MAP antigens have various merits in terms of antigen production and human safety. Recombinant *Nir A* product of MAP showing immune reactivity with bovine antiMAP antibodies facilitates its use as a diagnostic candidate to improve the specificity of MAP diagnosis.

Research Category: Recombinant antigen production – *Mycobacterium*

Abbreviations

Bp	-	Base pair
$CaCl_2$	-	Calcium chloride
° C	-	Degree centigrade
DMSO	-	Dimethyl sulphoxide
ELISA	-	Enzyme linked immunosorbant assay
hrs	-	Hours
IPTG	-	Isopropyl thiogalactoside
kDa	-	Kilo dalton
ng	-	Nanogram
µg	-	Microgram
µl	-	Microlitre
MHC	-	Major histocompatability
mins	-	Minutes
mM	-	Millimolar
MAP	-	*Mycobacterium avium* subsp. *Par atuberculo*sis
OADC	-	Oleicacid-albumin-dextrosecatalase
PCR	-	Polymerase chain reaction
PMSF	-	Phenyl methyl sulfonyl fluoride
pTB	-	Paratuberculosis
SDS-PAGE	-	Sodium dodecyl sulphate-Poly acrylamide gel electrophoresis

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***Research Guide or Chairperson of research: Prof Dr K VIJAYARANI**
University: Tamil Nadu Veterinary and Animal Sciences University, Chennai, 600051, Tamil Nadu

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Author Contributions: All author equally contributed

Author statement: All authors read, reviewed, agree and approved the final manuscript

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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