The Pharma Innovation Journal 2022; SP-11(9): 680-682

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(9): 680-682

www.thepharmajournal.com
Received: 15-06-2022
Accepted: 16-07-2022

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Cloning, sequencing and expression of FIS (Factor for Inversion Stimulation) gene of *Pasteurella Multocida*

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Abstract
Fis is a transcriptional regulatory protein which regulates the expressions of the capsules in *Pasteurella multocida* was amplified cloned, sequenced and expressed in prokaryotic expression system. Transformation in *E. coli* BL21 strain is confirmed by restriction digestion and colony PCR. Cloned Fis gene was also sequenced and BLAST analysis show 100 similarities with Fis gene sequence of other *P. multocida*. Cloned product was also expressed in *E. coli* BL21 and characterized by SDS-PAGE and western blotting which can be further used for functional study of Fis gene.

Keywords: *Pasteurella multocida*, Fis, cloning, expression, western blotting

Introduction
Pasteurella multocida is a gram-negative, cocco-bacillus, facultative anaerobe bacteria cause hemorrhagic septicemia in cattle. On the basis of capsular composition divided into five serogroup A, B, D, E, or F and on the basis of lipopolysaccharide (LPS) antigens further classified into 16 Huddleston serotypes. Serogroup A and rarely serogroup F strains cause fowl cholera outbreaks in birds, serogroup B and E strains cause bovine hemorrhagic septicemia, and toxigenic serogroup D strains cause atrophic rhinitis in swine ((Dabo *et al.*, 2007) [1]. Indian livestock industry represents enormous number of Bovine population in world. Hemorrhagic septicemia is an economically important endemic disease in India and cause of the high mortality in infected cattle and buffaloes (Ryan and Feder, 2019) [2].
Capsule, lipopolysaccharide and protein outer membrane, fimbriae and iron utilization are the virulence factors of *P. multocida* (Steen *et al*., 2010; He *et al.*, 2020) [3-4]. Fis is a transcriptional regulatory protein which regulates the expressions of the capsules in *P. multocida* (Steen *et al*., 2010) [3]. It is a nucleoid associated protein and found in many different bacterial species. High level of Fis proteins is expressed in bacterial cells during active growth phase. While during stationary phase of bacterial growth the expression of this protein becomes extremely low. This protein is also express in low level in nutrient starvation condition (Steen *et al*., 2010) [3]. Fis protein is concerned with transcriptional regulations of certain genes encoding for virulence factors in E. coli (Saldana *et al*., 2009) [5] and Salmonella (Kelly *et al*., 2004) [6]. In *P. multocida* Fis protein binds to a promoter region which is flanked by the phy A and hya E genes and accountable for the biosynthesis of the polysaccharide hyaluronic acid. The lack of efficient Fis protein results in to failure of capsule expression in *P. multocida*. The existence of efficient Fis protein is also required for the expressions of various virulence genes in *P. multocida* (Steen *et al*., 2010) [3]. There are a number of information available related to the molecular biology of the Fis protein in different bacterial species but there is scanty report available to Indian isolate of *P. multocida*. Considering above fact this study is design to know the molecular characterization and expression of Fis gene *of P. Mutocida.*

Material and Methods
Amplification of Fis gene
P. Multocoda P52 strains were grown routinely in brain heart infusion (BHI) broth (Himedia) or on BHI agar containing 5% sheep blood. The genomic DNA was isolated following the method of Wilson (1987) [7]. Fis gene was amplified using forward primer 5' GAATTCGAACAACAACGTAATCCTGC 3' and reverse primer 5' AAGCTT AATGGTCCGTTAACCCATAC 3' with HindIII and EcoRI restriction site. PCR amplification of Fis gene was carried out through a reaction mixture containing primers,

dNTPs, High Fidelity PCR Enzyme Mix (Fermatas), reaction buffers, magnesium chloride and genomic DNA. Amplification was carried out at 95 °C for 5 minutes' initial denaturation subsequently 35 cycles of 95 °C for 30 seconds denaturation, 48 °C for 45 seconds annealing and 72 °C for 30 seconds extension and final extension at 72 °C for 5 minutes. The amplified product was visualized on agarose gel.

Cloning and expression of Fis gene

The amplified product was eluted from gel using JET gel extraction kit (Thermo fisher scientific) and digested with HindIII and EcoRI (Fermatas) restriction enzymes. The digested product is purified from agarose gel and ligated to HindIII and EcoRI digested pET32a (+) vector using T4 DNA Ligation kit (Fermatas). Ligated product was used to transform in *E. coli* BL21 strain and plated on LB-ampicillin plates, plasmid was purified from positive clone and digested with EcoRI and HindIII to confirm presence insert of desired size. The reading frame of the inserted genes was confirmed by custom sequencing. Nucleotide BLAST was performed to know the similarity with published sequence.

The positive clones were selected for expression study and Fis protein expression was induced by 1.0 mM for 6 hours. Results indicated maximum expression at 1.0 mM IPTG and at 6hr incubation. The expressed Fis was purified by nickel chelating affinity chromatography and visualized on SDS-PAGE by coomassie blue staining.

Characterization of protein

The protein bands from SDS-PAGE were transferred on to PVDF membrane following semi-dry blotting method. The membrane was washed in distilled water to remove SDS and then blocked with 3% skim milk powder in PBS-T for 1 hour at room temperature. Then membrane was washed twice with PBS-T and then incubated with Ni-NTA HRP conjugate diluted 1:4000 (Thermo Scientific) for one hour at room temperature. The membrane was washed thrice with PBS-T. Finally, the membrane was developed with DAB solution (Amresco) for five minutes.

Result

Amplification of Fis gene

The Fis gene was amplified by PCR using designed primers and the product size of 303 bp was obtained (figure1).

Cloning and expression of Fis gene

The amplified Fis gene was double digested with EcoRI and HindIII restriction enzyme. Digested product was purified from agarose gel and ligated with pET32a (+) vector (Fig 11a and 11b) digested with same restriction enzymes. Ligated product was transformed in to *E. coli* BL21 and plasmid was purified from positive clone and digested with EcoRI and HindIII, product of desired size (303 bp) was released upon digestion (Fig 12). The reading frame of the inserted genes was confirmed by custom sequencing. Nucleotide BLAST analysis show 100% similarities to publish sequences. Sequenced data was submitted to NCBI with accession number KM887845.

Characterization of protein

Pet32-Fis plasmid was transformed in expression host *E. coli* BL21 strain. Cultures were grown at 37 ^{0}C with constant shaking until OD_{600} reached 0.4-0.6. Different concentrations of IPTG 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM and 1.0 mM was used for induction and samples were collected after 2 hr, 4 hr, 6hr, 8 hr, indicated maximum expression at 1.0 mM IPTG and after 6 hours.

The purified protein by nickel chelating affinity chromatography was subjected to western blotting using Ni-NTA HRP conjugate revealed a specific band at expected size of 29 kDa for Fis gene.

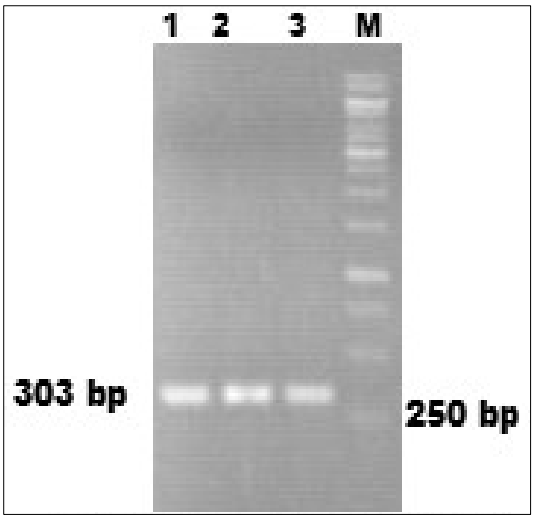
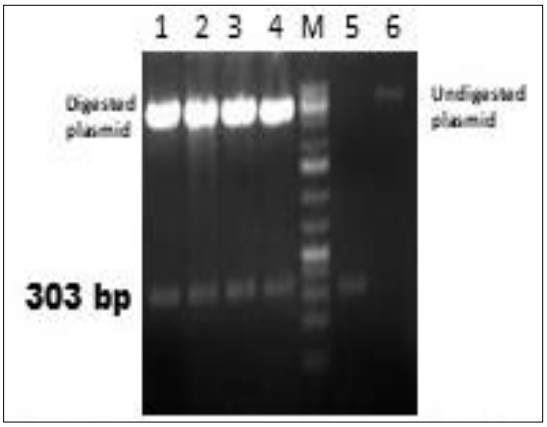
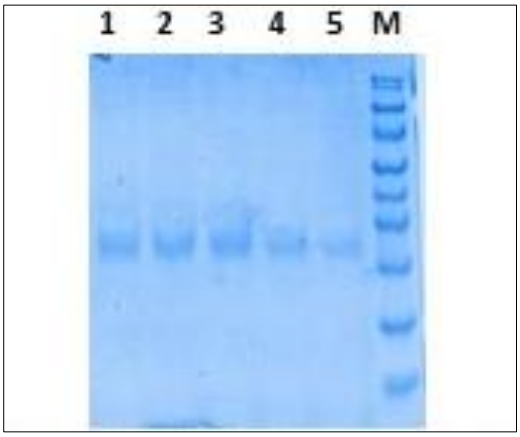


Fig 1: Amplification of Fis gene lane 1-3: amplified Fis gene lane M: 1KB Ladder



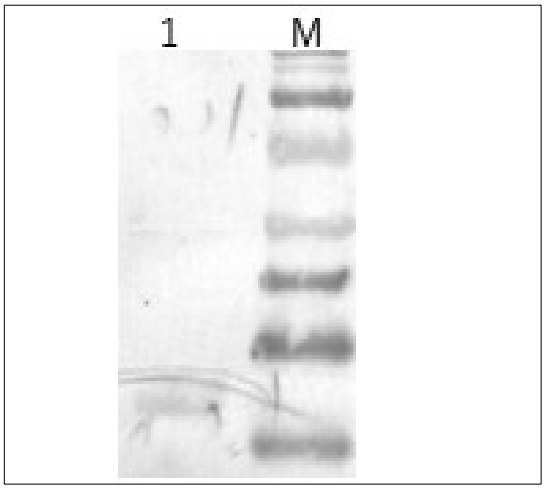
Lane 1-4: Screened transformed product Lane M:1 KB plus ladder
Lane 5: PCR product of positive transformed clone

Fig 2: Screening of positive done by restriction digestion and PCR



Lane M: Protein molecular weight marker
Lane 1-5: Ni-NTA column purified recombinant Fis protein

Fig 3: SDS-PAGE of Ni-NTA column purified recombinant Fis protein



Lane 1: Expressed Fis protein
Lame M: Pertained 10Kda protein marker

Fig 4: We stem blot of Fis with of Ni-NTA HRPO conjugate

Discussion

Haemorrhagic Septicemia is one of the most important livestock diseases of Asian and African countries in terms of economic losses due to high morbidity and mortality rate in cattle and Buffalo (De Alwis, 1992; Verma and Jaiswal, 1997) [8-9] caused by *P. multocida*. Polysaccharide capsule is play critical role in virulence of *P. multocida* and Fis gene regulating the capsule biosynthesis. It is DNA binding protein and was firstly recognized as factor for DNA inversion. Consequently, its various roles have been identified as positive and negative regular of gene expression. In *P. multocida* it regulates 42 genes generally acts as a transcriptional activator (Steen *et al*., 2010) [3]. To know more about role of Fis gene in capsular synthesis and in regulation of other virulence factor cloning and sequencing and expression are initial attempt which will be further helpful for its functional characterization.

Conclusion

Haemorrhagic Septicemia caused by *P multocida* is important disease of livestock. Fis gene play important role in regulation of virulence factor including capsular synthesis. Upon BLAST 100 similarity with Fis gene sequence of other *P. Multocida* strain indicating that Fis sequence is conserved.

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