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Pheno-genotypic profile of *Vibrio cholerae* hemolysin (hlyA) isolated from shrimp and shellfish at the Kedonganan fish market, Bali-Indonesia

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ABSTRACT

Vibrio cholerae is a bacteria that causes foodborne disease and have several virulence factors in causing the disease. This study aims to find out the phenotypic and genotypic profiles of *V. cholerae* hemolysin (hlyA) isolated from shrimp and shellfish at Kedonganan Fish Market, Bali. The research begins by rejuvenating the stock culture of *V. cholerae* on TCBS medium. Hemolysis test was performed on medium blood agar (BA) with 5% sheep blood addition, hemolysis profile in BA was then confirmed using Polymerase Chain Reaction (PCR) test. The results showed that 4 out of 9 isolates (44%) were positive producing hemolysin characterized by a hemolytic zone around bacterial colonies grown in BA, whereas based on PCR results obtained 5 of 9 isolates (55%) positive carrying the hlyA gene. A mismatch of hemolysin profile was found in one isolate of UPK12, this isolate did not hemolyze red blood cells but was detected molecularly carrying the hlyA gene. It can be concluded that *V. cholerae* isolated from Kedonganan Fish Market is phenotypically and genotypically detected carrying hlyA and has potential in causing disease.

Keywords: Vibrio cholerae, foodborne disease, hemolysin (hlyA), Polymerase Chain Reaction
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INTRODUCTION

Cholera is a foodborne disease caused by a bacterial infection of *Vibrio cholerae*. Until now, cholera is still a health problem, especially in developing countries. It has been reported eight pandemics that claimed thousands of lives worldwide.[1] Transmission of cholera generally through drinks or food especially contaminated seafood.[2] *V. cholerae* infection is characterized by severe diarrhea resembling rice water stool, which can rapidly lead to dehydration and even death if left untreated.[1,3,4]

V. cholerae is a very heterogeneous species with 206 serotypes of O antigen.[5] *V. cholerae* can be divided into two types based on its pathogenicity, namely *V. cholerae* serogroup O1 / O139 and *V. cholerae* serogroup non-O1 / non-O139. *V. cholerae* O1 consists of the classical biotype and El Tor, both of those biotypes consisting of Inaba, Ogawa, and Hikojima serotypes.[1,6]

The genotypic characteristics of *V. cholerae* pathogen, are characterized by several genes such as cholerae toxin (ctx), zonula occludens toxin (zot), accessory cholera toxin (ace), toxin-coregulated pilus (tcpA), and hemolysin (hlyA) and other genes. Acting as a virulence factor.[7,8] The hlyA gene is one of the virulence factors that play a role in disease progression and has the ability to damage the host tissue.[9] In a previous study, *V. cholerae* isolated from shrimp and shellfish was positive based on the ompW gene but did not carry the ctxA gene.[10] Based on these results, the local isolates of Bali have not been able to infer the nature of pathogenicity because there are other genes that have not been identified. This study aims to further test the phenotypic and genotypic profile of *V. cholerae* hlyA, so it is expected to reveal the pathogenicity pattern of *V. cholerae* isolates from isolated from shrimp and shellfish at Kedonganan Fish Market, Bali.

MATERIALS AND METHODS

Sample

This study used eight isolates of *V. cholerae* from shrimp and one isolate of shellfish origin which has been isolated in previous research from Kedonganan Fish Market, Bali.

Rejuvenation and Isolate Confirmation

V. cholerae bacteria were re-cultured on TCBS agar medium, incubated at 37° C for 24 hours. The growing *V. cholerae* colonies show the yellow color of 2-3 mm.[11]

Test of Hemolysin Production

The production of hemolysin is determined by the presence of a hemolytic zone formed by the isolate *V. cholerae*. The isolates were cultured on a blood agar base (Oxoid CM55) with 5% sheep blood added, then incubated at 37° C for 24 hours. Visible clear zones around the colony are considered a positive result of hemolysin production.[8]

Genomic DNA Isolation

Isolation of genomic DNA using Boil Cell Extraction (BCE) method adapted from Marlina et al. (2007) with modifications.[12] Bacterial colonies were fed into 200 µl TE. Then heated for 10 minutes at 100°C. Further cooled using ice for 1-3 minutes and then centrifuged 8,000 rpm for 1 minute. The supernatant is transferred to a new Eppendorf tube and stored at -80° C until being used.

Amplification of *hlyA Vibrio cholerae* Gen

Polymerase Chain Reaction (PCR) to detect the hlyA gene was performed using a pair of primer hlyA-F (5'-GAGCCGGCATTCATCTGAAT-3') and hlyA-R (5'-CTCAGCGGGCTAATACGGTTTA-3') with a product length of 481 bp.[8] The PCR machine was programmed at 95° C for 2 minutes, followed by 25 cycles of 95° C for 1 minute, annealing 50° C for 1 minute, extension 72° C for 1 minute and final extension 72° C for 10 minutes.

Electrophoresis of PCR Results

Electrophoresis was performed using a 1.5% agarose gel which added 1 µL Biotium Gel Red™ Nucleic Acid. Electrophoresis using TBE 0.5 X at 50 volts for 60 minutes was then visualized using UV Transilluminator.

RESULTS

Nine isolates of *V. cholerae* consisting of 8 isolates of shrimp origin and one isolate of shellfish have been successfully regenerated. The results of the isolation of *V. cholerae* on TCBS medium showed yellow colonies with a diameter of 2-3 mm, smooth surface, opaque middle, and slightly brighter edges.

The results of testing the hemolysis profile on Blood Agar showed 4 of 9 isolates (44%) positively produce hemolysin characterized by the presence of hemolysis zone around the growing bacterial colonies.

The hemolysis profile test on Blood Agar was further genotypically confirmed using PCR technique by detecting the presence of the hlyA gene. Based on PCR results, five isolates (55%) were obtained positive for carrying the hlyA gene of UPK1, UPK3, UPK8, UPK12, and UPK24 isolates.

Summary of phenotypic and genotypic hemolysis test results on *V. cholerae* isolated from Shrimp of Kedonganan Fish Market presented in Table 1.

Based on the summary in the table above, the number of isolates that positively hemolyze red blood cells on the media Blood Agar amounted to 4 isolates, whereas based on PCR results obtained five positive isolates carrying the hlyA gene. Phenotypically UPK12 isolates do not hemolyze red blood cells but are detected molecularly carry the hlyA gene, the rest on the other isolates obtained the suitability between phenotypic and genotypic properties.

Table 1 **Phenotypic and Genotypic Profile of hlyA *V.cholerae***

Number	Isolate Code	Production of *Hemolysin (Blood Agar)*	PCR of *hlyA* Gen
1	UPK1	(+)	(+)
2	UPK3	(+)	(+)
3	UPK8	(+)	(+)
4	UPK11	(-)	(-)
5	UPK12	(-)	(+)
6	UPK21	(-)	(-)
7	UPK22	(-)	(-)
8	UPK24	(+)	(+)
9	KPK17	(-)	(-)

Legend: UPK = *Pasar Kedonganan Shrimp,* KPK = *Pasar Kedonganan Shellfish*

DISCUSSION

The results showed that four isolates of *V. cholerae* (44%) isolated from Kedonganan Fish Market, Bali, were phenotypically hemolyzed on Agar Blood media and based on PCR test found five positive hlyA isolates (55%). This is in accordance with the results of research conducted by Sedaghat et al. (2013) found that 95% of *V. cholerae* isolates from clinical samples showed hemolysis properties in A good Blood medium while PCR results showed that the whole isolate carries the hlyA gene.[8] In this study, there was a phenotypic incompatibility with genotypic profiles in UPK12 isolates. In the Blood Agar medium, isolate did not show hemolytic properties but based on PCR results detected carrying the hlyA gene. According to Sedaghat et al. (2013) phenotypic and genotypic hlyA phenotypic discrepancy is due to a factor that can prevent the expression of the hlyA gene.[8]

The hlyA gene is a gene encoding virulence factor owned by *V. cholerae* associated with disease progression processes and can cause tissue damage. Usually, *V. cholerae* biotype El-Tor is known to produce and secrete hemolysin toxin into the medium.[9,13,14] Hemolysin tests are often used to distinguish between El-Tor biotypes and classical biotypes.[15] Nevertheless, it has been reported that several isolates of *V. cholerae* El-Tor biotype are nonhemolytic.[16] The finding of El-Tor biotypes in this study closely corresponds with the seventh case of pandemic cholera that occurred in Sulawesi in 1961 caused by *V. cholerae* O1 El-Tor biotype.[1]

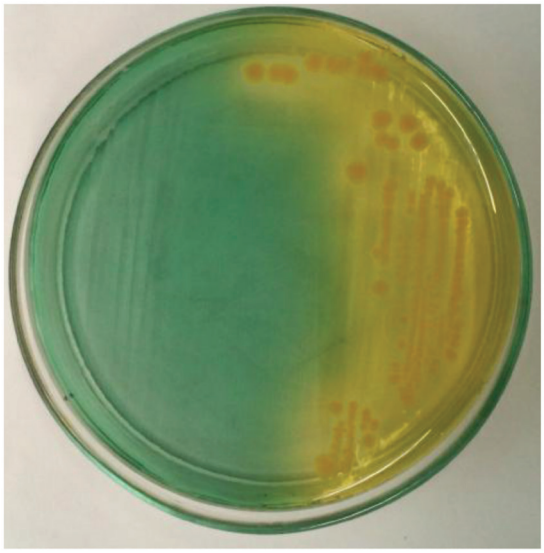

Figure 1 Growth of *V. cholerae* in TCBS medium

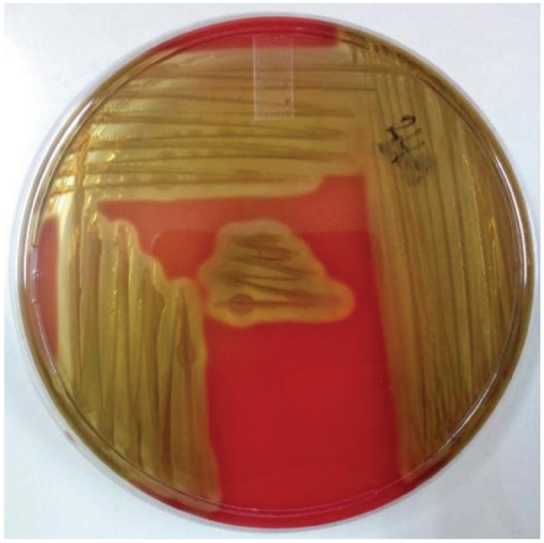

Figure 2 Hemolysis of *V.cholerae* in agar medium

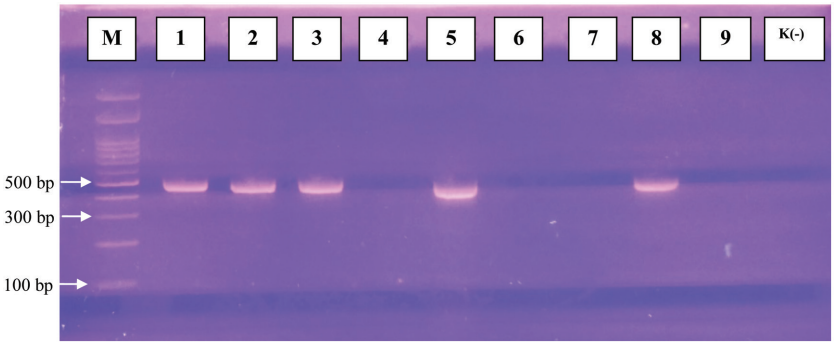

Figure 3 Electrophoregram as the result of hlyA *V. cholerae* gene PCR
Legend: M = Marker of 100bp DNA ladder, 1-9 = sample,
K (-) = negative control.
The result of 481 bp *hlyA* gene amplification.

Hemolysin is one of the most common pore-forming toxins (PFT) called *Vibrio cholerae* cytolysin (VCC).[17] This toxin is capable of making pores in the cell membrane which can further impair cell permeability, lyse red blood cells, and be lethal in mice.[18,19] Based on research reports conducted in Latin America, V. cholerae carrying the hlyA gene but lacking the ctx, zot, tdh, and tcp genes are still capable of causing disease.[20] Research conducted by Cinar et al. (2010) show that *V. cholerae* hemolysin causes death and vacuolization of the intestine in *Caenorhabditis elegans*.[17] Saka et al. (2008) reported that hlyA could induce liquid accumulation and cause histological damage to rabbit illegal loop testing.[21] Other studies have shown that in vitro, hemolysin is associated with cellular degeneration events such as vacuolization, lysis, apoptosis, and necrosis.[18,22] Further Kanoktippornchai et al. (2014) successfully proved the role of hlyA in the apoptosis of Chinese Hamster ovarian cells by increasing Bax protein expression and activating caspase-3 and caspase-9.[23]

The results of this study have addressed the pathogenicity of *V. cholerae* local isolates that have been isolated from Kedonganan Fish Market, Bali. The detection of the hlyA gene in the local isolate of *V. cholerae* shows that the bacteria have the potential to cause disease. Surveillance of disease agents that can contaminate foods such as *V. cholerae* is very important to prevent future outbreaks.

CONCLUSION

V. cholerae isolated from Kedonganan Fish Market is detected phenotypically and genotypically carrying hlyA and has potential in causing disease.

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