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RESEARCH ARTICLE
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Cloning and sequence analysis of *hsf*, an outer membrane protein gene of *Pasteurella multocida* serotype B:2

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Abstract

Aim: The present study was undertaken to clone, sequence and analyze the *hsf*, an outer membrane protein gene of *Pasteurella multocida* serotype B:2

Materials and Methods: *hsf* gene was amplified from genomic DNA of *P. multocida*. Polymerase chain reaction (PCR) product was cloned in pET-32a vector and was characterized. *hsf* gene was sequenced, analyzed and phylogenetic tree was constructed taking sequences of other strains.

Results: Amplicon size was found to be 785 bp. Recombinant got characterized through colony PCR and restriction enzyme analysis.

Conclusion: *hsf* gene of *P. multocida* serotype B is similar to serotype A, but different from serotype D. Further work is needed to evaluate role of Hsf protein in protection studies and to study the antigenic properties of this recombinant protein as a candidate for vaccine.

Keywords: cloning, *hsf* gene, outer membrane protein, *Pasteurella multocida*, phylogenetic tree, sequencing

Introduction

Pasteurella multocida, a Gram-negative coccobacillus, non-motile, rod shaped, facultative anaerobic bacterium, is the causative agent of a various economically dreaded diseases such as hemorrhagic septicemia (HS), atropic rhinitis, fowl cholera, shipping fever, pneumonia and snuffles in various domestic and wild animals. *P. multocida* isolates are differentiated mostly basing on their capsular types (A, B, D, E and F) and somatic serotypes (1-16) [1]. Organisms belonging to serogroup B/E are responsible for a very fatal disease i.e. HS among the cattle population. Similarly, predominant fowl cholera strains belong to serogroup A [1], whereas strains causing diseases in swine (progressive atrophic rhinitis) belong to serogroup D [2].

HS is endemic in most tropical countries such as India and Africa causing high mortality in livestock [3]. A wide range of host such as cattle, buffalo, pig, sheep, goat, deer and camel are susceptible to this infection [4]. Though the uses of inactivated and live attenuated vaccines have led to a considerable reduction in overall livestock mortality, still the objective of complete protection has not been achieved [3].

Outer membrane proteins (OMPs) are involved in protective immunity against *P. multocida* infections. Virulence factors of *P. multocida* aid in the colonization, invasion, and its pathogenesis are surface located [5].

OMPs have been reported to confer various virulence attributes of the organism like colonization and invasion thus, are potential antigenic candidates [6]. Therefore, identification of OMPs is critical to make out the protective antigens and to develop novel diagnostics after proper evaluation of their antigenic potential and the ability to contribute to the host immunogenicity. OMPs of *P. multocida* based on functional characteristics were categorized and have been shown to stage pivotal role in host pathogen interaction and disease processes [7]. The key antigens of *P. multocida* B:2 that evoke protective immunity to HS in cattle have still not been well defined. Although sequencing of genes encoding various OMPs has been carried out, still for many genes no study has yet been done.

The present study was aimed to characterize important OMP *hsf*, a high molecular weight auto transporter adhesion protein, is a virulence factor. No systematic study on sequence analysis of *hsf* gene has been carried out till date, therefore, these proteins can be exploited for development of recombinant antigens based diagnostic test for detection of HS in bovines or as vaccine candidate for providing protection against the ailment.

Materials and Methods

Ethical approval

The Institutional Animal Ethics Committee (IAEC)/Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA) of

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the IVRI, Izatnagar had approved for immunization studies.

Extraction of total genomic DNA of *P. multocida* serotype B:2

Genomic DNA was extracted from culture of *P. multocida* serotype B:2 maintained in the Division of Bacteriology & Mycology, Indian Veterinary Research Institute, Izatnagar by CTAB method and was preserved for further use.

Polymerase chain reaction (PCR) amplification, cloning and its characterization

hsf gene was amplified with PCR using primers with *Bam*HI and *Sac*I restriction enzyme sites (HSF-F/ Upper primer: 5'- CATATCggatccAATATTGCGAT TGGTGATGGT-3' and HSF-R/Lower primer: 5'- G AATCGgagctcGTTTGTATTGCCTTTTGTATTTTT -3') to amplify 785 bp amplicon from *P. multocida* genomic DNA. Primers were designed taking the reference sequence available at GenBank (AE004439.1).

The PCR was carried out in 50 µl reaction volume containing 10 pmol of each primer, 0.1 mM of dNTPs, 1.5 mM of $MgCl_2$, 2 U of vent polymerase (NEB, UK) in 1X reaction buffer using 50 ng of genomic DNA as template. The reaction conditions were as follows; initial denaturation 95°C for 5 min. 35 cycles denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min followed by one cycle of final extension at 72°C for 10 min. The amplified products were analyzed by electrophoresis in 1.5% agarose containing ethidium bromide (0.5 µg/ml).

The amplified PCR product and pET-32a vector were double digested with restriction endonuclease enzymes *Bam*HI (Fermentas) and *Sac*I (Fermentas) and ligated using T4 DNA ligase (Novagen, USA). Recombinants were screened through colony PCR using gene specific primers as given above and further analyzed by electrophoresis in 1.5% agarose containing ethidium bromide (0.5 µg/ml).

Recombinant plasmid was also characterized through double restriction digestion analysis using *Bam*HI and *Sac*I. The digested products of recombinant plasmid were separated on 1.5% agarose gel.

DNA sequence analysis

The positive recombinant plasmid containing *hsf* gene fragments of *P. multocida* was sequenced using both forward and reverse primers of pET-32a vector. Sequence was analyzed and submitted to NCBI GenBank. *P. multocida* strains 3480 (CP001409.1), Pm70 (AE004439.1) and *hsf* gene of *Haemophilus influenza* (AJ277635.1) were retrieved from NCBI. Phylogenetic analysis and percent similarity/divergence were analyzed using Mega Align (DNASTAR, Madison, Wisconsin, USA) software.

Results and Discussion

The ~785 bp amplicon size as visualized in agarose gel electrophoresis indicated the amplification of *hsf* gene from the *P. multocida* genomic DNA (Figure-1). PCR product cloned in pET-32a vector was characterized by both colony PCR and restriction enzyme analysis. Colony PCR upon gel separation gave amplicon size ~785 bp that indicated the presence of *hsf* gene (Figure-2). Recombinant plasmids were also characterized by double restriction enzyme digestion analysis where release of insert of size ~785 bp confirmed the presence of the gene of interest in the recombinant plasmid (Figure-3). Sequencing results were analyzed and further submitted to NCBI GenBank (KM593283). Phylogenetic tree based on *hsf* nucleotide sequences of three strains of *P. multocida* (P-52, 3480 and Pm70) and one strain of *H. influenza* is indicated in Figure-4. The nucleotide alignment results of *hsf* gene of different strains of *P. multocida* taking P-52 as reference strain revealed 96.8% homology and 2.3% divergence from Pm70 (*P. multocida* serotype A:1 isolates from USA) strain whereas 75.6% homology and 29.1% divergence from strain

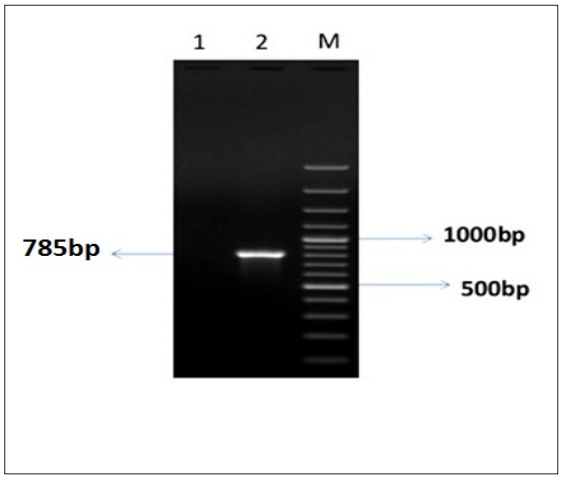

Figure-1: Polymerase chain reaction (PCR) amplification of 785 bp hsf gene. The PCR product was analyzed in 1.5% agarose gel containing 0.5 µg/mL ethidium bromide. Lane 1: No Template Control; Lane 2: PCR product; Lane M: 100 bp ladder.

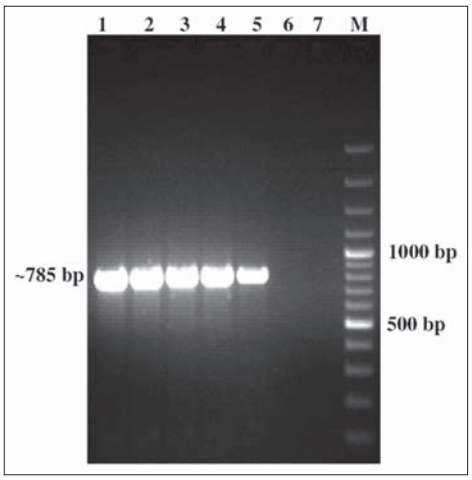

Figure-2: 1.5% agarose gel electrophoresis of colony polymerase chain reaction amplified using gene specific primers. Lanes 1-5: Recombinant clones; Lanes 6-7: Non recombinant clones; Lane M: 100 bp plus DNA ladder.

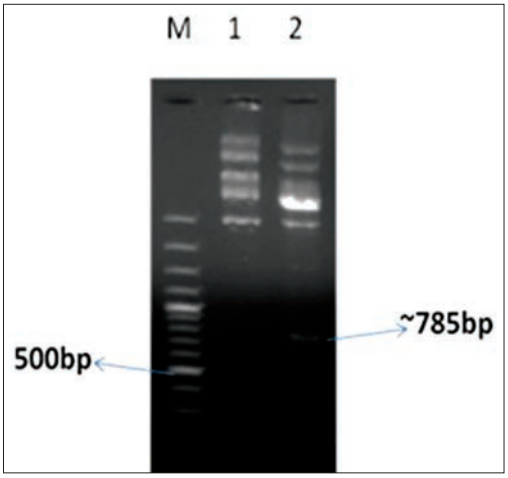

Figure-3: Restriction endonuclease analysis of recombinant plasmid, Lane M: 100bp ladder, Lane 1: Undigested recombinant plasmid, Lane 2: *Bam*HI and *Sac*I double digested recombinant plasmid

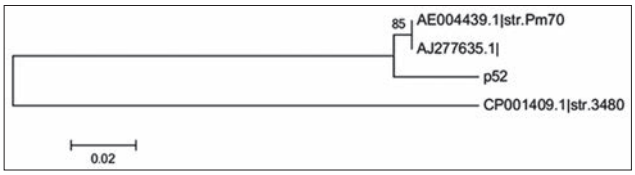

Figure-4: Phylogenetic tree based on nucleotide sequence illustrating the evolutionary relationship of *hsf* gene of P-52 strain with Pm70 (AE004439.1), 3480 strain (CP001409.1) of *Pasteurella multocida* and also with *Haemophilus influenza* (AJ277635.1). The phylogenetic tree was constructed using neighbor-joining analysis. Numbers represent bootstrap values (given as percentages) for a particular node.

3480 (*P. multocida* serotype D isolates from China). Phylogram suggested that *hsf* gene of *P. multocida* serotype B is similar to serotype A, but different from serotype D. As only one isolate was sequenced, so it is not clear whether the substitution from serotype D observed in this study carry signature sequences for capsular type specificity or due to host origin specificity or for geographical location. *hsf* is an adhesion related gene of *P. multocida* showed homology with *H. influenza* surface fibrils. The *H. influenza* type B Hsf is a vitronectin binding protein which contributes to resistance against antibody [8]. Transcriptional activation of *hsf* gene of *P. multocida* was also found to be escalated under nutrient deficient conditions [9]. These above two findings suggested the role of *hsf* gene in host resistance.

The *P. multocida* contains two types of *hsf* gene namely *hsf1* and *hsf2*. The frequency of *hsf* gene varies among serotypes with the gene *hsf2* being more prevalent. In porcine isolates the *hsf2* gene is present in 99.1% isolates whereas *hsf1* in 67% strains [10]. Among Indian small ruminant isolates *hsf2* gene was present in 90.9% isolates whereas *hsf1* in 51.1% isolates [11].

The sequence analyses for many other genes encoding OMPs have showed variable degree of homology that was reported by several authors. Recently, authors have reported that the *vacJ* gene had greater homogeneity (99.1-100%) among the *P. multocida* strains [12]. Singh *et al*., 2010 [13] compared *plpE* gene in different capsular types and observed more than 90% homology whereas Siju *et al*., 2007 [14] observed 78.4% of similarity between nucleotide sequence of *ptfA* gene in A:1 and B:2 isolates. Similarly Shivachandra *et al*., 2005 [15] reported 98.4% homology and 1.5% divergence in *tbpA* gene of *P. multocida* B:2 isolate from serotype A:1 isolates. Jain *et al*., 2013 [16] observed >95% homology among *hasR* and *hgbA* gene among different isolates. So this study highlights the need to exploit outer membrane genes and carry out multidimensional study in the field of diagnosis and prophylaxis.

Conclusion

HS is an important disease of cattle and buffaloes. For effective control of the disease, an efficacious and longer duration immunity vaccine is required. Identification and characterization of important immunogens of the bacteria would not only help in designing an improved vaccine but also Identification of the OMPs and exploiting it would serve as a candidate for development of diagnostics. As Hsf protein is one of the important virulence factors of the bacteria therefore further work is needed to evaluate its role in protection studies and to study the antigenic properties of this recombinant protein as a candidate for vaccine.

Authors' Contributions

AP carried out experimental execution, *in-silico* analysis, preparation of draft and revision of the manuscript. SK assisted in experimental executions. SKG and VPS participated in the conception and designing of experiment. KNV and RKA helped in trouble shooting during experiment and in the analysis of the final result.

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Competing Interests

The authors declare that they have no competing interests.

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