Cloning and Expression of Colicin N Exhibiting Activity Against Select Pathogenic *Vibrio* Species in Aquaculture

Ho Ta Giap[1,2*], Ngo Nguyen Vu[1,2], Tran Song Nguyen Khoi[1,2]

[1]Center for Hi-Tech Development, Nguyen Tat Thanh University, Saigon Hi-Tech Park, Ho Chi Minh City, Viet Nam
[2]NTT Hi-Tech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Viet Nam
[*]htgiap@ntt.edu.vn

Abstract

Antimicrobial resistance in aquaculture pathogenic bacteria is a prevalent threat that contributes to the increasingly uncontrolled misuse of antibiotics. Currently, bacteriocins have been selected as a viable alternative to antibiotics in aquaculture due to their high safety profile. In this study, Colicin N from *Escherichia coli* was cloned, expressed, and purified. Among seven tested *Vibrio* strains, recombinant Colicin at a concentration of 25 µg/mL exhibited inhibitory activity against *Vibrio parahaemolyticus* and *Vibrio owensii*. Furthermore, recombinant Colicin N showed a significant inhibitory effect against *Vibrio vulnificus* and *Vibrio harveyi* at a concentration of 12.5 µg/mL. The results of this study demonstrate the in vitro inhibitory activity of Colicin N against several pathogenic *Vibrio* species, highlighting its potential for further development as an antimicrobial agent in shrimp aquaculture.

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Keywords

AMR; Colicin N; Vibrio; AHPND; *Escherichia coli*.

1 Introduction

The sector of export-oriented aquaculture, particularly shrimp cultivation, is expanding annually on a larger scale. Nonetheless, aquaculture farming enterprises are constantly challenged by a high incidence of shrimp diseases primarily attributed to bacterial pathogens [1]. The transition from extensive to intensive farming practices, coupled with the frequent or uncontrolled use of antibiotics, has led to the widespread dissemination of antibiotic-resistant bacteria in shrimp farms [1-3]. The major pathogen groups consistently documented within the aquaculture environment and farming facilities include *Vibrio* spp., *Aeromonas*, *Enterobacterales* (such as *Escherichia coli* and *Klebsiella*), and *Enterococcus* [1-3]. Aquaculture dependent on antibiotics is the main reason for the rapid dissemination of these organisms, frequently harboring Antibiotic Resistance Genes (ARGs), which are typically located on plasmids and associated with mobile genetic elements (MGEs) [1]. This is concerning because the prevalent pathogens in shrimp ponds (*Vibrio* and *Aeromonas* species) are indigenous to the surrounding marine and estuarine environments, which enables drug-resistant bacterium strains to become dominant [1]. One strategy to mitigate antibiotic use is to identify and develop of novel antimicrobial agents.

Bacteriocins are ribosomally synthesized peptides or proteins secreted by bacteria into the environment, primarily functioning to eliminate competing bacterial strains [4]. The potential of bacteriocins to effectively inhibit antibiotic resistant bacteria represents a promising research direction for treating drug resistant bacterial diseases [4]. Colicins are a specific class of bacteriocins produced by *E. coli* that exhibit antimicrobial activity by piercing the cell membrane of target bacteria, leading to death by cytoplasm leakage [5]. Colicins use discrete structural modules that are designated to distinct functional steps in recognition, import, and killing. Each module's sequence and fold tune receptor specificity and the mechanism of target inactivation. T-domain mediates translocation across the outer membrane and interacts with import machinery or pores on the target cell surface. R-domain binds specific outer-membrane receptors to dock the Colicin. C-domain executable toxic activity results in bactericidal activity [6].

Colicin activity depends on inter-domain mechanics, surface electrostatics, catalytic motifs, and the immunity-binding architecture. The opening of a hinge or extension of the translocation segment is required for some Colicins to enter or plug an outer-membrane channel, thereby enabling import or channel occlusion [6]. Additionally, amphipathic helices and membrane-inserting C-terminal segments specifically form conductive pores in pore-forming Colicins. Full length architecture and surface charge distribution can modulate potency, as increasing the positive surface charge on colicin N enhanced cytotoxicity in experimental mutagenesis [7].

Among the various types of Colicins, Colicin N is noted for having the smallest molecular size, which potentially enhances its ability to penetrate target cells [8]. Furthermore, Colicin N has been demonstrated to possess the capability to inhibit lung cancer cells [9]. In this study, we hypothesized that the structural compactness and positive surface charge of Colicin N enabled it to overcome barriers specific to a genus and target conserved outer membrane porins in Vibrio species. Because colicin N is the smallest pore-forming colicin that has been reported [10]. To test this, we cloned and expressed recombinant Colicin N to evaluate its efficacy against diverse *Vibrio* isolates from Vietnamese shrimp farms. The potency of Colicin N was systematically assessed based on MIC thresholds and its comparative activity across different pathogenic strains.

2 Materials and Methods

2.1 Bacterial strains and reagents

Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio cholerae, Vibrio vulnificus, Vibrio harveyi, Vibrio owensii, and *Vibrio mimicus* strains were kindly provided by Dr. Vu Van Van from NTT Hitech Institute, Nguyen Tat Thanh University, Viet Nam. *Escherichia coli* BL21 (DE3) and *E. coli* DH5α were kindly provided by Dr. Nguyen Hoang Dung from the Institute of Life Sciences, Viet Nam Academy of Science and Technology, Viet Nam.

NdeI (R0111L), XhoI (R0146L), *Taq* 2X Master Mix (M0270L), T4 DNA Ligase (M0202L), Monarch® DNA Gel Extraction Kit (T1020L), 1 kb DNA Ladder (N3232L), Color Prestained Protein Standard (P7719L), and Monarch® Plasmid Miniprep Kit (T1010L) were purchased from New England Biolabs, USA. Ni Sepharose™ 6 Fast Flow (GE17-5318-02), Imidazole (I202-500G), sodium phosphate (S9763-500G), isopropyl β-D-1-thiogalactopyranoside (IPTG) (I6758-1G), and Sodium chloride (S9888-10KG) were purchased from Merck, Germany. PD-10 desalting columns packed with Sephadex™ G-25 resin were purchased from Cytiva, USA. Bacterial culture media were purchased from BD, Canada.

Primers for PCR reaction were purchased from Integrated DNA Technologies, USA.

Colicin N DNA sequence (GenBank: CP027259) was synthesized by the gene synthesis service of GenScript, China.

2.2 Primer design

Based on the sequences of the colicin N gene (GenBank: CP027259), the primers NdeI-colicin (5'-TATATACATATGGGTAGTAATGGCGCAGATA-3') and XhoI-colicin (5'-TATATACTCGAGTCATCGAATAACACTAGATA-3') were designed for the purpose of amplifying the Colicin gene, introducing a restriction site for the enzyme NdeI at the 5′end and a restriction site for XhoI at the 3′ end. The size of the amplified gene fragment is approximately 1185 bp. The primers Pet28bF (5’-CCTGGTGCCGCGCGGCAGCC-3’) and Pet28bR (5’- TCAGTGGTGGTGGTGGTGGT-3’), which amplify the cloning site on the pET-28b(+) vector, were designed for use in a Colony PCR reaction. The size of the amplified gene fragment is approximately 1213 bp.

2.3 Construction of the recombinant plasmid pET28 b(+)-colicin

pET-28b(+) plasmid and PCR product amplifying the colicin gene were double-digested using NdeI and XhoI, followed by ligation using T4 DNA Ligase. The recombinant plasmids were transformed into *E. coli* DH5α, and the positive clones were screened by kanamycin resistance. Colony PCR was utilized to screen bacterial colonies that carry the Colicin gene inserted into the pET-28b(+) plasmid. The recombinant plasmid pET-28b(+)-colicin was recovered from *E. coli* DH5α colonies via plasmid extraction and subsequently transformed into *E. coli* BL21 (DE3) for protein expression.

2.4 Expression of Colicin protein

To express Colicin protein, a colony of *E. coli* BL21 (DE3) carrying the pET-28b(+)-colicin plasmid was inoculated into 10 mL of Luria Broth (LB) medium supplemented with kanamycin (50 µg/mL) and incubated at 37 °C while shaking at 250 rpm for 3 h. Subsequently, the entire 10 mL culture was transferred into 500 mL of LB medium supplemented with kanamycin (50 µg/mL) and cultivated at 37 °C with shaking at 250 rpm until the bacterial density reached an OD_{600} of 0.5 (approximately 5×10^8 CFU/mL). Afterward, we evaluated the impact of (0.1, 0.2, 0.4, 0,8 and 1) mM IPTG (final concentration) on protein expression at 20 °C. After 18 h of induction, *E. coli* cells were harvested by centrifugation, and the resulting pellet was collected. The recombinant Colicin protein was then lysed using a Sonicator (operating at 20 kHz and 150 W). Colicin protein was purified using a Ni-NTA agarose column, and subsequently, the purity of the protein was verified by SDS-PAGE and Coomassie Brilliant Blue staining. Following Ni-NTA purification, the recombinant Colicin N was concentrated by Vivaspin column and stored in Tris pH = 7.0 with 15% glycerol at −20 °C. Before performing the MIC assays, the protein underwent a secondary purification step using PD-10 desalting columns and replacing the storage buffer with 0.1% NaCl. The protein concentration was determined based on the spectrophotometric absorbance value at a wavelength of 280 nm, utilizing the Beer-Lambert Law equation [11]:

$$A = \varepsilon c l.$$

Where:

A: the absorbance value measured at a wavelength of 280 nm.

ε: the molar absorptivity (or extinction coefficient) of the protein, typically expressed in units of $[mg/mL]^{-1} cm^{-1}$ or $[M]^{-1}cm^{-1}$.

c: the protein concentration, expressed in mg/mL or M (Molar).

l: the path length of the cuvette (cm).

2.5 Antibacterial assay

The antimicrobial efficacy of recombinant Colicin N was evaluated against a panel of Vibrio species, including *V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae*, *V. vulnificus*, *V. harveyi*, *V. owensii*, and *V. mimicus*. All strains were isolated from the pond water of whiteleg shrimp (*Litopenaeus vannamei*) aquaculture facilities. Minimum inhibitory concentration (MIC) was determined using the resazurin microtiter assay to provide a quantifiable and metabolic endpoint. The assay was performed in plates with 96 wells using Mueller Hinton Broth (MHB) adjusted with cations supplemented with 1.5% NaCl to support the optimal growth of *Vibrio*. Bacterial inocula were standardized to approximately 10^6 CFU/mL. A serial dilution of Colicin N was prepared in the culture medium, resulting in ten final concentrations: (25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.39, 0.195, 0.098, and 0.049) µg/mL. The plates were incubated at 35 °C for 24 hours. Following incubation, 0.02% (w/v) resazurin solution was added to each well and incubated for an additional 2 hours. The MIC was defined as the lowest concentration of Colicin N that prevented the color transition from blue to pink, indicating a complete inhibition of bacterial metabolic activity. To ensure reproducibility and robustness, all experiments were conducted in three independent biological replicates, with each sample tested in triplicate.

3 Results and Discussion

3.1 Cloning, expression, and purification of Colicin N

The PCR product of the Colicin gene, using NdeI-colicin and XhoI-colicin primers, was double-digested with the restriction enzymes NdeI and XhoI. The pET-28b(+) plasmid was processed similarly. Both the digested gene fragments and the vectors were then subjected to agarose gel electrophoresis for size verification and purification prior to the ligation step (Figure 1A). After correct sequence verification, the Colicin gene was cloned into the pET-28b(+) plasmid. The Colony PCR method was used to verify the presence of the recombinant plasmid pET-28b(+)-colicin (Figure 1B). The observed size of the Colicin fragment on the electrophoresis gel was approximately 1.2 kb. The recombinant plasmid, following purification, was transformed into *E. coli* BL21 (DE3) for Colicin protein expression and purification.

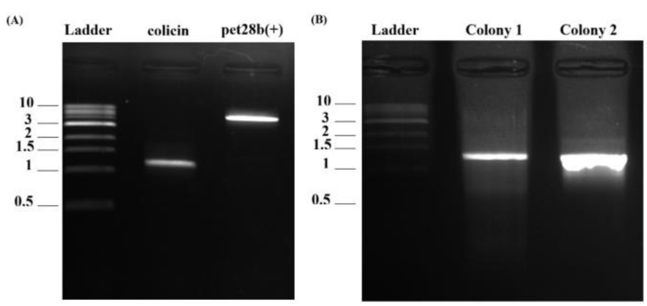

Figure 1 Agarose gel electrophoresis of Colicin fragment, recombinant plasmid pET-28b(+), and colony PCR products. (A) The colicin fragment and pET-28b(+) were double-digested with NdeI and XhoI. (B) PCR amplification of the colicin gene from two *E. coli* BL21 (DE3) colonies.

Recombinant Colicin was overexpressed using (0.1, 0.2, 0.4, 0.8, and 1) mM of IPTG for 18 h at 20 °C (Figure 2A). The results of induction across various concentrations of IPTG showed no significant differences, indicating that Colicin N can be expressed efficiently at a concentration as low as 0.1 mM. Therefore, all subsequent experiments to produce recombinant Colicin N utilized a concentration of 0.1 mM. The wash fraction using the NPI-10 buffer did not contain Colicin (Figure 2B, lane 7), indicating that the protein was bound to the Ni-NTA column. Then, Colicin was eluted with NPI-60, NPI-90, NPI-120, NPI-200, NPI-300, and NPI-500 buffer as a single band. Purified recombinant Colicin was quantified using the Beer-Lambert Law equation. The purified Colicin was concentrated using a Vivaspin 20 ml concentrator (50K MWCO) and subsequently diluted to specific concentrations for the MIC test to evaluate its antimicrobial activity.

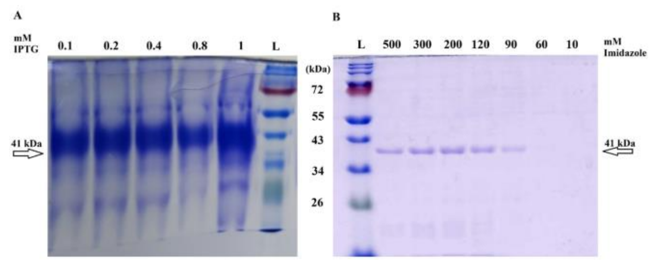

Figure 2 Colicin was analyzed by SDS-PAGE electrophoresis. (A) Colicin overexpression. (B) Colicin purification using Ni-NTA agarose.

3.2 Assessment of Colicin's inhibitory activity against *Vibrio* strains

The susceptibility of the seven isolates of *Vibrio* to Colicin N is presented in Figure 3. The assay used serial dilutions with a ratio of one to two, starting from a maximum concentration of 25 µg/mL down to 0.05 µg/mL. Among the tested isolates, Colicin exhibited inhibitory activity against four species: *V. parahaemolyticus* and *V. owensii* showed a MIC of 25 µg/mL, while *V. vulnificus* and *V. harveyi* demonstrated higher sensitivity with a MIC of 12.5 µg/mL. In contrast, no inhibitory effect was observed for *V. alginolyticus*, *V. cholerae*, and *V. mimicus* even at the highest concentration tested (Table 1). While the purification process included a secondary desalting step using PD-10 columns to eliminate residual imidazole and low molecular weight components, the absence of an empty vector protein control is a recognized limitation of this study. However, it is noteworthy that *E. coli* BL21 (DE3) is not known to endogenously produce bacteriocins or peptides with specific inhibitory activity against *Vibrio* species under the tested conditions [12-13]. Therefore, the observed antimicrobial effects are highly likely attributable to the purified recombinant Colicin N. Nonetheless, future studies should incorporate an empty vector control to provide a more definitive validation of protein specificity.

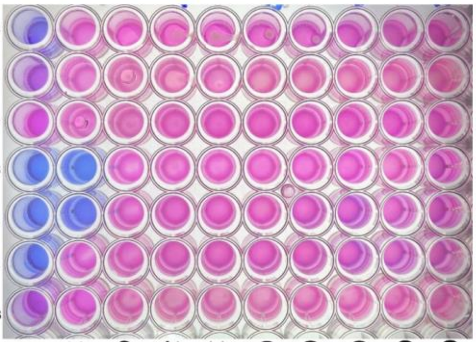

Figure 3 MIC for Colicin N on seven strains of *Vibrio*

This variation in susceptibility suggests that the antibacterial action of Colicin N is dependent on specific biological interactions with each *Vibrio* species. The resistance observed in *V. alginolyticus*, *V. cholerae*, and *V. mimicus* base on compatible receptors on the outer membrane, or a low efficiency in the translocation machinery required for the protein to reach its target [10]. Conversely, the higher sensitivity of *V. vulnificus* and *V. harveyi* might reflect a more effective binding affinity to their surface proteins or a more rapid penetration through the periplasm. These differences highlight the importance of the molecular structure of the cell envelope in determining the range of activity for Colicin N across the *Vibrio* genus.

Table 1 Determination of the MIC by Resazurin aided microdilution method of Colicin N against seven *Vibrio* strains

Bacteria	MIC reported in this study (µg/mL)
V. parahaemolyticus	25
V. alginolyticus	-
V. cholerae	-
V. vulnificus	12.5
V. harveyi	12.5
V. owensii	25
V. mimicus	-

4 Conclusion

In conclusion, recombinant Colicin N was successfully expressed in *E. coli* BL21 (DE3) and purified using Ni-NTA affinity chromatography. The purified Colicin N showed specific *in vitro* inhibitory activity against four different *Vibrio* spp.. The MIC values of 12.5 µg/mL for *V. vulnificus* and *V. harveyi*, 25 µg/mL for *V. parahaemolyticus* and *V. owensii* were recorded. However, no inhibitory activity was observed against *V. alginolyticus*, *V. cholerae*, and *V. mimicus* at the maximum concentration (25 µg/mL).

This study also has certain limitations, particularly the absence of a negative control in the MIC assays. Further research is needed in considering its potency in combating Colicin N *in vivo* experiments, also checking its safety concerning microbiota in the host. These investigations are necessary to determine its potential applicability in shrimp farming.

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Tạo dòng và biểu hiện Colicin N có hoạt tính kháng một số loài *Vibrio* gây bệnh phân lập từ môi trường nuôi thủy sản

Hồ Tá Giáp[1,2,*], Ngô Nguyên Vũ[1,2], Trần Song Nguyên Khôi[1,2]
[1]Viện Kỹ thuật Công nghệ cao NTT, Trường Đại học Nguyễn Tất Thành, Thành phố Hồ Chí Minh
[2]Trung tâm Phát triển Công nghệ cao Đại học Nguyễn Tất Thành, Khu Công nghệ cao Thành phố Hồ Chí Minh
[*]htgiap@ntt.edu.vn

Tóm tắt Kháng kháng sinh ở vi khuẩn gây bệnh trong nghành thủy sản là một mối đe dọa phổ biến, phát sinh từ việc lạm dụng kháng sinh ngày càng thiếu kiểm soát. Bacteriocin hiện đang được quan tâm nghiên cứu như một giải pháp thay thế khả thi cho kháng sinh trong ngành thủy sản nhờ vào tính an toàn cao. Trong nghiên cứu này, Colicin N nguồn gốc từ vi khuẩn *Escherichia coli* đã được nhân dòng, biểu hiện và tinh sạch. Trong số bảy chủng *Vibrio* nguồn gốc từ ao nuôi tôm được thử nghiệm, Colicin N tái tổ hợp ở nồng độ 25 µg/mL cho thấy hoạt tính ức chế đối với *Vibrio parahaemolyticus* và *Vibrio owensii*. Đối với hai chủng *Vibrio vulnificus* và *Vibrio harveyi* thì Colicin N thể hiện hoạt tính ức chế ở nồng độ 12,5 µg/mL. Kết quả của nghiên cứu này chứng minh hoạt tính ức chế *in vitro* của Colicin N đối với *Vibrio* gây bệnh, gợi mở tiềm năng ứng dụng trong việc chống lại bệnh do *Vibrio* trong nuôi tôm.

Từ khóa AMR; Colicin N; *Vibrio*; AHPND; *Escherichia coli.*