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Cloning and expression analysis of *nagJ* hyaluronidase gene of *Clostridium chauvoei*

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Clostridium chauvoei, a Gram-positive, spore forming anaerobe, causes a fatal black quarter disease in ruminants. Mostly young animals like cattle of 6–24 months age are affected. Though it is proposed that *C. chauvoei* produces many toxins like oxygen-stable hemolysin, DNase, hyaluronidase and oxygen labile hemolysin, the only well characterized virulence factors of *C. chauvoei* are neuroaminidase (Vilei *et al.* 2011), cctA (Frey *et al.* 2012) and flagellin (Kojima *et al.* 2000). Hyaluronidases of *C. chauvoei* are not yet characterized in detail. Bacterial hyaluronidases breakdown hyaluronate, and thus help in spread of bacteria and toxins. The genome analysis of *C. chauvoei* showed the presence of 2 genes for hyaluronidases, *nagJ* and *nagH* (Frey and Falquet 2015, Thomas *et al.* 2017). Currently, the information available regarding the hyaluronidases of *C. chauvoei* is very scanty. In the present study, as an initial step in characterization of its role in virulence, we cloned and expressed hyaluronidase (*nagJ*) gene in prokaryotic expression system.

For genomic DNA isolation, *Clostridium chauvoei* strain ATCC 10092 (ATCC, USA) was cultured in ATCC 2107 media at 37°C for 48 h under anaerobic conditions and genomic DNA was extracted using Genomic DNA extraction kit (Thermo Scientific, USA) as per manufacturer's instructions. The identification of *C. chauvoei* was carried out by PCR using the specific primers (IGSCS and 23UPCH primers) targeting 16S–23S rDNA spacer region (Sasaki *et al.* 2001). *nagJ* gene was amplified using the primers (For- CGCGAACAGATTGGAGGTA CAATAACAA AAGAAGGGGAA; Rev- GTGGCGG CCGCT CTATTATACTCCTGTTTGTGC TTTTGG) at 58°C annealing temperature using a programme consisting of denaturation at 94°C/5 min followed by 34 cycles of denaturation at 94°C/1 min with annealing temperature of 58°C/1 min and extension of 72°C/1 min. Final extension was carried out at 72°C for 10 min.

The amplified *nagJ* gene was cloned into pRham-N-His-SUMO-K an expression vector (Lucigen, USA) as per manufacturer's instructions. The recombinant clones were screened by colony PCR and the positive clones were induced for expression using L-rhamnose. SDS-PAGE analysis was carried out as per the protocol described elsewhere (Laemmli *et al.* 1970) to check the expression. Recombinant *nagJ* protein was purified under denaturing conditions in urea using Ni-NTA affinity chromatography (Qiagen, USA) as per manufacturer's protocol. Hyperimmune sera raised in chicken against recombinant *nagJ* protein (IgY Immunologix, India) was used to check the reactivity of the recombinant protein by Western blot analysis (Towbin *et al.* 1979).

PCR confirmation of culture was done by using primers specific for 16S–23S rDNA spacer region, which yielded specificamplificons of 522 bp confirming the identity of *C. chauvoei* (Fig. 1A). *nagJ* gene of *C. chauvoei* was amplified with an intense amplification at 1082 bp (Fig. 1B) as expected. PCR amplified *nagJ* gene was cloned in pRham-N-His-SUMO Vector and transformed into *E. cloni* 10G cells. Colonly PCR with *nagJ* specific primers was used to screen recombinants, which showed amplicons of 1082 bp size as expected (Fig. 1C) confirming the cloning of *nagJ* gene in the vector.

Expression of *nagJ* protein was analyzed by SDS-PAGE analysis, which showed the *nagJ* protein expression at 54 kDa (Fig. 2A) as expected. Recombinant protein *nagJ* purified under denaturing conditions showed a very intense 54kDa protein band (Fig. 2B). Recombinant *nagJ* protein concentration was 0.690 mg/ml. Furthermore, purified recombinant *nagJ* was confirmed by immunoblotting using anti-rnagJ antibodies (Fig. 2C).

Black quarter is an important bacterial disease of ruminants responsible for major economic losses in different parts of the world. *C. chauvoei* secretes exotoxins which are responsible for the pathogenesis of black quarter. Recently, whole genome sequence was analyzed for the *C. chauvoei* ATCC 10092 (Thomas *et al.* 2017), and few genes

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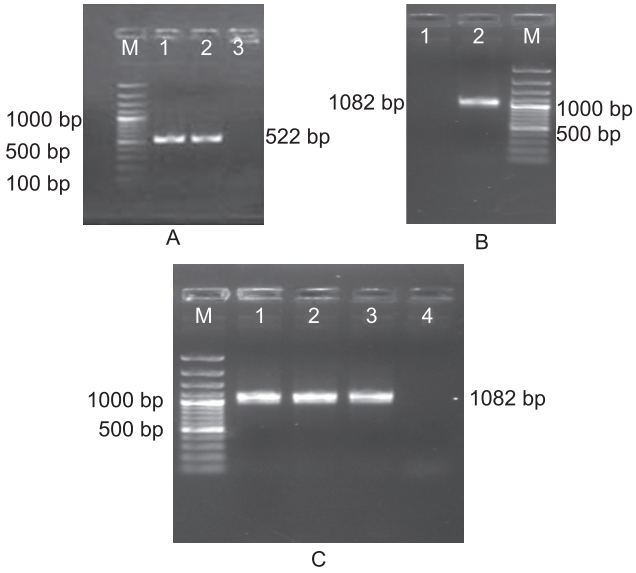

Fig. 1. Cloning of *nagJ* gene. (A) Confirmation of *C. chauvoei* by PCR based on 16S–23S rDNA spacer gene. Lane M, 100 bp DNA ladder; lanes 1 & 2, PCR product; lane 3, negative control. (B) PCR amplification of *nagJ* gene of *C. chauvoei*. Lane M, 100bp plus DNA ladder; lane 2, PCR product of *nagJ* gene; lane 1, negative control. (C) Confirmation of the recombinant clones by colony PCR. Lane M, 100bp plus DNA ladder; lanes 1–3, *nagJ* amplicon of 1082bp; lane 4, negative control.

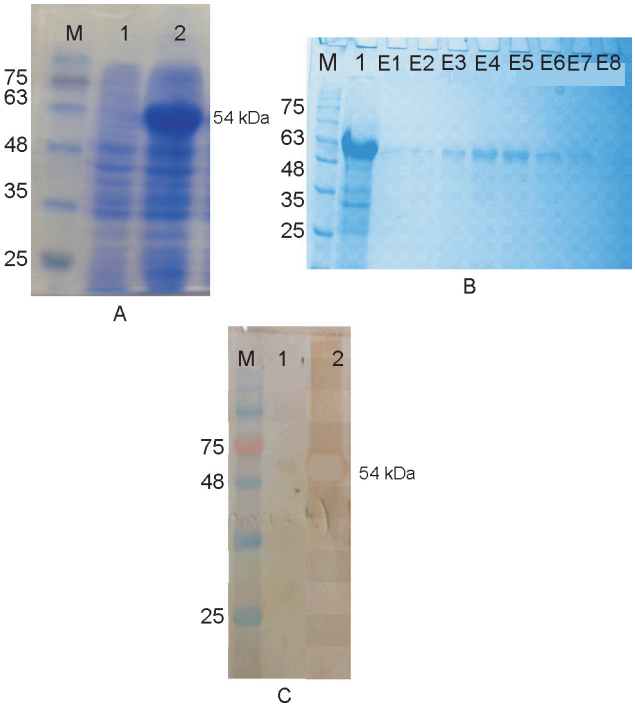

Fig. 2. Expression of *nagJ* gene. (A) Confirmation of expression of r-nagJ by SDS-PAGE analysis. Lane M, Protein ladder; lane 1, Uninduced cell lysate; lane 2, induced cell lysate. (B) SDS-PAGE profile of purified His-tagged recombinant *nagJ*. Lane M, protein ladder; lane 1, lysate; lanes E1-E8, different fractions of elutes. (C) Confirmation of r-nagJ by western blotting. Lane M, protein ladder; lane 1, uninduced cell lysate; Lane 2, induced cell lysate.

such as cctA (Frey *et al.* 2012, Sophia *et al.* 2013), sialidase NanA (Vilei *et al.* 2011) and NAD-dependent beta hydroxybutyryl CoA dehydrogenase (Dangi *et al.* 2014) were cloned, sequenced and expressed. Though it was proposed long back that the *C. chauvoei* produces hyaluronidases (Princewell and Oakley 1976, Hynes and Walton 2000), only recently it has been found that the genome of *C. chauvoei* harbours two different hyaluronidase genes, *nagH* and *nagJ* (Frey and Falquet 2015). Hyaluronidase enzyme affects the intracellular matrix of the host and thus favours the spread of *C. chauvoei* in the infected host. Hence, in an attempt to characterize the hyaluronidases of *C. chauvoei,* we selected *nagJ* for cloning and expression in prokaryotic system.

In the present study, *nagJ* gene was expressed in prokaryotic expression system and recombinant protein was characterized by SDS-PAGE analysis. Recombinant *nagJ* protein was characterized by western blotting analysis, thus confirming the hyaluronidase protein encoded by *nagJ* gene of *C. chauvoei.* Several other bacteria including *Clostridium* species express variants of hyaluronidases. *C. perfringens* genome sequence reveals 5 hyaluronidase genes (*nagH, nagI, nagJ, nagK,* and *nagL*) and the products of these genes differ in length, though their N-terminal amino acid sequences are similar (Shimizu *et al.* 2002). All of the *nag* genes have been shown to have N-terminal signal sequences, suggesting that they encode secretory enzymes (Nakai and Horton 1999). Another *Clostridium* species, *C. paraputrificum* has been shown to harbour *nag84A* gene, which encodes for hyaluronidase, though no hyaluronidase activity was demonstrated (Li *et al.* 2003).

SUMMARY

Black quarter caused by *Clostridium chauvoei*, a Gram positive anaerobe, is an important bacterial disease of ruminants. Though role of virulence factors such as cctA, sialidase and flagellin is well documented, hyaluronidases of *C. chauvoei* are not yet characterized. So, the present study was aimed at cloning and expression of hyaluronidase (*nagJ*) gene of *C. chauvoei*. The *nagJ* gene of *C. chauvoei* was amplified by PCR and cloned and expressed in prokaryotic expression system. Further, expressed recombinant hyaluronidase protein was purified and the expression was confirmed by SDS-PAGE and Western blotting analysis. Further investigation is needed to evaluate its functional activity and role in virulence of *C. chauvoei.*

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