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COMPREHENSIVE PROFILING OF *VIBRIO ALGINOLYTICUS* ISOLATED FROM *LITOPENAEUS VANNAMEI*: MORPHOLOGICAL AND MOLECULAR IDENTIFICATION, GROWTH DYNAMICS, AND VIRULENCE

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Abstract

This study provides a comprehensive characterization of *V. alginolyticus* isolated from diseased shrimp. A gram-negative, rod-shaped bacterium known as *Vibrio alginolyticus* was isolated. From the hepatopancreas of sick white leg shrimp also known as *Litopenaeus vannamei* provided by the University Malaysia Terengganu FPSM hatchery, bacteria were successfully isolated. Tests for isolates were conducted to ascertain their biochemical and physiological features. All of the biochemical and physiological characteristics are different, with the exception of salt tolerance testing, TCBS colony colour, and colony shape. Because certain isolates could haemolyse red blood cells, they were classified as virulent. The methods used to identify the bacterial strains included morphological and biochemical techniques, PCR, and sequencing of a conserved region of the 16S rRNA gene. The results showed that the isolates belonged to a single species. That grows in 1 to 10% NaCl, at 25 to 37°C, and on TCBS (thiosulfate-citrate-bile sucrose) agar. Non lactose fermenters and positive catalyse, the identification *of V. alginolyticus* was verified 99% the sequence identity of *V. alginolyticus* provided by the 16S rDNA sequence (GenBank accession number NR_PP980560.1). Via PCR analysis. The isolated strain's estimated LC_{50} dose was 1×10^7 colony forming units (CFU/ml), and an inhibition zone was found around Penicillin (10 mm), Amoxicillin (5 mm), Chloramphenicol (15 mm), Tetracycline (1 mm), and Ampicillin (5 mm). Based on antibiotic susceptibility testing. The findings contribute to a better understanding of the virulence, resistance patterns, and ecological impact of *V. alginolyticus,* with implications for disease management in shrimp aquaculture.

Keywords: Vibrio Alginolyticus, Virulence, White Leg Shrimp, Isolation, Litopenaeus Vannamei.

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1. INTRODUCTION

A Gram-negative opportunistic pathogen of marine aquatic species, *Vibrio alginolyticus* kills a large number of shrimp, shellfish, fish, and coral reefs in captivity (1). A major concern for the seafood industry has been identified as *Vibrio* spp. (2). *Vibrio* species are facultative, motile, gram-negative, straight, curved, or comma-shaped bacteria that are members of the *Vibrionaceae* family. They are widely distributed in aquatic environments across the world, including aquaculture settings, marine coastal waters, sediments, and estuaries. Twelve *Vibrio* species are classified as pathogenic to humans, primarily causing intestinal and extra-intestinal ailments. The majority of *Vibrio* species are considered non-pathogenic, with only a small number causing infectious diseases in aquatic plants, animals, or humans. These bacteria include *Vibrio cholerae*, *Vibrio parahaemolyticus, Vibrio vulnificus, Vibrio mimicus,* and *Vibrio alginolyticus* (3). Because of faster pathogen proliferation in natural water and increased susceptibility to environmental stress due to global climate change, aquatic life is more likely to experience mass death events (4). *Vibrio alginolyticus* is a halophilic organism that was previously classified as *V. parahaemolyticus* biotype 2. It doesn't grow on CLED agar; however, it does well in the presence of 10% sodium chloride. On TCBS, it grows huge, yellow (sucrose-fermenting) colonies. On non-selective solid media, there is a lot of swarming (5). When *V. alginolyticus* colonies were close together, they formed white colonies 1 to 2 mm in diameter, but when isolated, some produced small pink centres. (6) Haemolysin is a pathogenic factor found in *Vibrio* species that has hemolytic activity against erythrocytes. It's linked to a variety of *Vibrio* species that cause wound infection or intestinal infection as a clinical symptom. *V. vulnificus* and *V. alginolyticus* are well-known wound-infecting species. It's linked to a variety of *Vibrio* species that cause wound infection or intestinal infection as a clinical symptom. *V. vulnificus* and *V. alginolyticus* are well-known wound-infecting species (7). Standard media, such as blood and MacConkey agars, can support the growth of most *Vibrio* species. They are typically lactose-free fermenters (8). In the identification of *Vibrio alginolyticus*, biochemical characteristic detection techniques can be inaccurate. *Vibrio* species can be detected using PCR-based approaches, which are routinely utilized in research settings but not in clinical laboratories (9).

The most well-known group of microorganisms that cause major losses in shrimp culture is bacteria, which have disastrous economic consequences for damaged farms. In penaeid shrimp growing systems, bacterial infections, primarily *Vibrio,* have been reported (10). The principal agents of this disease are currently known to be opportunistic *Vibrio* species (including *V. alginolyticus, V. harveyi, V. parahaemolyticus,* and *V. vulnificus*). Moribund shrimp with acute bacteria and septic hemocytic nodules on the lymphoid organ, heart, and loose connective tissues are among the clinical symptoms (11). Shrimp larvae afflicted with vibriosis have necrosis of appendages, enlarged chromatophores, an empty gut, no fecal strands, and impaired sensation. Caused by *V. alginolyticus, V. parahaemolyticus,* and *V. parahaemolyticus,* cumulative mortalities can reach up to 80% after a few days. Microscopical demonstration of motile bacteria in the body cavity of moribund shrimp larvae was used to make the diagnosis, and standard

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microbiological methods were used to validate the isolation and identification of pathogenic bacteria (12).

2. MATERIALS AND METHODS

Isolation of Bacteria: The hepatopancreas of two diseased *L. vannamei* shrimps (n = 2) was removed and bacteria were isolated .The dissected organ was crushed with 0.9% NaCl, 0.1 ml of the crushed solution was plated in 10 ml of tryptic soy broth TSB (Merck, Germany), and swabbed and spread on TCBS agar thiosulfate citrate bile salt (Merck, Germany) at room temperature for 24h. Yellow colonies were picked and saved on tryptic soy broth TSB (Merck, Germany) containing 1.5% NaCl and stored for further studies (13).

Biochemical Tests. Salts Tolerance Test: Every isolated bacterium will grow in varying salt concentrations. 1.5%.3%.6% and 10% NaCl, *Vibrio alginolyticus* is a halophilic bacteria that tolerates high salt concentrations; as salt concentrations rise, so does the bacteria's growth (14)**. Hemolysis Test:** The *Vibrio alginolyticus* strain will be streaked on Oxoid blood agar (UK) for 24 to 48 hours after being incubated at room temperature. If there is a clear zone surrounding the bacteria at the streaking sites, it will be considered hemolytic. Isolates that lack a clean zone surrounding the bacteria at the streaking point are known as non-hemolytic isolates **(15). Fermentation Test:** A bacterial colony was streaked on McConkey agar and incubated at 28°C for 24 hours. On McConkey agar, lactose fermenters turn red or pink; nonfermenters do not **(16)**. **Gram Staining:** For microscopic observation, one colony from each sample is spread out on a microscope slide and stained using the Gram stain method, which involves applying crystal violet stain for one minute, adding Grams Iodine for one minute, quickly decolorizing with ethanol for thirty seconds, and counter staining with safranin for one minute (17). **Catalyse Test:** A single colony was added to the petri dish and one drop of 3% hydrogen peroxide (H_2O_2) was added straight with mixing to ensure the reaction could be observed correctly. After adding hydrogen peroxide the rapid bubble formation, just as in the case of catalytic reaction, was seen through this arrangement for careful observation (18).

Bacteria Identification: A total of five bacterial isolates were isolated from shrimp and specifically identified based on colony morphology, colour on TCBS, and size and shape. These had been sub-cultured on TSA with 1.5% NaCl .*Vibrio alginolyticus* was differentiated from *L. vannamei* by PCR amplification of the 16S rRNA gene with a universal primer. Isolates were sub-cultured on blood agar at 37° C for confirmation of the PCR results and identification with the BD Phoenix system (19).

Antibiotic susceptibility: Bacterial solutions were consistently swabbed onto the Muller Hinton Agar (MHA) with 3% NaCl (Oxoid, England) using sterile cotton buds. The antibiotics Ampicillin (AMP2), Ampicillin (AMP 10), Amoxicillin (AML 2), Penicillin (MY 2), Lincomycin (MY2), Chloramphenicol (C30), and Tetracycline (TE 30), obtained for the study's *Vibrio alginolyticus* isolates. And incubated for 24 hours at 30 °C. The diameter of the inhibition zone surrounding the discs was measured for this test (20).

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Lethal concentration LC_{50}: To determine the LC_{50} of *Vibrio alginolyticus* in white leg shrimp, six groups of ten shrimp each were fasted for 24 hours and then exposed in 20 µL tanks at 30 °C. Groups 1–5 were immersed for one hour in bacterial suspensions at concentrations of 1×10^8 to 1×10^4 CFU/ml, prepared through tenfold serial dilutions. Group 6 served as a control and was exposed only to TSB. Mortality was monitored daily for five days (21). The experiment was conducted in triplicate, and the LC_{50} was calculated using probit analysis in SPSS 20.0.

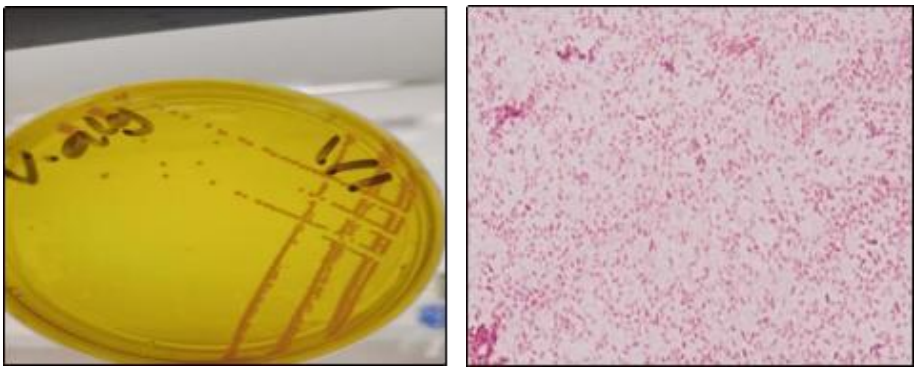
Growth Curve of Isolated *V. alginolyticus* and bacteria strength: The growth curve of *Vibrio alginolyticus* was determined at 8 h, optical density (OD) and colony-forming unit per ml (CFU/ml) were measured in tryptic soy broth (TSB) containing 1.5% salt. 5 universal tubes with one colony each were kept at 37°C to measure OD every 1 h. To determine the CFU/ml, bacteria were plated on TCBs, after serial dilutions, and incubated at 37°C for 24 hours. The number of colonies was used to calculate the CFU/ml (22).

For bacterial strength determination for LC_{50} determination, *Vibrio alginolyticus* was grown in TSB, 10ml at 37°C for 24h. Serial dilutions were done and 100 µL from individual dilutions were spread on TCBS agar. The numbers of colonies were then counted, and the CFU/ml was determined from the equation. Where N_{Col} the number of colonies was DF was the dilution factor, and V_{CP} was the volume of the culture plate.

$$(CFU/ml) = \left(\frac{N_{Col} \times DF}{V_{CP}}\right) \times 100\%$$

3. RESULTS

The isolated bacteria from the shrimp hepatopancreas TCBS agar plate were identified as *V. alginolyticus colonies*) yellow, convex; Figure 1). This is a characteristic colony morphology, which is important for bacterial identity (23). Microscopic examination (Figure 1 (and Gram staining showed that this organism was Gram-negative and rod-shaped as well as *V. alginolyticus*.



(a) **(b)**

Figure 1: (a) The *V. Alginolyticus* colony morphology (yellow convex colony), (b) gram-negative *V. alginolyticus* microscopic image with rod-shaped (40X)

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Vibrio alginolyticus is a Gram-negative rod that forms beta-hemolytic colonies on blood agar, thus it can lyse red blood cells (RBC). It ferments lactose on MacConkey agar, which causes the colonies to turn pink or red, and demonstrates positive catalyse activity by quickly disintegrating hydrogen peroxide. Together these tests verify its morphology, biochemistry, and pathogenesis (24). Since *V.alginolyticus* is a halophilic ,it is capable of growing at various concentrations of NaCl, and its growth is predominantly promoted at 1.5% and 3% NaCl. Growth is significantly reduced at high salinity, noticeably at 6 and 10%, when metabolism/reproduction is repressed, but the bacteria are still alive. The survival of bacteria demonstrates the stress resistance. Elevated NaCl conditions can evoke physiological responses, such as higher antibiotic resistance probably a reflection of the ability of the bacterium to overcome harsh conditions with a possible ecological and biotechnological importance (25), and a positive catalyse reaction due to producing bubbles after reaction with H_2O_2.

The growth curve of *Vibrio alginolyticus* based on OD and CFU/ml Figure 2 shows the changes in the population number of *V.alginolyticus* populations in OD, and CFU/ml.

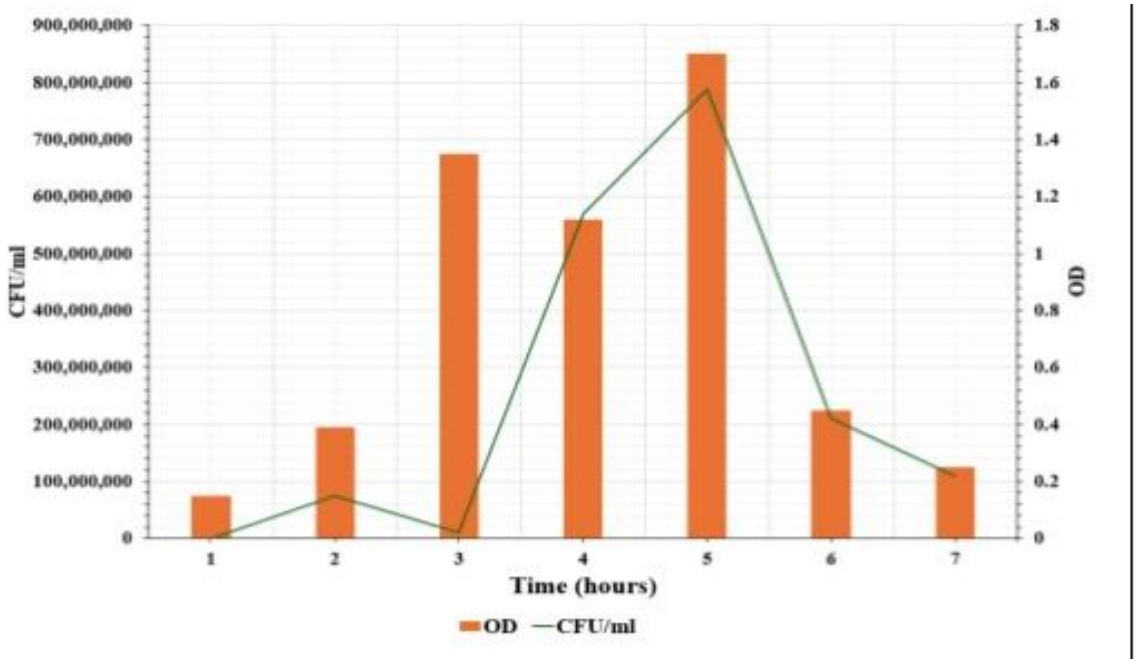

Figure 2: Growth curve for bacteria over time using OD and CFU

It displays the typical S-shaped curve with four phases: a lag phase (1 h) during which bacteria adapt and exhibit low OD and CFU; an exponential phase (1–5 h) characterized by rapid cell division and an increase in OD and CFU; a stationary phase at around 5 h, during which growth stabilizes with the number of CFU reaching a maximum at approximately 900 million and OD at approximately 1.6; and a death phase (5-7 h), during which both OD and CFU decrease due to bacterial death (26).

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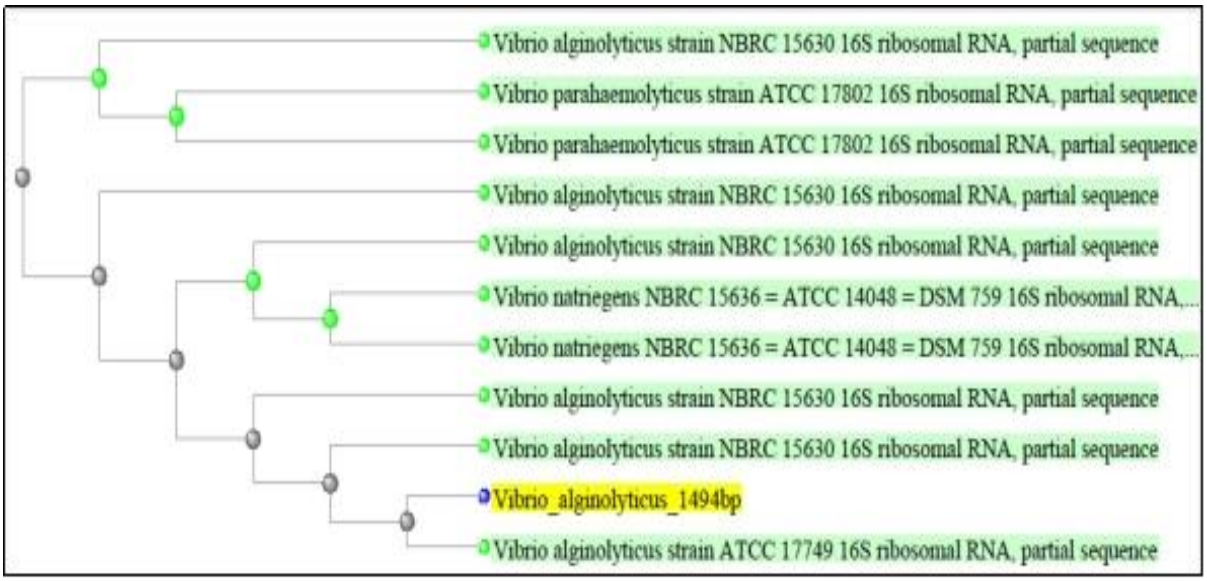
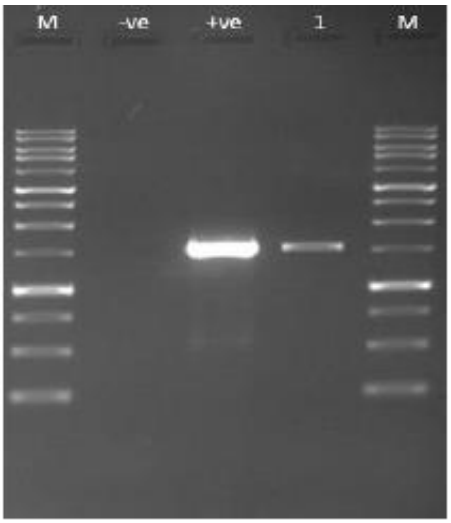
Specific regions of DNA of interest by the causative bacterial agent were amplified using chromosomal 16S rRNA included in the genetic profile of the organism. The result of PCR (Figure 3) indicated a high similarity between the sample and the strain of *Vibrio alginolyticus* .This, however, needed more confirmation from BD Phoenix .



(a)



(b)

Figure 3: a) 16S rRNA chromosomal PCR sequence for identification. b) A gel photo of 1 µl of PCR was run on 1% TAE agarose gel at 100 V for 60 min

The BD Phoenix system enabled us to rapidly and precisely identify *Vibrio alginolyticus* , with 99% sequence identity, substantiating its taxonomy and previous names (“Oceanomonas alginolytica” and “Beneckea alginolytica”). This marine bacterium is

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associated with human infections that include wound infections, cellulitis, otitis, conjunctivitis, meningitis, and diarrhea but is not commonly linked to diarrhea. This identification was confirmed using computerized biochemical and antibiotic susceptibility testing.

The LC 50assay showed that 1×10^{7}CFU/ml of *Vibrio alginolyticus* was the minimal lethal concentration that brought about 50% mortality within 5 days of experiment. This value was corroborated by survival ratio inter group analysis between the test and the control group, with the control shrimp presenting 100% of survival and the exposed to *V.alginolyticus* through water shrimp ranging from 70 to 90%.

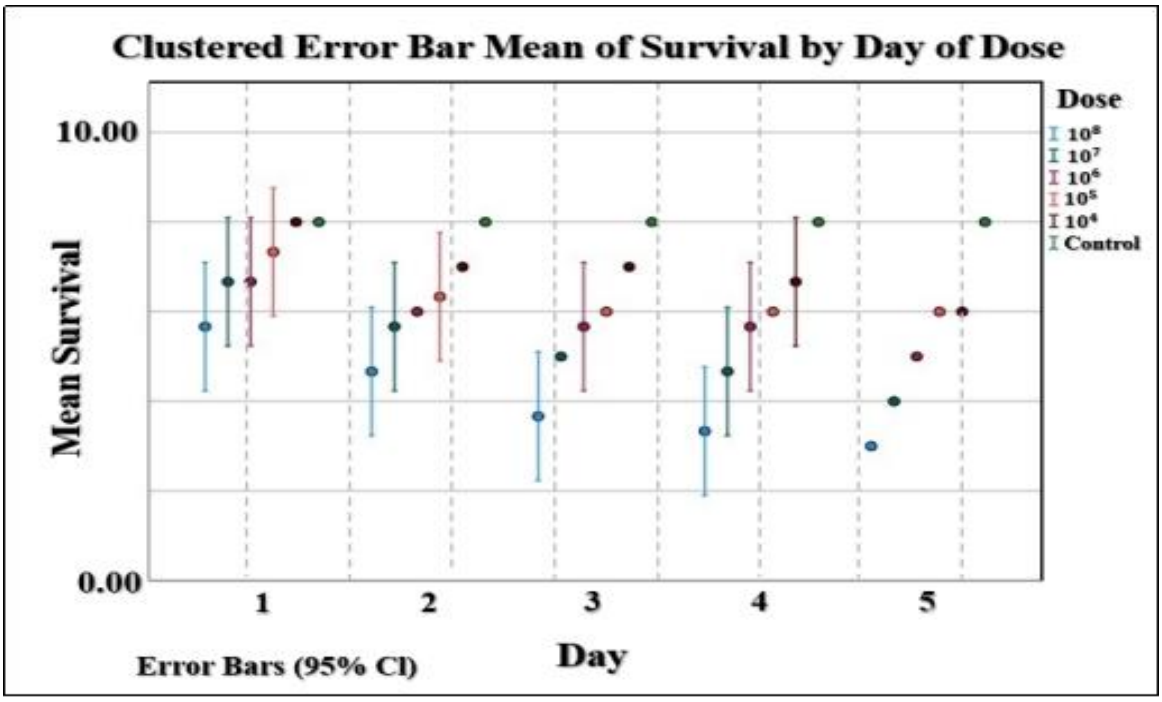

Figure 4: The critical outputs related to the clustered error bar mean of survival referring to the day of dose

The results of regression analysis revealed a highly significant dose-response relationship (R (0.945 = ²and confirmed the model's importance, meaning that with increasing bacterial concentrations, shrimp mortality was higher. These data highlight the critical need to evaluate dose-response relationships in the assessment of the toxicity of bacteria, particularly given differences among organisms and the possibility of severe effects at high doses. Previous studies give a range of doses (1×10^4–1×10^8 CFU/ml) for pathogenic *V.alginolyticus* in different types of shrimp (27). Antibiotic susceptibility is famous in biological contexts and medical technologists, since it can help researchers identify which antimicrobial regimen would be the most effective for *V. alginolyticus* testing correlated with antibiotic susceptibility, it can be observed that the Chloromphenicol C 30 (15 mm), Tetracycline TE 30 and Penicillin P 10 (10 mm), Amoxicillin AML 2 and Ampicillin AMP 2 (5mm), which have, as well, resistance to ampicillin and lincomycin.

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4. DISCUSSION

Vibrio alginolyticus is a gram-negative, rod-shaped bacterium, which has been successfully cultured and identified by morphological, biochemical, and molecular techniques. This bacterium is characterized by the capability of growth in saline and moderate temperature conditions, and it is highly pathogenic to a wide-range of marine organisms. Confirmation of *V. alginolyticus* is frequently based on PCR and 16S rDNA sequencing and exhibits high sequence homologies with the respective strains. *V. alginolyticus* has been recovered from a number of marine animals, including shrimp and fish, with TCBS agar and other selective media. It is a halophilic and mesophilic organism, growing in Salts 1-10% NaCl and 25-37°C (28). Molecular detection is performed by PCR using gene specific primers and sequence analysis of the 16S rDNA and appears to be closely related to the reference strains (99%) (29). The bacterium exhibits high pathogenicity with low LC_{50} of approximately 1 x 10^7 CFU reflecting its high virulence in marine animals such as shrimp and fish. Infected animals usually present with signs such as retarded growth, in appetence and tissue necrosis with high mortality in aquaculture (30), some antibiotics are highly resistant in *V. alginolyticus*, such as lincomycin and streptomycin, while some are sensitive, for example, imipenem and oxytetracycline (28). It`s different according to strains as in oyster isolates in Korea are completely resistant to ampicillin and vancomycin and partially resistant to cephalothin, erythromycin, and rifampin (20). Although *V. alginolyticus* is a major pathogen in aquaculture, it is also widely distributed in the natural marine environment. It can be found in seawater and seafood, suggesting its wide existence and possible influence on oceanic environment (31).The knowledge of its pathogenesis and resistance patterns is important for controlling its effects on the aquaculture and survival of the marine organisms.

5. CONCLUSION

Vibrio alginolyticus was effectively isolated and determined morphologically, biochemically, and by molecular methods. This isolate was able to grow at 1–10% NaCl and at 25–37°C, on TCBS agar, and was catalyse positive and non-lactose fermentation. Beta-hemolysis were revealed by haemolysis testing. The identity was verified by PCR, and 16S rDNA sequencing, and a 99% similarity to *V. alginolyticus*. The bacterium demonstrated significant pathogenicity, with an LC_{50} as low as 1×10^7 CFU/ml in shrimp, and its growth followed a typical bacterial curve. Its clinical and environmental significance is further highlighted by resistance to important antibiotics like ampicillin and lincomycin. Given its virulence, stress tolerance, and changing resistance patterns, the results highlight the necessity of continuous surveillance of *V. alginolyticus* in aquaculture settings. These findings advance our knowledge of the dangers posed by bacteria to marine life and point to possible ramifications for public safety, shrimp health, and sustainable aquaculture methods.

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