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RESEARCH ARTICLE OPEN ACCESS

Identification, Cloning, and Expression of A Major Thermolabile Hemolysin Gene from *Vibrio alginolyticus* ATCC17749

Raj Kamal Vibhuti[1,2] and Avijit Pramanik[1]*

[1]Department of Microbiology, School of Interdisciplinary and Applied Sciences, Central University of Haryana, Mahendragarh, India.
[2]Department of Medical Laboratory Sciences, Narayan Paramedical Institute and Allied Sciences, Gopal Narayan Singh University, Jamuhar, Rohtas, Bihar, India.

Abstract

***Vibrio alginolyticus*, an opportunistic pathogen in humans, obtains iron from various iron-containing compounds, including hemin, a heme derivative. This process may be an important virulence-associated adaptation for evolution into a human pathogen. The easiest way for a pathogen to acquire iron from a host is through erythrocyte lysis by hemolysins, which increases the available iron pool *in vivo*. Hemolysins are important virulence factors in many bacterial pathogens. In this study, a DNA fragment containing the 1281 bp ORF of the hemolysin gene from *V. alginolyticus* was identified by isolating a hemolytic-negative mutant from a random mutagenesis library. The external DNA sequence from the transposon inserted during random mutagenesis was identified by DNA sequencing. The hemolysin gene was amplified and cloned into the expression vector pET28a. The expression vector containing the hemolysin gene was transformed into *Escherichia coli* BL21(DE3), and the overexpressed protein had approximately 47 kDa molecular weight, as predicted by SDS-PAGE. The hemolytic activity of whole-cell lysate with overexpressed protein AGV17462.1 was tested, and it shows hemolysis slightly less than the positive control, which means 100% hemolysis. No hemolysis was observed after heat treatment at 100 °C for 10 min. Therefore, it is considered a thermolabile hemolysin. In conclusion, transposon-induced gene disruption leads to the loss of the hemolytic phenotype in the isolated mutant, in which the DNA sequence has been identified and characterized as a new hemolysin gene.**

Keywords: Hemolysin Gene, Random Mutagenesis, Hemolysis, Vibrio Alginolyticus, Transposon Insertion

***Correspondence:** avijit@cuh.ac.in

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INTRODUCTION

Marine aquatic animals suffer from high mortality due to vibriosis, and *Vibrio alginolyticus*, a gram-negative halophilic bacterium prevalent in marine ecologies such as estuaries and coastal regions, is one of the causative agents.[1] It is ubiquitous in seawater, can cause ear and wound infections, has been identified as the most prevalent pathogen in infected fish, and displays signs of bacterial septicemia. It is also responsible for ulcer diseases in marine animals including fish.[1] In 1973, it was isolated from a patient with gastroenteritis, calf wound, and purulent discharge from the conjunctivae. It is an opportunistic pathogen in humans.[2-4] *V. alginolyticus* infection is more common in summer than in winter.[5-7] Present knowledge of vibriosis is mostly based on the role of toxins in pathogenesis after extracellular protease (ECP), lipopolysaccharide (LPS), and outer membrane protein (OMP) isolation and purification.[8-10] The *V. alginolyticus* infection and transmission mechanism remains unclear, but indicates transmission through seawater.[11] Previous studies have strongly recommended that *V. alginolyticus* has a pool of virulence genes that are well-known in other species of *Vibrio.*[12] These genes may play important roles in establishing infections in host organisms including humans.[13-15] The first virulence gene, similar to *trh*, was identified in *V. alginolyticus* from the Alaskan region and Tunisia.[16,17] The scientific communities in China, Europe, and the United States of America have also highlighted *V. alginolyticus* virulence.[18-21] According to some studies, *V. alginolyticus* infection is associated with fatalities in immunocompromised patients.[22]

V. alginolyticus uses hemin, a heme derivative, as its sole iron source, which strongly suggests that *V. alginolyticus* produces hemolysin.[23-25] Hemolysin is a pore-forming exotoxin that lyses the membranes of erythrocytes and other mammalian cells. In addition to hemolysis, it induces various cytotoxic effects that often result in viability loss.[26,27] The main role of hemolysin is to liberate hemoglobin from erythrocytes for the utilization of hemoglobin iron, which plays an important role in establishing the infection process in *Vibrio* spp.[28] Thermostable direct hemolysin (TDH), HlyA (El Tor), TLH (Thermolabile hemolysin), δ-VPH (Thermostable hemolysin), and novel hemolysin gene (HLX), are the representative hemolysins present in the *Vibrios*.[29] Two of these, HlyA and TDH, have been investigated extensively, and are closely related to virulence.[29,30] However, TLH, δ-VPH, and HLX hemolysins have not been fully understood, and require further research.[31] In *V. alginolyticus*, the thermostable TDH-related hemolysin TRH has been reported, but has not characterized till now.[16,30] These hemolysins have been identified as human virulence factors that can cause septicemia, gastrointestinal illnesses, and even death.[32-34] TDH and other hemolysins of *Vibrio* spp. and their virulence mechanisms in diarrhea have been studied, and detailed information on the structural and functional characteristics of TDH-encoding genes is well represented in the international DNA and amino acid databases of *Vibrio parahaemolyticus*. However, very few reports have focused on *V. alginolyticus*-encoded hemolysin, which is lethal to mice and aquatic animals.[30,35-38] In this study, we identified a major thermolabile hemolysin gene with nucleotide and amino acid sequences different from those of previously reported hemolysins. The hemolysin gene identified in *V. alginolyticus* was heterologously overexpressed in *Escherichia coli* BL21(DE3) and its hemolytic activity was studied. The hemolytic-negative *V. alginolyticus* mutants did not lyse erythrocytes and may not be pathogenic or less pathogenic than the wild type. This finding may help to develop tools to regulate or block hemolysin expression, which ultimately inhibits erythrocyte lysis and reduces *V. alginolyticus* pathogenicity. This finding will also help in designing new drugs that can be used as antibiotics.[39]

MATERIALS AND METHODS

Chemicals and reagents

Agarose, ethanol, 25:24:1 phenol-chloroform-isoamyl alcohol (PCI), and potassium acetate were purchased from Sigma-Aldrich. Tris-base, hydrochloric acid, sodium hydroxide, phenol, chloroform, isopropanol, proteinase K, RNase, ethidium bromide, EDTA, calcium chloride, magnesium chloride, glucose, and sodium chloride were purchased from HiMedia Life Sciences.

Defibrinated sheep blood was obtained from KDT Biotechnologies (India). Chemicals and enzymes for molecular biology, including Taq polymerase, dNTPs, nuclease-free water, restriction enzymes, 10× standard buffer for PCR, and T4-DNA ligase were purchased from New England Biolabs (NEB) Ltd.

Bacterial strains, plasmids, and media

V. alginolyticus was grown in Luria Bertani medium (LB) containing 2% sodium chloride for routine culture and genomic DNA isolation. *E. coli* containing plasmid pET-28a was grown in LB containing 20 µg/mL kanamycin (Km), while *E. coli* DH5α and *E. coli* BL21(DE3) without plasmid were grown in LB without antibiotics. After transformation, the cells were grown on LBA plates containing 20 µg/mL Km for 24 h. The transformed colonies appeared on kanamycin plates and were grown in LB containing Km.

Transposon mutagenesis and mutant isolation

A streptomycin-resistant *V. alginolyticus* ATCC17749 colony was isolated as the recipient strain for conjugation mating. Donor strain SM10 λ *pir* pSC189 and recipient strain *V. alginolyticus* ATCC 17749 Str^R were directly inoculated from a glycerol stock on LB containing Km and LB containing streptomycin (Sm) + 2% sodium chloride, respectively. After overnight growth of donor and recipient cells at 37 °C and 200 rpm, cells from each tube were freshly inoculated in 10 mL respective broth with antibiotics and allowed to grow at 37 °C and 200 rpm for 3 h. Both donor and recipient cells were centrifuged at 4000 rpm for 3 min, washed two times with LB, and suspended in 200 µL LB. Both cells were mixed gently and spread on 0.45-µm filter paper placed on an LB agar plate without antibiotics and again grown for 4 h at 37 °C. The mating was then scraped up and resuspended in 10 ml LB containing 2% sodium chloride and 15% glycerol. The glycerol stock was stored at -80 °C and further used as a mutant library. Briefly, 100 µL mutant library was plated on LB agar containing 2% sodium chloride and 20 µg/mL Km and Sm and incubated at 37 °C overnight; the next day 200-230 colonies were observed. Every colony was picked and transferred to selective LB sheep blood agar plates containing 1% sodium chloride, 20 µg/mL Km, 20 µg/mL Sm, and 5% RBC suspension. Nearly 10,000 hemolytic-negative colonies were observed, and six hemolytic-negative phenotype mutants were further studied. Selected hemolytic-negative mutants were further confirmed by a hemolytic assay in a liquid broth.

Genomic DNA isolation

The bacterial cells were harvested from overnight grown cultures in LB containing 2% sodium chloride. Cell pellets were collected from 3.0 mL culture by centrifugation at 4000 rpm for 1 min, dissolved in 400 µL TE (pH-8.0) by vortex mixing, and incubated at 37 °C for 1 h after adding 50 µL 10% SDS and 50 µL 20 mg/mL proteinase K in TE (pH-7.5). Then, 500 µL 25:24:1 PCI was added to the reaction tube and the two phases were mixed by gentle inversion. The aqueous phase was extracted with 500 µL chloroform three times after centrifugation at 13000 rpm at 4 °C. The DNA-containing aqueous supernatant was transferred into new 1.5 mL microcentrifuge tubes and 0.8 volume chilled isopropanol was added. The tube was kept on ice for 30 min for DNA precipitation and centrifuged at 13000 rpm for 30 min at 4 °C. The supernatant was discarded; the DNA pellet was washed twice with 70% chilled ethanol, air-dried, re-precipitated after RNase treatment at 37 °C for 30 min, centrifuged at 13000 rpm, air-dried, and finally dissolved in 40 µL 10 mM Tris buffer.

Mutant DNA sequencing

The genomic DNA isolated from hemolysin-negative mutants was digested with BamHI for 6 h at 37 °C. The digested product was precipitated with 0.8 volume chilled isopropanol and dissolved in 50 µL 10 mM Tris-HCl. The BamHI-digested genomic DNA product was ligated with T4-DNA ligase overnight at room temperature to create a circular DNA-like plasmid with R6K γ ori, containing the inserted sequence of the transposon. The designed primers sequenced the region outward from where the transposon was inserted. The sequencing reaction was run at the BRIPL (India) using primers P1: 5'-TAATCACCGTCATGGTCTTTGTA-3' and P2: 5'-GCCTTCTTGACGAGTTCTTCTGA-3'. DNA sequences were investigated individually and alignment and sequence similarity were determined using NCBI nucleotide BLAST tools.

Bioinformatic analysis

The NCBI BLAST tool was used for DNA sequence search and alignment. For the protein, AGV17462.1 bioinformatic characterization was done using the ProtParam tool hosted by Expasy. The SignalP server (version 5.0) was used to identify the signal peptide for secretion. PSORTb version 3.0.3 was used for cellular localization.

Hemolysin gene amplification by PCR

The gene ORF identified by the *V. alginolyticus* ATCC17749 mutant DNA sequence analysis was amplified by PCR using specific primers AGV17462F: 5'-AACCATATGGACGACATCTCTA-3' and AGV17462R: 5'-CTAACTCGAGATTCTGTTGAGA-3'. PCR was done in 50 µL reaction mixture, containing 25 ng genomic DNA, 0.2 µM each primer, 200 µM each dNTP, 2.5 U Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. The amplification protocol of the hemolysin gene consisted of an initial denaturation at 96 °C for 5 min; followed by 30 cycles of amplification with denaturation at 96 °C for 20 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 2 min; and a final elongation at 72 °C for 10 min. The PCR product was purified using Monarch® PCR Cleanup Kit (NEB).

Hemolysin gene cloning into *E. coli*

Amplified DNA sequences of the gene of interest and pET28a were digested with NdeI and XhoI for 3 h. The digested PCR-amplified DNA was mixed with the digested plasmid, precipitated with 0.8 vol chilled isopropanol, and dissolved in 50 µL 10 mM Tris-HCl. T4 DNA ligase was used to ligate the DNA mixture into a plasmid during overnight incubation at room temperature. The resultant ligated product was used to transform *E. coli* DH5α. LB agar plate supplemented with 20 µg/mL Km was used to select the transformed colonies.

The protocol used for the purification of genomic DNA, plasmid DNA, and agarose gel electrophoresis has been described previously, with some modifications.[40-42] *E. coli* DH5α and *E. coli* BL21(DE3) was transformed as described by Lederberg and Cohen, with some modifications.[43]

Cloned gene overexpression in *E. coli*

The pET28a expression vector was used to clone the hemolysin gene and transform it into *E. coli* BL21(DE3) strain to express it. The transformed cells were inoculated in 10 mL LB containing 20 mg/mL Km and incubated at 37 °C and 200 rpm overnight. This culture was inoculated in 1000 mL LB containing Km and allowed to grow for 2.5 h or until the OD_{600} value reached 0.6. Hemolysin expression was induced with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) at 16 °C overnight. The cells were harvested from 250 mL culture by centrifugation at 4000 rpm for 10 min after overexpression. The cell pellet was washed twice with 1× PBS and the cells were collected by centrifugation at 4000 rpm for 10 min. The pellet was resuspended in 10 mM Tris-Cl buffer at three times the cell pellet volume. SDS was added to the resuspended cells at 2% (w/v) final concentration and 1 mM PMSF final concentration was also added. The samples were sonicated for 15 rounds of sonication, each consisting of 30 s exposure at 40% energy, separated by 30 s cooling between sonication cycles. The sample containing overexpressed protein was centrifuged at 12000 rpm for 20 min and protein-containing supernatant was collected in new microcentrifuge tubes and stored at -20 °C for further utilization.

Hemolytic assay

AGV17462 cloned under the T7 polymerase promoter-transformed *E. coli* BL21(DE3) was grown overnight in 10 ml LB broth. The supernatant was collected by centrifugation; then, 800 µL supernatant was added to 200 µL washed sheep erythrocytes, incubated at 37 °C for 2 h, and then centrifuged to remove all unlysed erythrocytes, according to a protocol developed by modifying that reported by Mercurio and Manning.[44] The supernatant absorbance (A_{540}) was recorded to determine the number of lysed erythrocytes. Adding 800 µL sterile deionized water to 200 µL washed sheep erythrocytes lysed all erythrocytes (100%) and taken as a positive control. *E. coli* BL21(DE3) cultures grown in an LB medium were used as negative controls.

RESULTS

Random mutant library and hemolysin-negative mutant isolation

A 20 µg/mL Sm-resistant spontaneous

V. alginolyticus ATCC17749 mutant was isolated by plating on LB agar medium containing 2% NaCl and 20 µg/ml Sm. Hemolytic *V. alginolyticus* ATCC 17749 Str^R strain was subjected to random mutagenesis by transposon insertion via conjugal mating with SM10 λ*pir* pSC189. A pool of transposon insertion mutants in a library with approximately 6× coverage of *V. alginolyticus* ATCC 17749 functional genes (23000 mutants) was obtained. Approximately 10000 mutants were screened for an apparent hemolytic activity loss on blood agar plates. Six phenotypically non-hemolytic mutants were isolated for further hemolysis characterization in *V. alginolyticus* ATCC 17749. Mutants HM-1-6 had hemolytic activity on sheep blood agar plates (Figure 1). In the broth assay experiments, adding deionized water achieved total lysis and a sterile medium was used as the negative control. HM-1-6 did not lyse the erythrocytes present in the reaction mixture (Figure 2).

Hemolysin gene identification by DNA sequencing

The plasmids with R6K γ *ori* isolated from mutant genomic DNA were isolated and sequenced away from an inserted transposon. The nucleotide

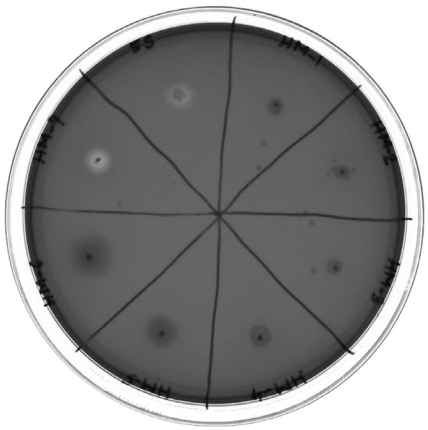

Figure 1. Phenotypically hemolysis negative mutant of *Vibrio alginolyticus* ATCC 17749 and wild type *Vibrio alginolyticus* ATCC 17749 on Blood Agar Plate

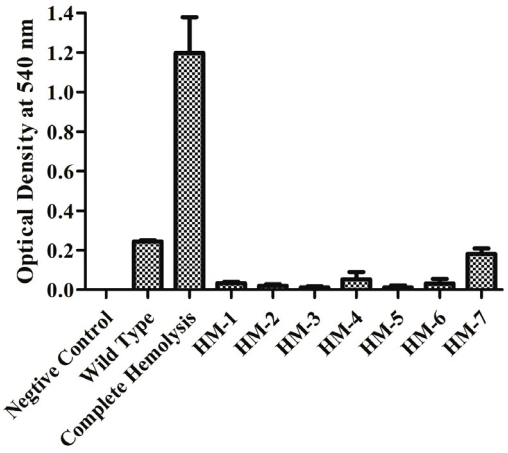

Figure 2. Hemolytic activity is expressed as the A_{540} of supernatant after incubation with 5% Red Blood Cells. All values shown represent the mean and standard deviation of triplicate

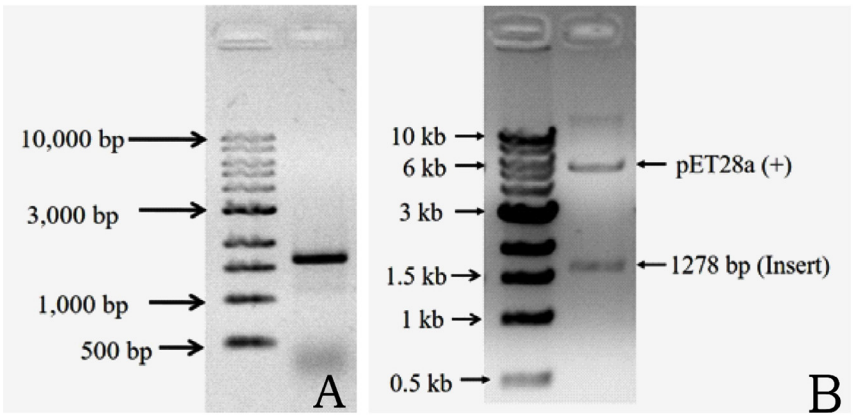

Figure 3. Amplification of hemolysin gene from *V. alginolyticus* ATCC 17749 with the primer P1 and P2. Lane 1: DNA marker and lane 2: amplified product of the gene (3A). Double digestion of cloned plasmid confirmed gene of desired insert cloned successfully. Lane 1: DNA marker and lane 2: pET28a (+) and desired gene (3B)

sequences in the hemolytic mutant DNA sequences were analyzed using the online tool nucleotide BLAST (NCBI). This confirmed that the mutant strain carrying the transposon insertion into a 1281 bp ORF (accession number CP006718.1) encodes a 426 amino acid protein (accession number AGV17462.1). The protein sequence was subjected to pBLAST against a non-redundant database. The best hit was the CNNM domain-containing protein (accession number WP_005379948.1) from *Vibrio* with 100% identity and 100% query coverage. In *V. alginolyticus*, it showed the best hit against the DUF21 domain-containing protein (accession number EGQ9715304.1), with 99.77% identity at 100% query coverage. It also showed 99.77% identity at 100% coverage with *Vibrio* sp. JCM 18904 protein hemolysin and related proteins containing the CBS domain (accession number GAJ70819.1). This is a novel thermolabile hemolysin gene found in *V. alginolyticus*. The nucleotide and amino acid sequences differed from those previously reported for hemolysins. The protein is 47.6 kDa with a theoretical pI of 5.73.

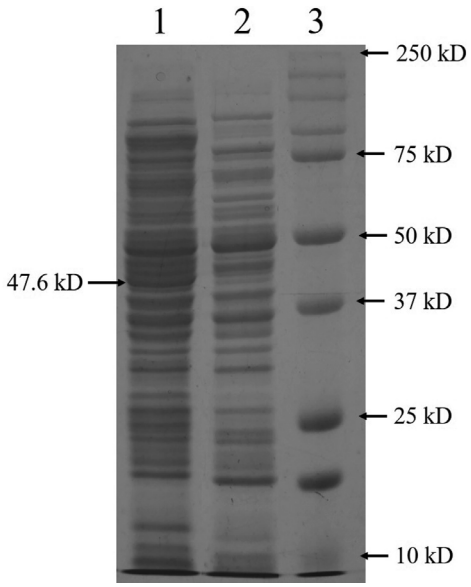

Figure 4. SDS-PAGE analysis of the overexpressed hemolysin protein in *E. coli* BL21(DE3). Lane 1: Whole protein from cloned cells including hemolysin; lane 2: proteins from wild type BL21(DE3); lane 3: molecular weight protein marker

V. alginolyticus hemolysin gene cloning into *E. coli* and overexpression

The complete DNA sequence of the hemolysin gene is a 1281 base pair that encodes a 426 amino acid protein. The primers amplified a 1.28 kb DNA sequence containing the complete hemolysin gene sequence (Figure 3a). The amplified product from *V. alginolyticus* was cloned into *E. coli* BL21(DE3) cells using the pET28a vector. Plasmids were isolated from the

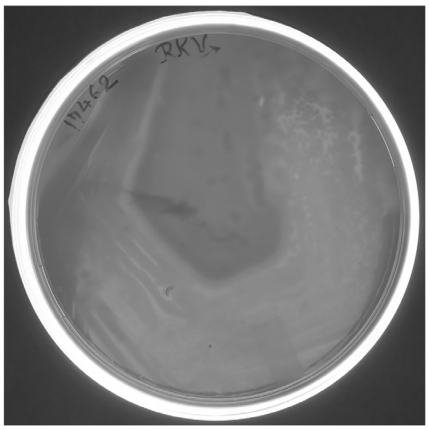

Figure 5. Overexpression of cloned in *E. coli* BL21(DE3) hemolysin gene AGV17462.1 on sheep blood agar plate. A clear hemolysis zone visualized around the growing colonies

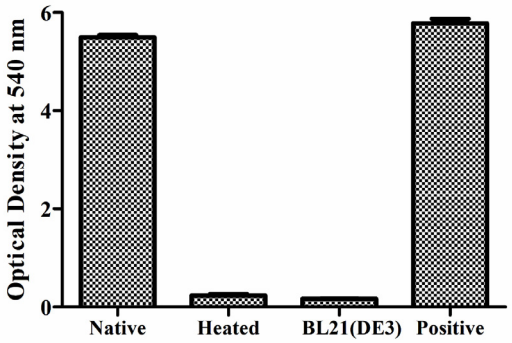

Figure 6. Detection of hemolytic activity of overexpressed hemolysin before and after heating. Proteins were incubated with washed sheep erythrocytes for 2 hours at 37 °C. Hemolysis were determined by optical density at 540 nm. Native is non heat treated, heated is heat treated at 100 °C for 20 minutes, and BL21(DE3) is from *E. coli* taken as negative, and complete hemolysis was achieved by deionized water taken as positive

transformed colonies and digested with the restriction enzymes NdeI and XhoI, followed by agarose gel electrophoresis. The presence of a double band (Figure 3b) confirmed the presence of an insert in the clone *E. coli*. Final confirmation was performed by sequencing. The hemolysin gene could overexpress by the induction with IPTG in *E. coli* BL21(DE3) at 25 °C. Total protein was extracted using the SDS whole-cell lysate method, and SDS-polyacrylamide gel electrophoresis analysis showed that the molecular weight of the overexpressed hemolysin was 48 kDa (Figure 4). We employed SignalP 5.0, which failed to detect the Sec or Tat system signal peptide for secretion. A slight signal was detected for lipoprotein signal peptides, and a strong signal was detected for other signal peptides. Therefore, a novel mechanism may underlie its secretion. PSORTb CMSVM, ModHMM, and SCL-BLAST suggested that it was a cytoplasmic membrane protein. No secretory signalling peptides were detected. Hemolysis was observed on a plate away from the colony.

Hemolytic activity of heterologously expressed hemolysin gene

E. coli BL21(DE3) cells with pET28a -AGV17462.1 were induced with IPTG and whole-cell protein was extracted by sonication. Hemolytic activity of the protein was detected in whole-cell lysate crude extract with overexpressed hemolysin, but not *E. coli* BL21(DE3) proteins used as a negative control; *E. coli* BL21(DE3) cells with PET28a-AGV17462.1 were plated on a blood agar plate that showed hemolysis (Figure 5); *E. coli* BL21(DE3) pET28a was used as a negative control. The hemolytic activity of protein AGV17462.1 was 5.49 in terms of the optical density at 540 nm, whereas that of the positive control was 5.78. The thermostability of the overexpressing hemolysin was tested at 100 °C for 20 min. The hemolytic activity of AGV17462.1 crude extract was abolished after heat treatment, rendering it thermolabile (Figure 6).

DISCUSSION

Present knowledge of the role of hemolysin in *Vibrio* spp. mainly focuses on TLH, VHA, Vp-TDH, HlyIII, VLLY, VHH, HlyA purification, characterization, and pathogenicity.[29,37,45-55] The predicted molecular weight of the hemolysin was 47.6 kDa. The protein AGV17462.1 contains 426 amino acids encoded by a 1281 bp DNA sequence present on chromosome 1 of *V. alginolyticus*. The amino acid sequence of the protein was highly similar to that of *Vibrio* proteins. *Vvh* from *Vibrio* sp. JCM was 99.77% identical with 100% coverage with AGV17462.1 from *V. alginolyticus* ATCC17749. The hemolysin gene was cloned into a vector plasmid in *E. coli* DH5α and further cloned in *E. coli* BL21(DE3) for expression.

The amount of hemolysin secreted in the medium from *E. coli* BL21(DE3) recombinant plasmid was lesser than that released into *V. alginolyticus* ATCC 17749 supernatant. Similar to *V. cholerae* enterotoxin synthesis in *E. coli*, it accumulates in the periplasmic space.[56] Another study also recommended that the *V. fluvialis* hemolysin is secreted into the extracellular environment after N-terminal region cleavage.[55] No secretion signals for the Sec or Tat system in AGV17462.1 hemolysin protein was observed. Pure TLH showed strong phospholipase activity against egg yolk emulsions. Thus, TLH protein may be a phospholipase similar to the VHH hemolysin identified in *V. harveyi*.[57,58] Both hemolysin from *V. cholerae* O139 and the lecithin-dependent hemolysin from *V. parahemolyticus* have phospholipase activity.[59,60] Extracellular phospholipase A2 and lysophospholipase from *V. vulnificus* demonstrated hemolytic activity in sheep and mouse erythrocytes, whereas PhlA from *V. mimicus* showed strong hemolytic activity in rainbow trout and tilapia erythrocytes.[61,62] The hemolysin gene was overexpressed in *E. coli* BL21(DE3) cells. SDS-PAGE displayed that the molecular mass of the overexpressed protein was 48 kDa. The hemolytic activity of protein was approximately equal to that of the positive control (100% sheep erythrocyte lysis). Heating the protein at 100 °C for 10 min abolished the hemolytic activity, making it thermolabile. The mechanism by which this helps in *V. alginolyticus* virulence has not been determined yet.

CONCLUSION

In conclusion, we report the identification, molecular cloning, and overexpression of a

new hemolysin gene from *V. alginolyticus* ATCC 17749. The nucleotide and amino acid sequences of this hemolysin differed from those of known hemolysins. The protein with accession number AGV17462.1 is the major hemolysin protein present in *V. alginolyticus* ATCC17749. The transposon was inserted into a gene, and this disruption led to the loss of hemolytic activity of the isolated mutant; the nucleotide sequence of the disrupted gene was identified and characterized as a hemolytic gene. This study aids in understanding the relationship between hemolysin and virulence factors. These findings may ultimately aid in the diagnosis and treatment of infections caused by *V. alginolyticus* and other *Vibrio* species.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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