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RESEARCH ARTICLE
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Polymerase chain reaction amplification and cloning of immunogenic protein NAD-dependent beta hydroxybutyryl CoA dehydrogenase gene of *Clostridium chauvoei*

Saroj K. Dangi[1], Ajay P. Singh[1], Satyaveer S. Dangi[2], Prasad Thomas[1], Santosh K. Gupta[1], Rajesh K. Agarwal[1] and K. N. Viswas[1]

1. Division of Bacteriology & Mycology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India;
2. Division of Physiology & Climatology, Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India.

Corresponding author: K. N. Viswas, e-mail: vkn111@gmail.com, SKD: sarojsvet@gmail.com, APS: drajay_vet@yahoo.co.in, SSD: dangivet@gmail.com, PT: prasadthomas99@gmail.com, SKG: skgupta15@rediffmail.com, RKA: grace_bly@yahoo.com

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Abstract

Aim: The present study was aimed at polymerase chain reaction (PCR) amplification and cloning of NAD-dependent beta-hydroxybutyryl coenzyme A dehydrogenase (BHBD) gene of *Clostridium chauvoei*.

Materials and Methods: *C. chauvoei* was cultured and confirmed by 16-23S rDNA spacer region primers. The primers for *nad-bhbd* gene of *C. chauvoei* were designed to aid in cloning into pRham-N-His SUMO-Kan vector, and *nad-bhbd* gene was amplified by PCR. The amplified *nad-bhbd* gene was purified and cloned into pRham-N-His SUMO-Kan expression vector. The recombinant plasmid was transformed into *E. cloni* 10 G cells and the clone was confirmed by colony PCR using the pRham-SUMO-NAD-For and pRham-SUMO-NAD-Rev primers and also by sequencing.

Results: PCR amplification of *nad-bhbd* gene yielded a product length of 844 base pairs which was cloned into pRham-N-His SUMO-Kan vector followed by transformation into *E. cloni* 10G chemically competent cells. The recombinant clones were characterized by colony PCR, sequencing, followed by basic local alignment search tool (BLAST) analysis to confirm the insert.

Conclusions: Immunogenic protein NAD- dependent BHBD of *C. chauvoei* was cloned and the recombinant clones were confirmed by colony PCR and sequencing analysis.

Keywords: black quarter, *Clostridium chauvoei*, NAD-beta-hydroxybutyryl coenzyme A dehydrogenase.

Introduction

Black quarter is one of the important bacterial diseases of domestic animals, which affect mainly cattle, buffaloes, and sheep. It is caused by *Clostridium chauvoei*, a Gram-positive, motile, spore-forming bacilli. It is the second most important bacterial disease in India next only to hemorrhagic septicemia in causing death among bovids. *C. chauvoei* is one of the most pathogenic *Clostridium* species causing considerable losses in livestock production [1]. According to the report by Department of Animal husbandry, Fisheries and Dairying, Government of India, there were about 417 outbreaks across the country in which 2676 cattle and buffaloes were affected and 900 animals died in the year 2011 alone [2]. Although *C. chauvoei* is generally considered to be specific for ruminants, there are reports of black quarter in nonruminants such as pig, mink, fresh water fish, whales, frogs, elephant, and humans [3-8]. Ruminants are exposed to the pathogen by ingestion of *C. chauvoei* spores present in the soil, which then induce cell necrosis, edemic lesions and fever followed by lameness and death [9-11]. Treatment is of limited value, considering the acute nature of the disease, and the vaccination remains the preferred control measure in the field conditions. Whole inactivated bacteria and chemically toxoided culture supernatants were most commonly used for the prophylaxis of blackleg [12]. *C. chauvoei* flagella plays an important role in virulence and are the only particular antigens for which there is any evidence of involvement in inducing protective immunity against blackleg [13-15]. Besides, *C. chauvoei also* produces four toxins viz., oxygen-stable hemolysin, oxygen-labile hemolysin, DNase and hyaluronidase, and also a neuraminidase [16-19]. A 27 kDa hemolysin was purified from *C. chauvoei* strain C6H but was not further characterized [18]. Recently, another toxin gene, *C. chauvoei* toxin A (*cctA*), which is both cytolytic and hemolytic, has been cloned, sequenced, and expressed [20].

In a quest for the protective antigens of *C. chauvoei*, using 2D-gel electrophoresis and mass spectrometry approaches, several immunogenic proteins were identified in our laboratory (unpublished data). Among these, one important immunogenic protein identified was NAD-dependent beta-hydroxybutyryl-coenzyme A dehydrogenase

(NAD-BHBD). The 3-hydroxybutyryl-CoA dehydrogenase was identified as immunogenic protein molecule of *Flavobacterium columnare,* which causes columnaris disease in fish, and it may be a candidate molecule for the development of vaccines against columnaris disease [21].

Currently, the information available regarding the NAD-BHBD is very scanty. As a first step in the characterization of immunogenic potential of this protein, in the present study, we identified and amplified the *nad-bhbd* gene, and cloned in prokaryotic expression vector.

Materials and Methods

Ethical approval

There was no use of live animal in the study.

Bacteria

The bacteria *C. chauvoei* American type culture collection (ATCC 10092) used in the present study was obtained and procured from LGC promochem pvt. Ltd., Bangalore.

Genomic DNA isolation

The bacterials strain were cultured in ATCC 2107 media (1% tryptose, 1% beef extract, 0.3% yeast extract, 0.5% dextrose, 0.5% NaCl, 0.1% soluble starch, 0.05% L-cystine HCl, and 0.03% Na acetate) at 37°C for 48 h and the genomic DNA was extracted by CTAB-NaCl method [22]. About 3-5 ml of late logarithmic bacterial culture was centrifuged at 9,300 g for 10 min to separate bacterial cells from supernatant. The bacterial pellet was washed twice with phosphate buffered saline. The pellet was again suspended in lysis buffer (200 µl of × 1 TE, 300 µl of 10% sodium dodecyl sulfate and 5 µl of proteinase K) and incubated at 37°C for 1 h. Resuspension of the cells in lysate solution (500 µl of 5 M NaCl and 100 µl CTAB) was done, and samples were kept in the water bath at 63°C for 10 min. Equal volume of chloroform: Isoamyl alcohol (24:1) was added, and centrifugation at 15,700 g for 10 min was done. To the supernatant, $1/10^{th}$ volume of 7.5 M ammonium acetate and 2 volume of chilled ethanol was added followed by centrifugation at 9,300 g for 20 min at 4°C. Resuspension of the pellet in elution buffer was done followed by storage at −20°C.

Confirmation of *C. chauvoei* by 16-23S rDNA spacer region primers

Polymerase chain reaction (PCR) amplification of 16-23S rDNA was performed as described by Sasaki *et al*. [23]. The identification of *C. chauvoei* was carried out using the specific primers (IGSCS and 23 UPCH primers) targeting 16S-23S rDNA spacer region.

PCR amplification of *nad-bhbd* gene of *C. chauvoei*

The primers were designed from available sequence of *nad-bhbd* gene of *C. chauvoei* in NCBI (accession number-CMBL 010000001.1), to aid in cloning into pRham-N-His small ubiquitin-related modifier (SUMO)-Kan vector and the sequence of the primers designed is given below.

pRham-SUMO-NAD-For-CGCGAACAGATTGGAGGTGAAAAGATTTTTGTAATTGGT

pRham-SUMO-NAD-Rev-GTGGCGGCCGCTCTATTATCTGCTATAGTCGTAGAATCC

PCR was carried out in the volume of 25 µl reaction mixture containing 10 ng of genomic DNA, 2.5 µl of × 10 thermopol buffer, 3 µl $MgSO_4$ (25 mM), 0.5 µl dNTPs (10 mM), 10 pmol each of the primer and vent polymerase. PCR was performed in thermocycler (Eppendorf, Mastercycler) using a program consisting initial denaturation temperature of 94°C for 5 min followed by 40 cycles of denaturation of 94°C for 1 min, annealing temperature of 59°C for 1 min and extension of 72°C for 1 min 30 s. Final extension was carried out at 72°C for 10 min.

Cloning of *nad-bhbd* gene and confirmation of the recombinant clones

The amplified *nad-bhbd* gene was purified by qiagen gel extraction kit (Qiagen, Germany) and cloned into pRham-N-His SUMO-Kan expression vector (Lucigen Corporation, Wisconsin, USA) using manufacturer's protocol. The recombinant plasmid was transformed into *E. cloni* 10 G cells and the clone was confirmed by colony PCR using the pRham-SUMO-NAD-For and pRham-SUMO-NAD-Rev primers (Lucigen Corporation, Wisconsin, USA) and also by sequencing. pRham-N-His SUMO-Kan-*nad-bhbd* construct was sequenced at a custom DNA Sequencing facility (Eurofins). Sequences were analyzed using DNASTAR Lasergene software (DNASTAR, Inc., USA) and subjected to analysis using basic local alignment search tool (BLAST) program of NCBI (National Centre for Biotechnology Information) against the draft genome sequence of *C. chauvoei*. Nucleotide sequence of *nad-bhbd* gene was submitted to NCBI nucleotide sequence database and has been assigned accession number, KM051572.

Results

Confirmation of *C. chauvoei* by PCR

PCR confirmation of culture was done using primers specific for 16S-23S rDNA spacer region [23] using *C. chauvoei* enomic DNA as template. Agarose gel electrophoresis analysis of the PCR product showed specific amplification product length of 522 bp (Figure-1) confirming the identity of *C. chauvoei.*

PCR amplification of *nad-bhbd* gene of *C. chauvoei*

nad-bhbd gene of *C. chauvoei* was amplified by self-designed gene-specific primers using *C. chauvoei* DNA as template. Amplified product was analyzed by 1.5% agarose gel electrophoresis which showed an intense amplification at 844 bp (Figure-2) as expected. The PCR amplified *nad-bhbd* gene was eluted using gel extraction kit. The yield of the eluted DNA was approximately 80% of the original PCR product, and

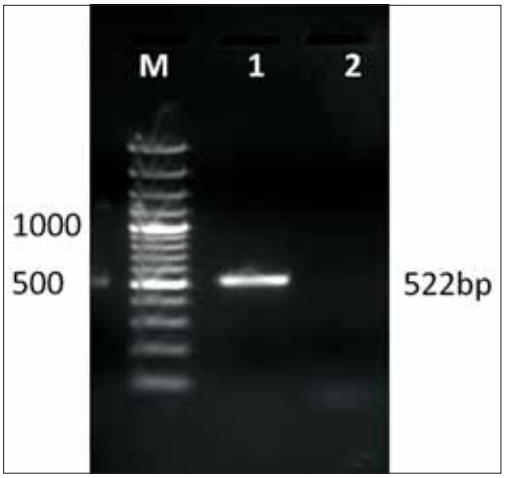

Figure-1: DNA electrophoresis of polymerase chain reaction (PCR) amplicons of 16S-23S rDNA spacer gene. M-DNA ladder, 1-PCR product, 2-Negative control.

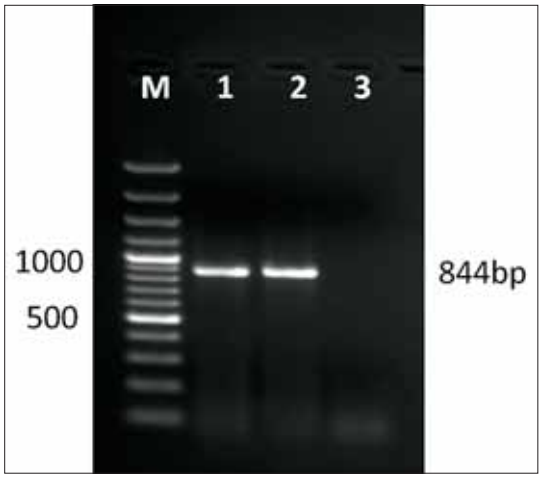

Figure-2: Electrophoresis of amplificated DNA of *nad-bhbd* gene of *Clostridium chauvoei*; M-DNA ladder, 1 and 2-*nad-bhbd* gene, 3-Negative control.

the DNA was obtained as a distinct band without any smearing.

Cloning of *nad-bhbd* gene of *C. chauvoei*

The PCR products having the SUMO flanking region were ligated with pRham N-His SUMO vector and transformed into *E. cloni* 10 G cells and plated on LB kanamycin agar, and incubated overnight at 37°C. LB kanamycin agar plate showed only two colonies. Both the colonies were inoculated into 5 ml LB broth containing kanamycin. LB broth containing single colony kept for overnight growth in a shaker incubator at 37°C and 180 rpm. pRham N-His-SUMO-*nad-bhbd* clones were then confirmed by colony PCR, which showed amplicons of 844 bp size as expected (Figure-3). Plasmid was isolated from these clones and sent for sequencing using sequencing primers specific for the vector backbone. Sequence analysis confirmed that the clones were indeed positive for *nad-bhbd* gene (Figure-4). Sequence was then analyzed against the nucleotide sequence database using BLAST program of NCBI. Based on the maximum

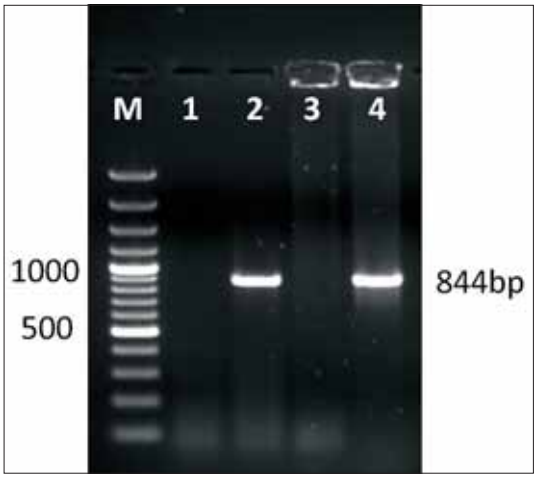

Figure-3: DNA electrophoresis of polymerase chain reaction amplicons of recombinant clones; M-DNA Ladder, 1 and 3-*nad-bhbd* negative clones, 2 and 4-*nad-bhbd* positive clones.

GAAAAGATTTTTGTAATTGGTGCTGGTACTATGGGAGCTGGAATTGTTCAAGCTTTTGCACA
AAAAGGTTATGAAGTTATCGTTAGAGATATAAAGGACGAATTTGTTGAAAAAGGAATTGCTG
GAATTAATAAAGGACTTAGCAAACAAGTAGCTAAAGGAAAGATGACTGAAGAAACTAAGGA
AGCTATATTATCAAGAATTACAGGAACAACTGATATGAAATTAGCAGAAGACTGTGATTTAGT
TATAGAAGCAGCAATTGAAAATATGCAAATCAAGAAACAAATATTTGCTGAATTAGATGAAAT
CTGTAAAGAATCAGCTATTTTAGCATCAAATACTTCATCATTATCAATAACTGAAGTTGCATCA
GCAACAAAGAGAGCAGATAAGGTTATAGGAATGCATTTCTTTAACCCAGCTCCAGTAATGAA
ATTAGTGGAAATAATCAGAGGGATGGCTACTTCACAAGAAACTTTTGATGCTGTTAAGGAATT
ATCAGTAGCAATTGGTAAAGAACCAGTAGAAGTTGCAGAAGCTCCAGGATTTGTAGTTAATA
GAATACTTATCCCAATGATTAATGAAGCAACATTTATTCTTCAAGAAGGAATTGCTTCAGCTGA
AGATATAGATAGTGCTATGAAATATGGTGCTAATCACCCAATGGGACCTTTAGGTTTAGGAGA
TCTTATTGGATTAGATGTTTGTTTAGCTATAATGGATGTTTTATATAATGAAACAGGAGATACTA
AATATAGAGCAAGTAGCCTACTAAGAAAATATGTTAGAGCTGGATGGCTAGGTAGAAAATCA
GGAAAAGGATtCTACGACTATAGCAGATAA

Figure-4: Nucleotide sequence of *nad-bhbd* gene.

identity score, insert sequence had 100% identity at nucleotide level with *nad-bhbd* gene of *C. chauvoei* strain JF4335, confirming that the gene cloned in the pRham N-His SUMO vector was indeed the *nad-bhbd* gene.

Discussion

Black quarter, caused by a Gram-positive, anaerobic spore-forming rod called *C. chauvoei*, is a dreadful disease affecting ruminant population. Several virulence factors have been proposed to be produced by *C. chauvoei*, including alpha, beta, gamma, and delta toxins, and neuraminidase [11]. However, most of this information is based on the studies conducted in a very closely related species, *C. septicum*. Very few studies have been conducted in *C. chauvoei* to establish the actual role of these proposed virulence factors. Recently, another toxin gene, *cctA*, which is both cytolytic and hemolytic, has been cloned, sequenced and expressed [20]. They showed that cctA protein had hemolytic and cytotoxic activity, and offered protection against virulent spores of *C. chauvoei*. However, the protective antigens of *C. chauvoei* are not yet fully characterized.

In a recent work in our laboratory, using the 2D-gel electrophoresis and mass spectrometry approaches, several immunogenic cell-surface associated proteins

of *C. chauvoei* were identified (unpublished data), among which NAD-BHBD, was one important immunogenic protein. Recently in other study, the 3-hydroxybutyryl-CoA dehydrogenase was identified as immunogenic protein molecule of *F. columnare*, which causes columnaris disease in fish, and it may provide candidate molecules for the development of vaccines against columnaris disease [21]. Hence, we choose to characterize this gene. Accordingly, *nad-bhbd* gene of *C. chauvoei* was amplified, cloned, and characterized by sequencing analysis.

Conclusion

In summary, in the present study, *nad-bhbd* gene was identified, amplified, and cloned in pRham N-His SUMO expression vector. The recombinant clone was confirmed by colony PCR using gene-specific primers and by sequencing of the insert. However, further research is required to express the *nad-bhbd* gene in *E. cloni* and to evaluate if the recombinant NAD-BHBD protein has immunogenic potential.

Authors' Contributions

PT and KNV have designed the plan of work. SKD and APS carried out the laboratory work, and KNV and RKA guided during the experiment. SKG and SSD drafted and revised the manuscript. All the authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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