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Cloning and Sequencing of *hly* Gene of Indian Isolate of *Listeria monocytogenes*

S. Das*, V. P. Singh, K. Ltu, S. Kathiresan and K.N. Bhilegaonkar[1]

Division of Bacteriology and Mycology, Indian Veterinary Research Institute Izatnagar- 243122, Bareilly

ABSTRACT

Listeria monocytogenes, a Gram- positive facultative intracellular organism, causes a number of important illnesses in human being and animals. In this study, *hly* gene of Indian isolate of *Listeria monocytogenes* was cloned, sequenced and results of sequence analysis showed 99% homology with *hly* gene of many strains of *L. monocytogenes*.

Keywords: *hly* gene, *Listeria monocytogenes*, sequence analysis

Introduction

Listeria monocytogenes causes a number of illnesses in human including abortion, meningo-encephalitis, arthritis, gastro-enteritis, peritonitis, endocarditis, etc. (Farber and Peterkin, 1991) and it is also responsible for abortion, circling calf syndrome, meningitis in animals (Vishwanathan and Ayyar, 1950; Dutta and Malik, 1981). The most important virulent gene of *L. monocytogenes* is *hly* gene, which encodes β hemolysin. It aids in intracellular survival of this pathogen (Kuhn *et al.*, 1988).

Mutants with *hly* gene deletion are generally avirulent, but can be reverted back to virulence by introducing cloned *hly* gene into these mutants (Cossart *et al.*, 1989). Presently, no sequence information of *hly* gene of the Indian isolates of *L. monocytogenes* has been reported. The sequence data of *hly* gene is helpful in future to design the primers for specific identification of *L. monocytogenes* and also for differentiation from the other species of *Listeria*.

***Corresponding author :** Central Institute of Fisheries Technology, Cochin, India. E-mail: sanjoy125@yahoo.co.in

[1]Division of Veterinary Public Health, Indian Veterinary Research Institute, Izatnagar

Keeping these in view, the present study was undertaken to sequence *hly* gene of Indian isolate of *L. monocytogenes*.

Materials and Methods

Bacterial strains

The bacterial cultures used in this study were *Listeria monocytogenes* (L-55) and *Escherichia coli* (DH5α). *L. monocytogenes* L-55 was maintained on tryptic soya agar slopes and stored at 4^{0}C. *E. coli* DH5 α (Invitrogen) was used as a host for the cloning.

Isolation of genomic DNA

Isolation of genomic DNA from *L. monocytogenes* (L-55) was carried as per the protocol of Liu *et al.* (2003) with a slight modification. The bacterial pellet was washed once with sterile normal saline before suspending in lysis solution. Extraction with phenol: chloroform: isoamyl alcohol was carried out twice. The purity and concentration of purified DNA was determined by measuring OD at 260 and 280 nm.

Primers

Primers *viz.* Hly-seqF (5'-ATGAAAAAAATAATGCTAGTTT-3') and Hly-seqR (5'-TTATTCGATTGGATTATCTACACTA-3') were

designed based on the published sequence of *hly* gene of *L. monocytogenes* ATCC 9525 (Gen Bank Accession No. AF253320) to amplify the whole 1590 bp CDS region of *hly* gene of *L. monocytogenes*.

Amplification of *hly* gene

Genomic DNA (100 ng) was used as template in 200 µl reaction mixture consisting of 20 µl of 10x PCR buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 0.08 % Nonidet P40), 1.5 mM $MgCl_2$, 80 p mole of each primer, 200 µM dNTP mix (Fermentas) and 4 units of *Taq* DNA polymerase (Fermentas). PCR was carried out with a programme of initial denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 30 sec (denaturation), 52°C for 1 min (annealing) and 72°C for 80 sec (extension). Final extension was carried out at 72°C for 5 min. The amplified products were checked on 1% agarose gel in 0.5 x TBE buffer (25 mM Tris-HCl, 50 mM boric acid, 5 mM EDTA, pH 8.2) and photographed under UV gel documentation system (Alpha Innotech Corporation, USA).

Cloning of *hly* gene

T/A cloning vector, pGEMT-easy (Promega), was used for cloning the *hly* gene. The DNA fragment of interest was excised, gel eluted using QIA quick gel extraction kit (Qiagen, Germany). The purified PCR product was checked again using Hly-seqF/Hly-seqR primers and cloned into pGEMT-easy vector in ligation mixture (1 µl 10x T 4 ligation buffer, 5 µl (200 ng) of purified PCR product, 1 µl of T4 DNA ligase, 1 µl of pGEMT-easy vector and 2 µl of nuclease free distilled water) along with one control having no vector DNA. It was incubated at 4°C for overnight.

White recombinant colonies were randomly picked up from the plate and incubated in 5 ml LB broth containing ampicillin (100 µg / ml) and incubated overnight at 37°C under constant shaking. The plasmids were extracted using HiYield™ Plasmid Mini Kit (Real Biotech Corporation, Taiwan) following manufacturer's protocol. The gene was released from the plasmid vector by RE (*Nco*I and *Sal*I) digestion. The digested samples were analyzed by 1% agarose gel electrophoresis.

Transformation and selection of clones

Ten milliliter of LB broth was inoculated with *E. coli* DH5 α culture and incubated at 37°C for 18 h with vigorous shaking (200 rpm) and then finally kept on ice for 10 min. Transformation was carried out by using 0.1 M $CaCl_2$ following the procedure of Sambrook and Russel (2001) with a little modification.

Selection of the recombinant clones was based on blue / white selection procedure on LB agar containing ampicillin (75 µg/ml), 20 mM IPTG and X-gal (80 µg / ml) (Sambrook and Russel, 2001). Randomly, 10 white colonies were picked up and stored on a master plate (LB agar with 75 µg /ml of ampicillin) at 4°C.

Confirmation of clones

For plasmid isolation, 5 white colonies were inoculated in 25 ml of LB broth containing ampicillin (75 µg/ml) and incubated at 37°C for 18 h with vigorous shaking at 200 rpm. Isolation of plasmid was performed by alkaline lysis miniprep method as per the protocol of Sambrook and Russel (2001).

Confirmation of clones was performed by (PCR) and restriction endonuclease analysis (REA). Plasmids isolated from different colonies were checked for the presence of desired insert by PCR using Hly-seqF/Hly-seqR primer pair. Approximately, 50 ng of plasmid was used for amplification, while PCR reaction mixture and programme were carried out as mentioned previously. The confirmation of clones and their orientation were done using the *Nco*I restriction enzyme. Approximately, 4 µg of plasmid was digested with 20 units of *Nco*I in 20 µl of reaction mixture containing 2 µl of 10x buffer Tango with BSA and incubated at 37°C for 3 h. in a water bath. Then, it wa s analyzed on 2% agarose gel and visualized under an UV gel documentation system.

Sequencing of DNA

Clone with desired and specific insert as

confirmed by PCR and R.E. digestion was stab inoculated in LB soft agar (1% agar) and sent to University of Delhi, South campus for DNA sequencing of *hly* gene. Sequencing was done by di-deoxy chain termination method.

Analysis of sequence

After receiving the sequence data from University of Delhi south campus, the analysis of sequence was carried out. The DNA sequence of *hly* gene of *L. monocytogenes* (L-55) was aligned with that of *L. monocytogenes* (ATCC 9525) using Clustal W2 software, which is available online and accessed through http://www.ebi.ac.uk/Tools/clustalw2/index.html, while deduced amino acid sequence data of β-hemolysin protein encoded by *hly* gene of *L. monocytogenes* were compared using Mega 4.1 software. The restriction map of *hly* gene of both *L. monocytogenes* ATCC 9525 and L-55 was deduced using NEB cutter V 2.0, accessed online through http://tools.neb.com/NEBcutter2/ . The nucleotide sequence of *hly* gene of *L. monocytogenes* L-55 was also compared with the reported sequences of *hly* gene of other strains of *L. monocytogenes* namely F5782 (U25446), NRRL 33056 (AY512427), NNRL 33125 (AY512451), NRRL 33178 (AY512473), M13 (DQ838551), NRRL B-33315 (DQ844127), NRRL B-33323 (DQ844130), NRRL B-33345 (DQ844138), NRRLB-33356 (DQ844142), FSL-E1-124 (EU372019), FSL-N4-289 (EU372022), FSL-F2-658 (EU372031), Lm26686 (DQ309974), HCC23 (AY878649), H4 (DQ054588), ATCC 15313 (AY750900), A12678 (DQ838565), 21385 (DQ838558), EGD (AL591974), Isolate 25064 (DQ838563), Isolate 21351 (DQ838557), NRRL 33215 (FJ030523), Strain 10403S (DQ054589), Clip80459 Serotype 4b (FM242711) and M2 (DQ838568) using Basic Local Alignment Search Tool (BLAST). The accession numbers of hly gene of those isolates have been mentioned in parenthesis. Only *hly* CDS portion of the sequences were into consideration for comparison. Based on these sequence analysis, one phylogenetic tree was created using Clustal W2 software.

Results and Discussion

The isolated genomic DNA of *L. monocytogenes* strain L-55 was found without any contamination as the purity was 1.8. The amplification of *hly* gene of *L. monocytogenes* was carried out using Hly-seq-F/ Hly-seq-R primers and product of 1590 bp size amplified and detected in agarose gel electrophoresis (Fig. 1).

Recombinant plasmid was constructed by ligation of PCR amplified *hly* gene into pGEMT-easy cloning vector and were transformed into *E. coli* (DH5á). Approximately, 30 white colonies (recombinants) were obtained after plating on LB agar containing IPTG, X-gal and ampicillin. No colonies were observed on the control plate.

Confirmation of clones containing desired insert was carried out by PCR and Restriction Endonuclease analysis (REA). Out of five clones, only four clones yielded specific 1590 bp amplified products as shown in Fig. 2. Two fragments of approximately 1500 and 3000 bp were obtained on 2% agarose gel using *Nco*I restriction enzyme in REA (Fig. 3).

The whole sequence was analyzed and the reverse complementary of the sequence was made as the orientation of the insert was found reverse. After locating the position of the forward and reverse primers, the open reading frame (ORF) of *hly* gene was found to be 1590 bp (Fig. 4) and sequence has been submitted to the GeneBank (Accession No. FJ263386). The sequence analysis of *hly* gene of *L. monocytogenes* (L-55) was carried out and it showed 98% homology with published sequence of *hly* gene of *L. monocytogenes* (ATCC 9525). On comparing with other published sequences of *hly* gene of different strains of *L. monocytogenes*, a 99% sequence homology was observed with other strains of *L. monocytogenes*. The phylogenetic analysis based on the *hly* gene sequences of *L. monocytogenes* L-55 as well as other strains have been created and has been depicted in Fig. 5. Based on the phylogenetic tree, it can be stated that the Indian isolate L-55 is closer to the NRRL strains (Northern Regional Research Laboratory,

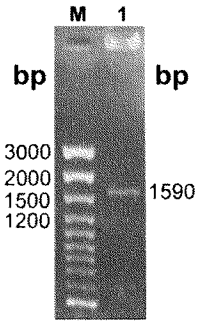


Fig.1. Amplification of *hly* gene of *L. monocytogenes*. Lane M: 100 bp DNA ladder plus; Lane 1: PCR amplified product hly gene

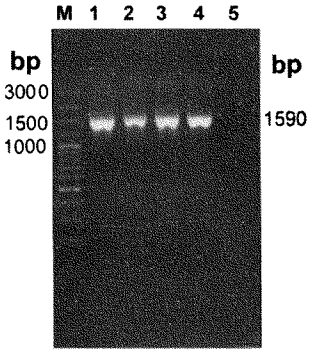


Fig. 2. Confirmation of clones by PCR. Lane M: 100 bp DNA ladder plus; Lane 1: Plasmid from colony no.1; Lane 2: Plasmid from colony no. 4; Lane 2: Plasmid from colony no. 2; Lane 3: Plasmid from colony no. 3; Lane 4: Plasmid from colony no. 4; Lane 5: Plasmid from colony no. 5.

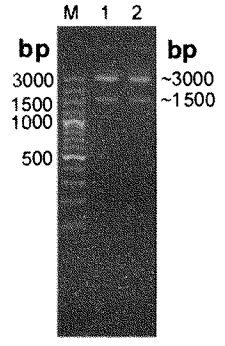


Fig.3. Confirmation of clones by restriction endonuclease (R.E.) analysis. Lane M: 100 bp DNA ladder plus; Lane 1: Plasmid from colony no.1; Lane 2: Plasmid from colony no. 4.

Fig. 4. *hly* gene of *L. monocytogenes* L-55

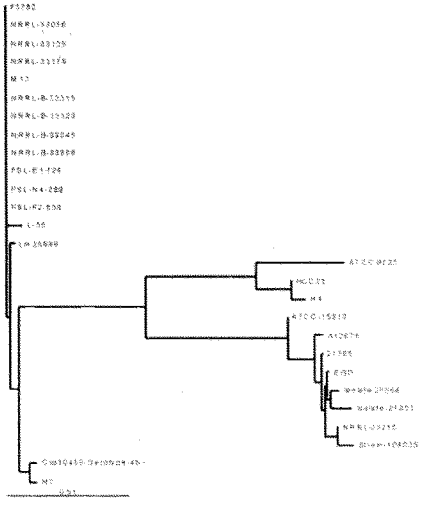
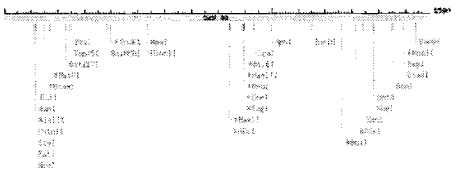
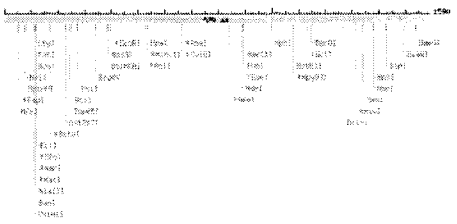


Fig. 5. Phylogenic analysis of Indian isolates (L-55) and other isolates of *L. monocytogenes*

The culture collection of Agricultural Research Service, USA). The restriction map of *hly* genes of *L. monocytogenes* ATCC 9525 and L-55 as deduced by NEB cutter V2.0 software has been depicted in Fig. 6. Based on the presence and absence of different restriction endonuclease (RE) recognition site, *hly* gene sequences of ATCC 9525 and L-55 strains possessed some variations (Fig. 6). When the deduced amino acid sequences



a. *L. monocytogenes* ATCC 9525



b. *L. monocytogenes* L-55

Fig. 6. Restriction maps of hly genes of *L. monocytogenes* ATCC 9525 and L-55.

MKKIMLVFITLIL**I**SLPIAQQTEAKDASAF**H**KE**D**LISSMAPF**T**SPPASEKTPIEKHA
DEIDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNNADIQV
VNAISSLTYPGALVKANSELVENQPDVLPVKKDSLTLSIDLPGMTNQDNKIVVKNATKS
NVNNAVNTLVERWNEKYAQAYPNVSAKIDYDDEMAYSESQLIAKFGTAFKAVNNSLNVN
FGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVNAENPPAYIS
SVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSFKAVIYGGSAK
DEVQIIDGNEGDLPDTLEKGATTNREFPGVPIAYTTNFLKDNELAVIKNNSEYIETTSK
AYTDGKINIDHSGGYVAQFNISWDEINYDPEGNEIVQHKNWGENNKSKLAHFTSSIYLP
GNARNINVYAKECTGLAWEWWRTVIDDRNLPLVKNRNISIWGTTLYPEYSNSVDNPIE

a. L. monocytogenes ATCC 9525

MKKIMLVFITLIL**V**SLPIAQQTEAKDASAF**N**KE**N**LISSMAPF**A**SPPASEKTPIEKHA
DEIDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNNADIQV
VNAISSLTYPGALVKANSELVENQPDVLPVKPDSLTLSIDLPGMTNQDNKIVVKNATKS
NVNNAVNTLVERWNEKYAQAYPNVSAKIDYDDEMAYSESQLIAKFGTAFKAVNNSLNVN
FGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVNAENPPAYIS
SVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIINNSSFKAVIYGGSAK
DEVQIIDGNEGDLPDTLEKGATTNREFPGVPIAYTTNFLKDNELAVIKNNSEYIETTSK
AYTDGKINIDHSGGYVAQFNISWDEINYDPEGNEIVQHKNWGENNKSKLAHFTSSIYLP
GNARNINVYAKECTGLAWEWWRTVIDDRNLPLVKNRNISIWGTTLYPEYSNSVDNPIE

b. L. monocytogenes L-55

Fig. 7. Amino acid sequences of β-hemolysin gene of *L. monocytogenes* ATCC 9525 and L-55

of *L. monocytogenes* L-55 and *L. monocytogenes* ATCC 9525 were compared using Mega 4.1 software, the polymorphism was observed in 4 positions *viz.* position 14, 31, 34 and 43, as shown in Fig. 7.

L. monocytogenes is a very important pathogen causing severe diseases in human being and animals (Dutta and Malik, 1981; Farber and Peterkin, 1991). Listeriosis in human is mostly foodborne disease and many outbreaks of this disease have been reported due to consumption of various kinds of food items (Schlech *et al.*, 1983; Fleming *et al.*, 1985; Ericsson *et al.*, 1997). Moreover, unlike many other foodborne pathogens, the growth of *L. monocytogenes* could not be prevented on storage of food products at low temperature as the organism can grow at wide range of temperature even at refrigerated temperature (Junttila *et al.*, 1988). The most important virulent gene *L. monocytogenes* is *hly* gene and it has been found that *hly* gene deleted mutant strain cannot survive in the intracellular environments and being readily phagocytosed (Kuhn *et al.*, 1988; Kuhn and Goebel, 1999). Using Hly-seq-F/Hly-seq-R primers, a 1590 bp amplified product was obtained in PCR. This indicated that the whole CDS of the *hly* gene has been amplified as the length of *hly* gene is 1590 bp.

On sequence analysis using BLAST tool, a 99% homology was observed with published sequences of *hly* gene of many strains of *L. monocytogenes* and 98% homology with *hly* gene of *L. monocytogenes* ATCC 9525. A higher degree of variation was observed on the comparison of the restriction maps of *hly* gene sequence of these two strains of *L. monocytogenes*. Polymorphism in amino acid sequence was observed only in 4 positions in the protein consisting of 529 amino acids. These findings indicate that *hly* gene of *L. monocytogenes* is much conserved.

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