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Gene cloning, expression and homology modeling of hemolysin gene from *Aeromonas hydrophila*

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ABSTRACT

Hemolysin is a significant toxin secreted by *Aeromonas hydrophila*, which contributes pathogenicity of fish to humans. The complete ORF of hemolysin gene (1886 bp) was amplified using PCR. It was cloned in TA and sub-cloned in pET28a vector then transformed into *Escherichia coli* BL21(DE3) codon plus RP cells expressed by the induction with 1.0 mM of IPTG. The expected size of expressed protein was 68.0 kDa estimated by migration in 12% SDS–PAGE. Anti-His monoclonal antibodies were used to substantiate the recombinant protein by Western blotting. The percent similarity between hemolysin of *A. hydrophila* with other hemolytic toxins revealed that the hemolysin/aerolysin/cytotoxin sequence varied from 99.35 to 50.40%. Homology modeling was used to construct 3-D structure of hemolysin of *A. hydrophila* with the known crystal 3-D structure (PDB: 1XEZ). This protein can be used for immunoassays and it is suitable for vaccine candidate against *A. hydrophila* infection.

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Aeromonas hydrophila is a ubiquitous bacterium, which causes infection and several diseases like hemorrhagic septicemia, fin and tail rot in fishes. *A. hydrophila* and *Aeromonas sobria* were isolated from infected fish and diagnosis of motile aeromonad septicemia was reported [1,2]. Motile aeromonads cause septicemia in channel catfish resulting in considerable economic loss [3]. Motile aeromonads were isolated from fish, milk, ice creams that cause gastroenteritis, meningitis, endocarditis and osteomyelitis in humans [4]. *A. hydrophila* secretes a wide range of extracellular enzymes which play vital role in the ecology, survival and pathogenicity. It also secretes various extracellular proteins viz. protease, enterotoxin, DNase, RNase, elastase, lecithinase, amylase, lipase, gelatinase, chitinase [5,6], cytotoxic enterotoxins [7], aerolysin and hemolysin [8].

The expression and characterization of hemolytic toxin [9–12], enterotoxin [13], outer membrane protein [14] of *Aeromonas* sp. have been reported earlier. Cloning and expression of aerolysin gene from *Aeromonas trota* has been investigated [15] and new hemolysin gene (hlyA) of *A. hydrophila* did not exhibit any homology with other known hemolysin and aerolysin genes detected in *Aeromonas* sp. [16]. The chitinase (chiA)[1] gene was cloned and expressed in *Escherichia coli* [17]. Amylase gene (amyB) of *A. hydrophila* JMP636 has been cloned and expressed in *E. coli* [18]. An extracellular protease in *A. hydrophila* SO2/2 and D13 was cloned and expressed in *E. coli* C600-1 [19]. *A. hydrophila* JMP636 strain encodes the DNase (nucH) gene which is cloned and expressed in *E. coli* showing the DNase activity [20]. The lipase gene (lip) was cloned and expressed in *E. coli*. It was purified from both *A. hydrophila* culture supernatant and the periplasmic fluids of *E. coli* containing the lip gene [21].

The 3-D structure of hemolysin protein of *A. hydrophila* is unknown so far. Homology modeling was used for constructing an atomic resolution model of a protein from its amino acid sequence [22]. The distribution of ϕ and ψ values presented in the Ramachandran plot is probably the most frequently used measure for the reliability of the experimentally derived model of a protein molecule [23]. The aim of the present study was to clone and express hemolysin gene from *A. hydrophila* in *E. coli*. Moreover, the 3-D structure of hemolysin of *A. hydrophila* was generated and validated through *in silico* strategies. Phylogenetic tree for hemolysin of *A. hydrophila* was constructed using the other homologous protein sequences.

Materials and methods

Strains, vectors, enzymes and chemicals

Aeromonas hydrophila AH14 was isolated from fish and it was characterized on the basis of 16S rDNA sequences and submitted

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[1] *Abbreviations used:* chiA, chitinase; IDT, integrated DNA technology; NB, nutrient broth; LB broth, Luria–Bertani broth;OD, optical density; IPTG, isopropyl thiogalactoside; PBS, phosphate buffered saline; RT, room temperature; BSA, bovine serum albumin; HRP, horseradish peroxidase; TMB, 3,3′,5,5′-tetramethylbenzidine.

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to NCBI under Accession No. EF681112. The isolates of *A. hydrophila* AH14 were also amplified and sequenced the conserved fragment of hemolysin gene [24]. *E. coli* DH5α, *E. coli* BL21(DE3) and *E. coli* BL21(DE3) codonPlus RP cells were preserved in laboratory; pTZ57R-T and pET28a were obtained (Fermentas; Novagen), Taq DNA polymerase, dNTPs, T_4 DNA ligase, BamHI, NdeI, X-gal, IPTG, DNA marker were also obtained (Fermentas) and other kits as well as reagents were obtained from standard commercial resources.

Designing and synthesis of hemolysin primers

The existing hemolysin nucleotide sequences of *A. hydrophila* were retrieved from the NCBI GenBank (www.ncbi.nlm.nih.gov/genbank). These sequences were aligned in ClustalX 2.0 and hemolysin primers were designed with restriction enzymes sites using Oligo 4.0, Gene runner and DNAStar. The stop codon did not include in designing the reverse primer that could be useful for purification of recombinant hemolysin. These hemolysin primers were synthesized from Integrated DNA Technology (IDT, USA) (Table 1).

Genomic DNA isolation and PCR conditions

Aeromonas hydrophila was revived from −80 °C in nutrient broth (NB) and was grown at 30 °C overnight. Genomic DNA was extracted as described in earlier method with little modifications [25]. The PCR mixture (50 μl) consisted of 10 ng of genomic DNA, 3.0 U of Taq DNA polymerase, 5 μl of 10× PCR amplification buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 200 μM dNTP and 10 pmol of each primer. Amplification includes initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing of primers at 50 °C for 1 min and extension at 72 °C for 1.30 min. A final extension at 72 °C for 20 min was used. Ten microliters of PCR products was analyzed in 1.2% agarose gel electrophoresis with ethidium bromide at 8 V/cm and was visualized under gel documentation system.

Construction and identification of hemolysin gene

The amplified product was purified using QIAquick™ gel extraction kit and ligated into pTZ57R-T vector using T_4 DNA ligase. This construct (pTZ57R-T-hemolysin) was transformed into competent *E. coli* DH5α prepared by $CaCl_2$ method [26]. The selection marker ampicillin was used with X-gal and IPTG. The plasmid DNA was extracted and digested with NdeI and BamHI restriction enzymes and sub-cloned in pET28a expression vector and transformed into *E. coli* BL21(DE3) codon plus RP competent cells. The recombinant hemolysin gene was identified and characterized by restriction digestion with NdeI/BamHI, PCR amplification and DNA sequencing.

Expression and identification of recombinant hemolysin

The positive clone of recombinant plasmid (pET28a-hemolysin) was inoculated into 5 ml Luria–Bertani (LB) broth containing kanamycin (30 μg/ml) and chloramphenicol (50 μg/ml). The culture was incubated at 37 °C with 200 rpm overnight. Next day, 200 μl of grown culture was inoculated into separate flasks each containing 100 ml of LB broth and again incubated until optical density (O.D. 600 nm) of culture reached up to 0.6. Bacterial cultures were induced using different concentrations of isopropyl thiogalactoside (IPTG 0.25–1.0 mM) and the culture harvested every 1 h up to 16 h. Similarly, the *E. coli* BL21(DE3) codon plus RP containing pET28a was induced up to 16 h as a negative control. The 1.5 ml bacterial cultures were centrifuged at 13,000*g* for 10 min at 4 °C. The bacteria were homogenized in 80 μl phosphate buffered saline (PBS pH 7.2) and added 20 μl 5× SDS sample loading buffer (0.225 M Tris·Cl, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT). It was mixed properly and boiled at 90 °C for 5 min and centrifuged at 13,000*g* for 10 min. The 10 μl lysates were loaded directly onto 12% SDS–PAGE following the procedure [26].

Table 1
The hemolysin gene specific primers of *A. hydrophila* used in this study.

Primers	Restriction site	Sequences (5′ ————————3′)
AHF8		5′ATGAAAAACAAAAAACCACGCAAATTCA3′
AHR8		5′TCACCCGTCTACGCTTGTCGGTA3′
AHF10	NdeI	5′catatgATGAAAAACAAAAAACCACGCAAATTC3′
AHR10	BamHI	5′ggatccTCACCCGTCTACGCTTGTCG3′
AH101R	BamHI	5′ggatccCCCGTCTACGCTTGTCG3′
AHR102	BamHI	5′ggatccGTGCTGGCCGGCCGGTGGC3′

Protein purification under denaturing conditions

Expressed recombinant hemolysin cells were kept in −20 °C and thawed the cell pellet for 15 min on ice and resuspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea, pH 8.0) at 5 ml per gram weight. Stir cells for 60 min at room temperature and centrifuged lysate at 10,000*g* for 30 min at room temperature (RT) and discarded the cellular debris. Add 1 ml of the 50% Ni–NTA slurry to 4 ml lysate and mixed gently by shaking 200 rpm for 60 min at RT. Loaded the lysate–resin mixture carefully into an empty column (Genei, Bangalore) and washed two times with 4 ml buffer C (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea, pH 6.3). Eluted the recombinant hemolysin four times with 0.5 ml buffer D (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea, pH 5.9), followed by four times with 0.5 ml buffer E (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M urea, pH 4.5). Collect all the fractions and analyzed by 12% SDS–PAGE.

Western blot analysis

After SDS–PAGE of expression hemolysin was transferred onto nitrocellulose membrane (Millipore) by Amersham Pharmacia Western blotting system following the procedure described [26]. The membrane was blocked using 3% bovine serum albumin (BSA) then dipped into 1:2000 diluted Anti-His antibody (QIAGEN, Germany). The horseradish peroxidase (HRP) conjugated rabbit anti-mouse antibody was diluted into 1:4000 and used as secondary antibody. The membrane was washed and detected by 3,3′,5,5′-tetramethylbenzidine (TMB) membrane peroxidase substrate (KPL) until dark purple bands appear.

Homology modeling and validation of 3-D structure

The protein sequence of hemolysin from *A. hydrophila* was searched for 3-D homology with protein data bank (PDB: www.rcsb.org/pdb/home/home.do) through BLASTP [27]. The high score and low E-value were to be considered. The hemolysin of *A. hydrophila* resembles the 3-D crystal structure of cytolysin on 2.30 Å resolution (1XEZ) of *Vibrio cholerae*. The homology modeling was used to generate the 3-D structure of hemolysin through Modeller [22]. The 3-D structure was evaluated and validated with the help of PROCHECK [23], favored amino acid residue in Ramachandran plot, its accuracy and G- factor were considered.

Sequence similarities and phylogenetic analysis

The protein sequence of hemolysin of *A. hydrophila* was searched with BLAST and homologous sequences were retrieved from NCBI GenBank. The two sequences were used to align in BLAST2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi)

and observed the percent identity of hemolysin sequences. All these hemolysin protein sequences were aligned in CLUSTAL X [28]. The computed alignment was manually checked and corrected. Pairwise evolutionary distances were computed using the passion correction algorithm employed in MEGA 4.0 [29] program and phylogenetic tree was constructed by neighbor-joining method. Total of 100 bootstrapped values were sampled to determine a measure of the support for each node on the consensus tree.

Nucleotide sequence and protein model accession number

The nucleotide sequences of *A. hydrophila* isolate AH14 hemolysin submitted to NCBI GenBank under EU009398 have been assigned. The 3-D structure of hemolysin of *A. hydrophila* isolate AH14 was submitted to protein model database (http://mi.caspur.it/PMDB/) and PM0075246 has been assigned.

Results

PCR amplification and cloning of hemolysin gene

In the present study, new primers designed for amplification of the complete ORF of hemolysin gene from *A. hydrophila* AH14 isolate were executed. The size of PCR product was 1886 bp at an optimal concentration of 1.5 mM $MgCl_2$ and primer annealing at 50 °C. The gel purified hemolysin gene was ligated in pTZ57R-T vector and then recombinant plasmid pTZ57R-T-Hem was successfully constructed. Ampicillin was used as a selection marker for clones. The plasmid DNA was extracted and confirmed by PCR amplification and by digestion with BamHI and NdeI restriction enzymes. The two bands were observed on gel: one for hemolysin containing the BamHI and NdeI restriction sites (1895 bp) and 2800 bp of cloning vector. The hemolysin gene of *A. hydrophila* AH14 was sequenced and showed the homology with hemolysin gene of NCBI GenBank. The G+C content of hemolysin gene ORF was 63.35%. The summary of hemolysin sequences' percent identity is given (Table 2).

The construction of expression plasmid, induction and identification of recombinant hemolysin protein

The pET28a-Hem clone was successfully constructed and used for expression in *E. coli* BL21 (DE3) codon plus RP cells. It was also confirmed by digestion with NdeI and BamHI restriction enzymes. The two bands 1895 bp of hemolysin insert also containing the BamHI and NdeI restriction sites and 5200 bp of vector (pET28a) were obtained. In this study, high level expression of hemolysin was obtained using 1.0 mM concentration of IPTG at 25 °C. The 68 kDa size of recombinant expressed protein was observed on 12% SDS–PAGE (Fig. 1).

Table 2
Similarity of hemolysin protein sequences (621 amino acids) of *A. hydrophila* AH14 isolate with other bacterial hemolytic toxins.

Strains from NCBI GenBank	Gene designation	Accession no.	Percent identity
A. hydrophila	Hemolysin	BAD90680.1	99.35
A. hydrophila	Hemolysin	BAD90679.1	98.71
A.hydrophila subsp. hydrophila ATCC 7966	Hemolysin	YP_856050.1	98.22
A. hydrophila	Hemolysin	BAD90678.1	97.90
A. hydrophila	Hemolysin	AAC38074.1	97.26
A. salmonicida subsp. salmonicida A449	Aerolysin	YP_001142615.1	91.62
A. hydrophila	Hemolysin	AAF45031.4	83.25
A. hydrophila	Hemolysin	ABS18368.1	76.00
AERSA Hemolysin 4 precursor	HLY4	Q08677.1	71.65
Photobacterium profundum 3TCK	Hemolysin	ZP_012185961	54.26
Listonella anguillarum	Hemolysin	AAW50398.1	51.50
Vibrio fluvialis	Hemolysin	AAN39121.1	52.49
V. mimicus	Hemolysin	AAB58399.1	51.36
V. cholerae	Hemolysin	BAA09545.1	50.56
V. cholerae V51	Hemolysin	EAZ50625.1	50.40
V. cholerae	Cytolysin	1XEZ	50.40

Protein purification and Western blot analysis

The expression of hemolysin was not seen in native condition. Total 100 ml of induced and uninduced cells was dissolved in 8 M urea solution. The lysate was directly used for purification using Ni–NTA agarose affinity chromatography column. The monomeric form of hemolysin protein was eluted in buffer E (pH 4.5). The confirmation of purified hemolysin was performed on 12% SDS–PAGE and obtained the 68 kDa recombinant protein (Fig. 2). The eluted fraction of hemolysin, uninduced and induced proteins was used for characterization using Western blotting and showed the 68 kDa band on nitrocellulose membrane (Fig. 3).

Analysis of the 3-D structure of hemolysin

The complete ORF of hemolysin from *A. hydrophila* encodes 621 amino acids; the 3-D structure shows the homology with 1XEZ. The sequence of hemolysin was 50% identical with E-value 2e-175 of cytolysin of *V. cholerae*. The positives of amino acid of hemolysin were similar with 67%. Two α helix and 26 β sheets were observed in the 3-D structure of hemolysin. The 3-D structure of hemolysin of *A. hydrophila* is shown (Fig. 4). The evaluation of

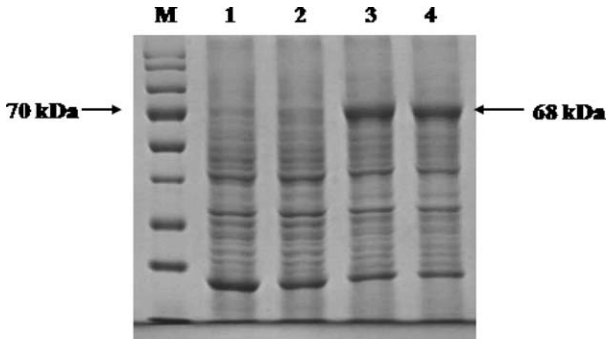

Fig. 1. SDS–PAGE for expression of recombinant hemolysin in *E. coli* BL21(DE3) codon plus RP cells. (Lane M) 10–120 kDa pre-stain protein marker (Fermentas); (lane 1) BL21(DE3) codon plus RP cells; (lane 2) BL21(DE3) codon plus RP cells with pET28a-hem uninduced; (lane 3) BL21(DE3) codon plus RP cells with pET28a-hem (induced by 0.25 mM IPTG) and (lane 4) BL2l(DE3) codon plus RP cells with pET28a-hem (induced by 1.0 mM IPTG).

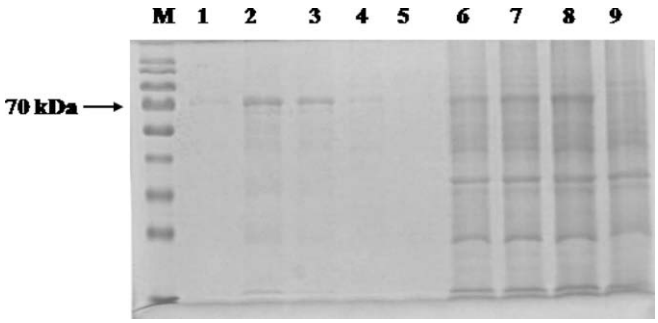

Fig. 2. Ni–NTA affinity chromatography used for purification of recombinant hemolysin. (Lane M) 10–120 kDa pre-stain protein marker (Fermentas); (lanes 1–4) purified hemolysin; (lanes 5–8) flow through and (lane 9) uninduced sample.

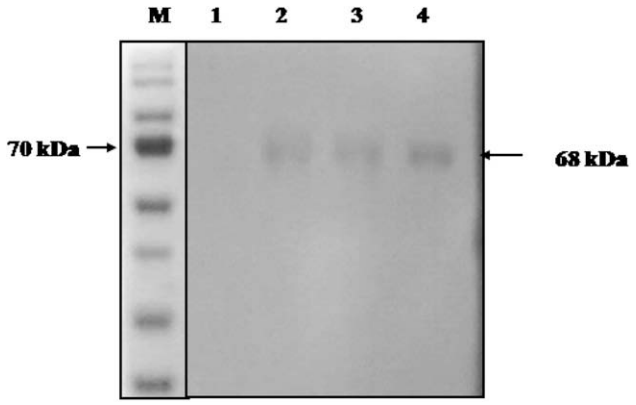

Fig. 3. Recombinant hemolysin fusion protein detected by Western blotting using anti-His monoclonal antibodies. (Lane M) pre-stain protein marker; (lane 1) uninduced *E. coli* BL21(DE3) codon plus cells; (lanes 2–3) induced *E. coli* BL21(DE3) codon plus cells and (lane 4) purified hemolysin.

the 3-D structure on the basis of minimum free energy was −46,063.62 kcal/mol. The validation of the structure on the basis of spatial arrangement of amino acid residue in most favored region of Ramachandran plot was observed. The torsion angles of 87.4% amino acid residue were in most favored region and only 1.1% amino acid residue in disallowed region. The overall G-factors were 0.5 at resolution of 2.30 Å.

Phylogenetic analysis of hemolysin

The phylogenetic tree has been based on deduced amino acid sequences of hemolysin from *A. hydrophila* and other related bacteria as well as aeromonads. The phylogenetic tree for hemolysin of *A. hydrophila* is shown (Fig. 5). Two major clades were obtained in the phylogenetic tree and one showed the hemolysin of *A. hydrophila* and other *Aeromonas* sp. as well as other bacteria containing the homologous sequences of hemolysin.

Discussion

The source isolate of *A. hydrophila* AH14 was recovered from fish with hemorrhage present on the body surface. In the pond cultured fish system of India, *A. hydrophila, A. sobria, Aeromonas caveii* , *Edwardsiella tarda* and *Flavobacterium* sp. are responsible for causing disease outbreaks. However, it is not easy to diagnose and identify these pathogens based on clinical symptoms. There is a need to develop an appropriate molecular and immunological diagnosis assay of *A. hydrophila* infection in fishes to humans. A more practical approach in the development of diagnostic method is to clone, express and purify the hemolysin protein from *A. hydrophila.* The size of PCR product was 1886 bp observed at an optimal concentration of 1.5 mM $MgCl_2$ and primer annealing at 50 °C. The primers targeting the complete ORF amplified aerolysin gene of *A. hydrophila* XS91-4-1. The size of PCR product was 1458 bp excluding the signal peptide sequence region. The aerolysin gene was cloned in pET32a vector [12]. The complete ORF of gene encoding the hemolytic protein of *A. hydrophila* has been cloned into pGEM-T vector and verified through DNA sequencing and showed the similar homology with *A. hydrophila* β-hemolysin gene [30].

Aeromonas trota AK2 produces the extracellular pore-forming hemolytic toxin aerolysin was mutagenized using transposon mini-Tn5Km1 to generate a hemolysin-deficient mutant (AK253). The nucleotide sequences of aerA gene located on the 1.8-kb ApaI–EcoRI fragment from genomic library were determined to consist of ORF with 1479 bp. *In vitro* coupled transcription–translation analysis of the 1.8-kb region suggested that the aerA gene codes for 54 kDa protein in agreement with nucleotide sequence data [15].

The two unrelated distinct virulence genes of *A. hydrophila* hemolytic toxins were assessed in a suckling mouse model. The hemolysin gene isolated from *A. hydrophila* A6 cosmid bank encoded a potential gene product of 621 amino acids and a predicted

Fig. 4. Three-dimensional structure of hemolysin of *A. hydrophila*.

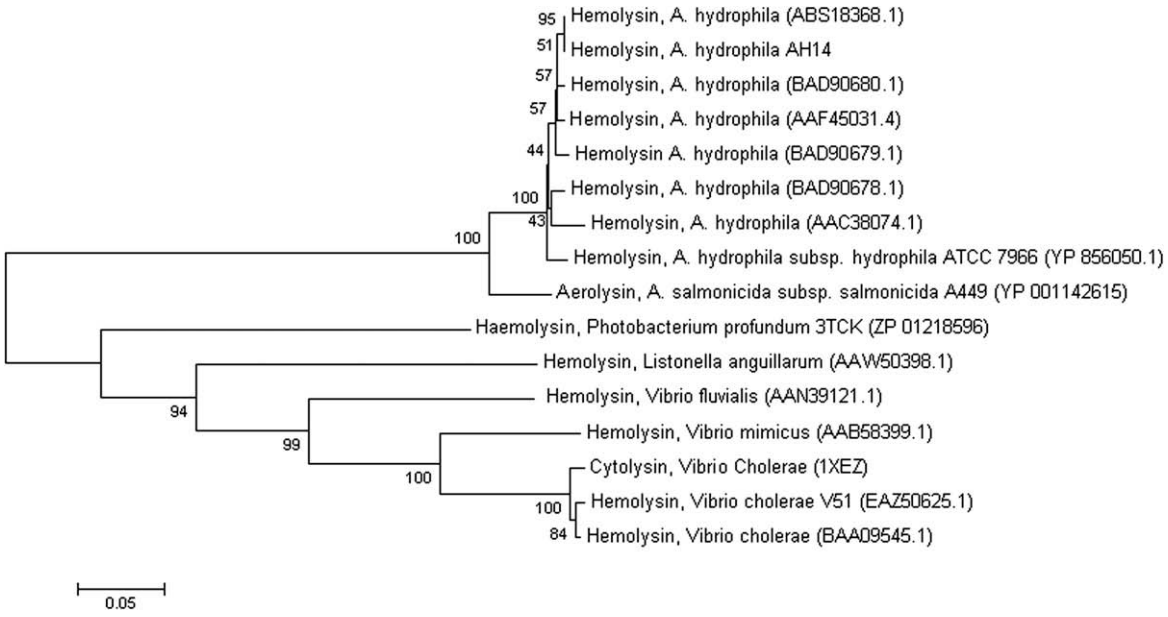

Fig. 5. Unrooted phylogenetic tree based on hemolysin amino acid sequences of *A. hydrophila* AH14 isolate and other related bacteria showing the similar homology. The bar represents 0.05 amino acid changes per site.

molecular size of 69.0 kDa. The aerolysin gene encodes a pore-forming toxin (β-hemolysin) which was partially cloned and sequenced for development of mutant. The mutant was generated using mutagenesis of hemolysin and aerolysin it showed lesser virulence in mouse model in comparison to wild strain [11]. Thirteen major virulence factors viz. aerolysin, hemolysin OMPA1, MepA, SerA, etc., of *A. hydrophila* were investigated in this emerging pathogen and help to diagnostics, vaccines and therapeutics [35]. Aerolysin (aerA) has expressed in *E. coli* under the control of bacteriophage T7 promoter. The coding region for aerA gene of *A. hydrophila* XS91-4-1 was cloned and expressed in *E. coli* BL21 cells excluding the signal peptide. The recombinant aerolysin showed hemolytic activity on agar diffusive hemolysis assay and Western blot analysis demonstrated good antigenicity [12]. In this investigation, total 621 amino acid sequences were also obtained in hemolysin of *A. hydrophila*. It is well known major virulence factor of *A. hydrophila* showing the sequences' similar homology with the earlier reported hemolysin. Therefore, it can be used as a suitable antigen based diagnostic of *A. hydrophila* infection and vaccine candidate for fishes.

The extracellular hemolysin (AHH1) gene of *A. hydrophila* ATCC7966 was cloned. The complete nucleotide sequences have been determined. *E. coli* carrying this gene expressed an extracellular heat-labile hemolysin for rabbit red blood cells. The nucleotide sequence of this region contained a single ORF of 1734 bp encoding the 577 amino acids with 63.658 kDa. The protein was analyzed using *in vitro* [35S] methionine-labeled proteins in *E. coli* CSR603 carrying the AHH1 plasmid suggesting that AHH1 gene codes for 60 kDa polypeptide [31]. The cytolytic enterotoxin of *A. hydrophila* was cloned and its DNA sequence was determined. N-terminal amino acid residues of the *Aeromonas* cytolytic enterotoxin were used as a probe to screen a genomic library constructed in EMBL3. All biological activities associated with the cytolytic enterotoxin were neutralized by rabbit homologous polyclonal antibodies. The confirmation of protein on SDS–PAGE and Western blot of cell lysate of *E. coli* (λ CH4) revealed a protein band of 52 kDa using antisera to the cytolytic enterotoxin or antibodies generated against a synthetic peptide to the toxin. DNA sequence analysis of ORF (1479 bp) encodes a protein of 54.5 kDa, a precursor form of the cytolytic enterotoxin, with a 23 amino acid leader peptide. The homology of cytolytic enterotoxin at DNA and protein sequences has resembled aerolysin of *Aeromonas* species but restriction patterns were different [7].

New virulence hemolysin gene from *A. hydrophila* produced a cytotoxic enterotoxin (Act) showing the cytotoxic, enterotoxic and hemolytic activities. It was identified and encoded 439 amino acid residues with 49 kDa. It was cloned, sequenced and overexpressed in *E. coli*. The hlyA gene exhibited 96% identity with its homolog found in a recently annotated genome sequence of an environmental isolate, namely the type strain ATCC 7966 of *A. hydrophila* subspecies hydrophila. The hlyA gene did not exhibit any homology with other known hemolysin and aerolysin genes detected in *Aeromonas* isolates [16].

In this study, high level expression of hemolysin was obtained using the 1.0 mM concentration of IPTG at 25 °C. The 68 kDa size of recombinant expressed hemolysin protein was observed on 12% SDS–PAGE (Fig. 1). The expression of hemolysin gene was under the control of strong T7 promoter. We have used two BL21(DE3) and BL21 (DE3) codon plus RP *E. coli* strains for expression of hemolysin. No expression obtained in lane 1 and lane 2 due to only cells and only pET28a plasmid containing cells were used. Lanes 3–4 containing recombinant hemolysin gene induced by 1.0 mM concentration of IPTG and showed the optimal expression (Fig. 1). Hemolysin did not express in *E. coli* BL21(DE3) cells due to lack of rare codons in the cells. However, hemolysin was successfully expressed in *E. coli* BL21 (DE3) codon plus RP cells because it has the coding genes for the rare codons. The expression of hemolysin was not seen in native form. It was aggregated within *E. coli* cells in the form of inclusion bodies. The monomer of hemolysin protein was eluted in buffer E (pH 4.5). The eluted fraction hemolysin, uninduced and induced proteins was used for characterization by Western blotting. Western blot analysis showed the 68 kDa protein on nitrocellulose membrane (Fig. 3). Uninduced hemolysin sample (lane 1) did not express but lanes 2–3 showed the binding of anti-His antibodies with recombinant expressed and purified (lane 4) fusion hemolysin. Anti-His antibodies were used for detection of recombinant hemolysin. The C-terminal region of expressed hemolysin fusion protein with 6X His-tag could

be useful for immunoblotting (Fig. 3). The recombinant aerA was expressed in a soluble form and purified using His-bind resin affinity chromatography [12].

The 3-D structure of hemolysin of *A. hydrophila* satisfied on the basis of energy and mostly the amino acid resides within the most favored region in Ramachandran plot. The stereochemical spatial arrangement of hemolysin was favored due to less amino acid residues lying within the disallowed region. Since, the studied hemolysin model has only 1.1% amino acid residue in disallowed region it would conclude that this structure fulfills the parameter provided for making a good model. The β-ketoacyl acyl carrier protein synthase (KAS) III plays an important role in the initiation of fatty acid in *Enterococcus faecalis*. The homology modeling method has been used to generate the 3-D structure of KASIII protein. The generation of 3D structure was done through the Modeller, quality and validation of structure was approved with PROCHECK. The identification of active site residue in this protein has been targeted with two antibacterial drugs [32].

Multidrug resistance protein 1 (MRR1/ABCC1) is a 190 kDa member of the ATP binding cassette (ABC) superfamily of transmembrane protein. The molecular modeling has been used to generate 3-D structure of protein by Modeller and validation of structure by PROCHECK and WHAT IF tools [33]. NAD^+ dependent DNA ligase is important enzyme which catalyzes the joining of nicks between the double stranded DNA in bacteria. Homology modeling has been used to generate the 3D structure of NAD^+ dependent DNA ligase of *Mycobacterium tuberculosis* and validation of 3D structure by PROCHECK. This study reported that several compounds could block the pathway of the ligase synthesis in the *M. tuberculosis* [34].

The hemolysin gene showed the homology with hemolysin gene of NCBI GenBank. The G+C content of hemolysin gene ORF was 63.35%. A relatively high G+C content of the hemolysin gene is a characteristic of aeromonads [15]. The percent similarity of 621 amino acids was studied with other bacteria encoding the similar proteins. The similarities among hemolysin and other sequences of hemolytic toxins revealed that the hemolysin/aerolysin/cytotoxin protein of *A. hydrophila* and other related bacteria is 99.35–50.40% (Table 2). The deduced amino acid sequence of aerA gene product of *A. trota* exhibited 99% homology with amino acid sequence of aerA product of *A. sobria* AB3 and 57% homology with the amino acid sequences of the products of the aerA genes of *Aeromonas salmonicida* 17-2 and *A. sobria* [15].

The phylogenetic tree was based on deduce amino acid sequences of hemolysin from *A. hydrophila* and other bacteria. Two major clades were obtained in the phylogenetic tree and one was hemolysin of *A. hydrophila* and other *Aeromonas* sp. and other bacteria having the hemolysin. *A. hydrophila* AH14 isolate was closely related to hemolysin of *A. hydrophila* GenBank sequences. Aerolysin from *A. salmonicida* was found in the same clade but another branch due to 91% similarity with *A. hydrophila* hemolysin. *V. cholerae* hemolysin and cytolysin were 50.4% similar and they were found in different clade.

Conclusion

Novel information about the cloning, expression, purification and molecular modeling of hemolysin gene from *A. hydrophila* isolated from fish was reported. The recombinant hemolysin of *A. hydrophila* could be produced and purified in adequate quantity to use as antigens in developing a convenient and economical diagnostic method. It will reduce the cost, transport and reproducibility problems associated with the present diagnostic tests, which require growth and purification. It can be used as diagnosis of *A. hydrophila* infection in fishes for control and prevention of the spread in new regions. The hemolysin can be used as a future vaccine candidate against *A. hydrophila*. The 3-D structure of hemolysin of *A. hydrophila* possibly helps to better understand the nature and also might use as potential drug targets against the active amino acid residue. The phylogenetic tree showed the homology of hemolysin present in various bacteria, which might be risk for fishes and other animals, respectively.

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