Detection of \( tlh \) and \( tdh \) genes in \textit{Vibrio Parahaemolyticus} inhabiting farmed water ecosystem used for \textit{L. Vannamei} aquaculture

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Abstract. \textit{Vibrio parahaemolyticus} is halophilic gram-negative bacteria that live as natural inhabitant in aquatic environment. All \textit{Vibrio parahaemolyticus} strain known to have \textit{thermolabile hemolysin} encoded by \( tlh \) gene as species marker. \textit{Thermostable direct hemolysin} encoded by \( tdh \) gene is responsible for regulating virulence factor in \textit{Vibrio parahaemolyticus}. Aim of this research is to detect \( tlh \) and \( tdh \) gene from water of \textit{L. vannamei} aquaculture in Rembang regency. Colonies of green-blueish bacteria grew from isolation of \textit{L. vannamei} aquaculture water in CD-VP media which was identified as \textit{Vibrio parahaemolyticus}. Colonies of \textit{V. parahaemolyticus} grew to be small and green-blueish bacteria colonies in TCBS agar. Result of molecular analysis showed that bacteria isolated from water sample are specifically identified as \textit{Vibrio parahaemolyticus} bacteria by the detection of \( tlh \) gene. \textit{Vibrio parahaemolyticus} isolated from water of \textit{L. vannamei} aquaculture detected as \( tdh \) negative that indicates \( tdh \) gene is not present in isolated bacteria. \textit{Vibrio parahaemolyticus} isolate were cultured in Wagatsuma agar for \( tdh \) gene confirmation test that showed Kanagawa negative result, which indicated that \textit{V. parahaemolyticus} did not produce \textit{thermostable direct hemolysin}. These results showed that \textit{Vibrio parahaemolyticus} isolated from aquatic environment of \textit{L. vannamei} aquaculture in Rembang regency did not show virulence factors.

Keywords: \textit{Vibrio parahaemolyticus}, \textit{tdh} gene, \textit{tlh} gene

1. Introduction
Development of vanname shrimp (\textit{Litopenaeus vannamei}) aquaculture is commercially followed by the increasing risk of disease infection caused by opportunistic pathogen. \textit{Vibrio parahaemolyticus} is halophilic gram-negative bacteria that live as natural inhabitant in aquatic environment [1]. \textit{V. parahaemolyticus} bacteria in aquatic environment are able to infect vanname shrimp (\textit{L. vannamei}) through wound in shrimps’s outer shell that will spread through hemolymph in \textit{L. vannamei} circulation sytem [2].

\textit{L. vannamei} is one of transmission media of \textit{V. parahaemolyticus} bacteria to human host in which specifically target human intestinal. Based on Alam [3], \textit{V. parahaemolyticus} is halophilic gram-negative bacteria that live as natural inhabitant in aquatic environment [1].
parahaemolyticus infection is one of the main causes of 20-30% gastrointestinal cases in humans that happen in several Asian countries such as Japan, Hong Kong, Thailand, and Indonesia. V. parahaemolyticus bacteria enter human gastrointestinal system by the consumption of raw or undercooked shrimps that have been infected by V. parahaemolyticus [4]. V. parahaemolyticus bacteria that have found optimal condition in human gastrointestinal will start colonizing the host organism to obtain nutrient for growth survival purpose [5].

V. parahaemolyticus produce hemolysin as enterotoxin that cause blood cell lysis in the infected organism. $thl$ gene encode thermolabile hemolysin as specific marker to identify V. parahaemolyticus species [6]. However, $thl$ gene is not specific to direct the virulence factor in V. parahaemolyticus [7]. V. parahaemolyticus bacteria have several virulence factors that include adhesin, thermostable direct hemolysin ($tdh$), and TDH related hemolysin ($trh$). $tdh$ gene encodes thermostable direct hemolysin that have function as one of the virulence factors in V. parahaemolyticus [8].

2. Method

2.1. Obtaining Sample

Obtaining water of L. vannamei aquaculture in Rembang regency was done in November when the aquaculture had been in ± 50 days. Water sample was obtained from one of vanname shrimp aquacultures located in Rembang regency. 500 ml water sample was obtained from the aquaculture then collected into sample bottles. Obtained water sample was kept into the ice box for transferring to the laboratory.

2.2. Vibrio parahaemolyticus Isolation

Isolation of Vibrio parahaemolyticus procedure aimed to obtain Vibrio parahaemolyticus isolates that will be further used for DNA extraction process. 1 ml of water sample with $10^2$ dilution was put in Compact Dry VP (CD-VP) media, then incubated in the temperature of 35°C ± 2°C within 24 hours. The growing green-blueish colonies then isolated by streaking-out from media to be re-cultured in thiosulfate citrate bile salts sucrose (TCBS) media and differentiated based on the color formation from the growing colonies. Cultured colonies resulted from the isolation was streaked to be transferred to 2 ml microtube. 500 µl sterile aquades was put into 2 ml microtubes, then homogenized with vortex for 30 seconds [9].

Positive control and negative control were cultured in CD-VP media. Positive control used was Vibrio parahaemolyticus from Major Fisheries and Estuarine Aquaculture Institution (BBPBAK) in Jepara. Negative control used was Bacillus pumilus from bacteriology laboratory of UPT Laboratorium Terpadu Undip. Gram coloring procedure was being done to understand bacteria type used in this research.

2.3. DNA Extraction

DNA of Vibrio parahaemolyticus bacteria was extracted based on Qiaamp DNA mini kit DNA extraction protocol (Qiagen, Hilden, Germany) for cultured bacteria that include destruction procedure, RNA and protein elimination procedure, and purification procedure.

Destruction procedure began by putting samples in room temperature. 500 µl AL buffer added into eppendofor tube that contain sample, sample then homogenized with vortex for 30 seconds, eppendorf tube then gently turned upside and downside for five times, then rehomogenized with vortex for 30 seconds. Samples were incubated for 5 minutes in room temperature. Then 200 µl cooled absolute ethanol was added to be homogenized with vortex for 30 seconds. ±500-600 µl solution transferred to DNA
collection tube (tube with filter), then put into centrifugation with speed of 8000 rpm for 30 minutes. Bottom solution under the filter then put away.

RNA, protein, and other components elimination procedure was started by adding 500 µl AW1 buffer, then centrifugated for 1 minute with speed of 8000 rpm. Solution inside the column tube was discharged. 500 µl AW2 were buffer added, then centrifuged for 3 minutes with speed of 14500 rpm. AW2 buffer was being used for total cleaning procedure. Solution inside the column tube then discharged. Tube centrifuged with the speed of 14500 rpm for 1 minute to dry out the filter. Filtered column tube moved into new DNA collection tube (eppendorf without filter).

Purification procedure was being done by adding 100 µl AE buffer. Column tube was incubated for 1 minute, then centrifuged for 1 minute at speed of 8000 rpm to obtain DNA. AE buffer was used to dilute DNA that would separate DNA from the filter. Samples were divided into working samples and stock samples. DNA was kept in temperature of 4°C for short-term storage and in temperature of -20°C or -80°C for long-term storage.

2.4. DNA Amplification

*tdh* gene amplification was being done with tdh86F forward primer (5'-CTGTCCCTTTTCTGCCCCCG-3') and tdh331R reverse primer (5'-AGGCCAGACCCGCTGCACT-3') [10]. *tlh* gene amplification was being done with tlh-f forward primer (5'-ATTACTCCCGCTTGTCTG-3') and tlh-r reverse primer (5'-GCAGACATAGGTATAGTGTGTT-3') [11]. PCR reaction mix contain 50 µl MyTaq HS Red DNA Polymerase PCR Kit (Meridian Bioscience Asia, Singapore, Singapore) that include 5x MyTaq Red Reaction buffer, 20 µM forward primer, 20 µM reverse primer, 1 U/µl MyTaq HS Red DNA Polymerase, and DNA template. Amplification procedure used DNA isolate of *Vibrio paraaemolyticus* from vannamei shrimp aquaculture and BBPBAP Jepara, and DNA isolate of *Bacillus pumilus* bacteria.

Thermal cycle process for *tlh* gene included pre-denaturation at 95°C for 3 minutes, followed by 35 amplification cycles that consisted of denaturation at 95°C for 30 seconds, annealing at 60°C for 45 seconds, and elongation at 72°C for 30 seconds, and final step was post-elongation at 72°C for 5 minutes [11]. Obtained PCR products were kept in the temperature -20°C for further use.

Thermal cycle process for *tdh* gene included pre-denaturation at 95°C for 5 minutes, followed by 35 amplification cycles that consisted of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute, and elongation at 72°C for 30 seconds, and final step was post-elongation at 72°C for 2 minutes [10]. Obtained PCR products were kept in the temperature -20°C for further use. Qualitative analysis for DNA products from PCR was done by electrophoresis procedure.

2.5. *tdh* Gene Confirmation Test

*tdh* Gene confirmation test was aimed to understand the characteristic of virulence factor in *V. parahaemolyticus* isolate from vannamei shrimp aquaculture water in Rembang regency with wagatsuma agar media. *V. parahaemolyticus* isolate from vannamei shrimp aquaculture water, *V. parahaemolyticus* isolate from BBPBAP Jepara, and *Bacillus pumilus* were incubated in the temperature of 35°C ± 2°C within 24 hours. *V. parahaemolyticus* that have virulence factors will show hemolytic activity that form clear area around bacteria colonies that grow in wagatsuma agar media known as kanagawa phenomenon. *Vibrio parahaemolyticus* that do not have thermo and direct hemolysin will not form clear area around bacteria colonies that grow in wagatsuma agar media [12].
3. Result
3.1. Isolation of Vibrio parahaemolyticus
Based on this research, water sample from *L. vannamei* aquaculture cultured on CD-VP media showed growing activity of green-blueish bacteria colonies. Positive control showed growing activity of green-blueish bacteria colonies on CD-VP media. However, negative control did not show any growing activity of bacteria colony on CD-VP media. Green-blueish bacteria colonies that grew on CD-VP media were identified as *Vibrio parahaemolyticus*. This according to the statement of Temamura [13] that green-blueish bacteria colonies that grow on Compact Dry VP media are identified as *Vibrio parahaemolyticus*.

According to Temamura [13], Compact Dry VP consist of selective chromogenic agar that include culture media (peptone, NaCl, bile salt, antibiotic) and two chromogenic enzyme specific for β-glucosidase and β-galactosidase that differentiated *V. parahaemolyticus* with other bacteria. According to Motarjemi [14], *V. parahaemolyticus* bacteria colonies produce β-glucosidase and β-galactosidase that grow to be green-blueish bacteria colonies on Compact Dry VP media. CD-VP media consists of bile salt and antibiotic that inhibit the growth of gram-positive bacteria. Based on this characteristic showed that CD-VP media as selective media to identify *V. parahaemolyticus*.

Single colony of *V. parahaemolyticus* from CD-VP media was isolated to be cultured in thiosulfate citrate bile salts sucrose (TCBS) agar media. Based on this research known that *V. parahaemolyticus* grew to be green-blueish colonies on TCBS media. According to Nordstrom [16], *V. parahaemolyticus* can not fermentate sucrose that form green-blueish bacteria colonies on TCBS agar media, meanwhile *Vibrio* bacteria that can fermentate sucrose will form yellow bacteria colonies on TCBS agar media.

3.2. Molecular Identification of Vibrio parahaemolyticus
Molecular identification was done to detect *tlh* (thermolabile hemolysin) gene and *tdh* (thermostable direct hemolysin) gene in *Vibrio parahaemolyticus* isolates. According to Nordstrom [16], *tlh* gene is specific marker for *Vibrio parahaemolyticus* species and *tdh* gene regulate virulence factor in *V. parahaemolyticus*. Molecular identification of *V. parahaemolyticus* from isolation of *L. vannamei* aquaculture water in Rembang Regency began with extraction of *V. parahaemolyticus* bacteria isolate cultures on TCBS media. DNA extraction method in this research used Qiamp DNA mini kit protocol (Qiagen, Hilden, Germany).

Quantitative analysis for *Vibrio parahaemolyticus* DNA extract from *L. vannamei* aquaculture water isolate showed DNA concentration of 971.7 ng/µl with DNA purification of 2.17 in ratio of A260/280. Quantitative analysis for *Vibrio parahaemolyticus* DNA extract from BBPBAP Jepara showed DNA concentration of 88.3 ng/µl with DNA purification of 2.11 in ratio of A260/280. Quantitative analysis for *Bacillus pumilus* (negative control) DNA extract showed DNA concentration of 70.1 ng/µl with DNA purification of 1.92 in ratio of A260/280. DNA purification and concentration analysis result showed that DNA isolation method in this research were able to isolate DNA from *V. parahaemolyticus* bacteria of *L. vannamei* aquaculture water, *V. parahaemolyticus* isolate from BBPAB jepara, and *Bacillus pumilus* isolate.
Figure 1. Visualization of tlh gene amplification products; (M) : Marker; (1) : V. parahaemolyticus BBPBAP Jepara isolate; (2): V. parahaemolyticus L. vannamei aquaculture water isolate; (3): Bacillus pumilus

tlh-F and tlh-R primers amplified tlh gene in DNA isolate of V. parahaemolyticus from L. vannamei aquaculture water and DNA isolate of V. parahaemolyticus from BBPAB Jepara in DNA band with length of 113 bp (figure 1). In which figure 1 shows visualization of tlh gene amplification products in 1% agarose gel with Kappa Universal Ladder 100bp DNA ladder. Based on research that was done by Vinoj [23], explained that DNA product with band length of 113 bp specific for tlh gene in V. parahaemolyticus. Two set of tlh primers resulted DNA amplification products as DNA single band with the right DNA band length for tlh gene of V. parahaemolyticus.

According to this result, DNA PCR product can be used for sequencing process. Result of PCR process for Bacillus pumilus DNA extract were detected as tlh negative. This showed that tlh gene were only detected in V. parahaemolyticus DNA isolates.

Sequencing DNA products of tlh gene amplified from V. parahaemolyticus isolates was aimed to obtain DNA nucleotides sequences. Obtained sequences were then aligned with Basic Local Alignment Search Tool (BLAST) program in National Center for Biotechnology Information (NCBI) website. Alignment of V. parahaemolyticus isolates resulted from amplification products with tlh gene primer showed that tlh gene in V. parahaemolyticus isolates had 84 bp Query length. Result of homologous analysis with BLAST showed that bacteria isolates from L. vannamei aquaculture water were identified as Vibrio parahaemolyticus bacteria. Query cover showed highest value on 70% in Vibrio parahaemolyticus strain VP1-1997. Highest similarity value was in tlh gene sequence of Vibrio parahaemolyticus strain VP1-1997 with the same max score and total score in value of 104. E-value of 3e-19 showed that significant alignment based on identical tlh gene sequence of Vibrio parahaemolyticus. Vibrio parahaemolyticus isolates have identical value of 98% with tlh gene of Vibrio parahaemolyticus sequences in gene bank. tlh gene specifically identified Vibrio parahaemolyticus bacteria species. According to Bhunia [17], tlh gene is found in all genoms of V. parahaemolyticus bacteria as specific marker to determine V. parahaemolyticus bacteria. However, tlh gene is not significant to characterize virulence factor in V. parahaemolyticus.

tdh gene amplification process with tdh86F and tdh331R primer in V. parahaemolyticus isolate from L. vannamei aquaculture water and V. parahaemolyticus isolate from BBPBAB Jepara did not result DNA product. This result showed TDH negative in which tdh gene that regulate thermostable direct hemolysin were not detected in Vibrio parahaemolyticus isolates. This result is in line with the statement from Levin.
[18] that certain \( V. \text{parahaemolyticus} \) strains produce thermostable direct hemolysin that regulated by \( tdh \) gene that determine bacteria virulence factor in host organism. \( tdh \) gene amplification result showed that \( V. \text{parahaemolyticus} \) isolate from \( L. \text{vannamei} \) aquaculture in Rembang regency and \( V. \text{parahaemolyticus} \) isolate from BBPBAP Jepara were detected to habe no \( tdh \) gene that encode \textit{thermostable direct hemolysin} as virulence factors in \( V. \text{parahaemolyticus} \).

3.3. \( tdh \) Gene Confirmation Test

According to Belkin and Collwell [19], clear zones that are formed in wagatsuma blood agar media show \( \beta \)-hemolysis activity caused by thermostable direct hemolysin from \( V. \text{parahaemolyticus} \) bacteria known as Kanagawa Phenomenon. Kanagawa phenomenon is an indicator for \( tdh \) gene confirmation test to determine virulence characteristic in \( V. \text{parahaemolyticus} \) bacteria. \( V. \text{parahaemolyticus} \) isolate from BBPBAP Jepara and \( L. \text{vannamei} \) aquaculture water showed Kanagawa negative result in which purple-greyish bacteria colonies grew with no clear areas formed in wagatsuma agar. According to Buller [20], kanagawa negative is shown with no clear/transparent area formed on blood cells around \( V. \text{parahaemolyticus} \) colonies in Wagatsuma agar media. \( Bacillus pumilus \) isolate did not show growing activity in Wagatsuma agar that consist of high composition of NaCl. According to Vaishnavi [21], high salt concentration with base pH media make Wagatsuma agar as selective growth media for \( V. \text{parahaemolyticus} \). Negative Kanagawa result indicated that \( V. \text{parahaemolyticus} \) isolates from BBPBAP Jepara and \( L. \text{vannamei} \) aquaculture in Rembang regency did not have \( tdh \) gene in which no hemolytic activity shown in Wagatsuma agar media. According to Yangihara [22], Wagatsuma agar consist of 5% of human blood cells to observe \( \beta \)-hemolytic activity of \( V. \text{parahaemolyticus} \) bacteria. \( \beta \)-hemolytic activity in Wagatsuma agar is one character of virulence factors as result of thermostable direct hemolysin in \( V. \text{parahaemolyticus} \). According to Hester and Harrison [23], \( V. \text{parahaemolyticus} \) in aquatic environment generally do not form \( \beta \)-hemolytic in wagatsuma agar. \( V. \text{parahaemolyticus} \) bacteria that do not have \textit{thermostable direct hemolysin} will not show any characteristic to regulate virulence factor in order to initiate colonization in aquatic environment.

4. Discussion

The possibility of \( V. \text{parahaemolyticus} \) in aquatic environment to have thermostable direct hemolysin found to be in very low percentage. This accordance to statement by Raghunanth [24] that \( V. \text{parahaemolyticus} \) pathogenic strain have high sensitivity to low oxygen concentration and organic component level in aquatic environment. Thereby, this condition limits the ability of \( V. \text{parahaemolyticus} \) pathogenic strain to survive in the environment with uncontrolled external factors. Various external factors in aquatic environment inhibit in regulating virulence factors in \( V. \text{parahaemolyticus} \). This become one reason \( V. \text{parahaemolyticus} \) that are detected with \( tdh \) negative are more common to be found in aquatic environment such \( L. \text{vannamei} \) aquaculture area in Rembang regency. Molecular identification and \( tdh \) gene confirmation test in this research proved that \( V. \text{parahaemolyticus} \) bacteria isolates from \( L. \text{vannamei} \) aquaculture water in Rembang regency did not have virulence factor to regulate virulence characteristic to initiate colonization in host organism that live in this aquatic environment.

\( L. \text{vannamei} \) is one of host organisms that tend to be vulnerable with \( V. \text{parahaemolyticus} \) infection in aquaculture environment. \( V. \text{parahaemolyticus} \) bacteria are able to infect \( L. \text{vannamei} \) through exposure of open wound in exoskeleton and during molting cycle that make \( L. \text{vannamei} \) have high risk of pathogen infection. According to Selvin [25], \( V. \text{parahaemolyticus} \) become opportunistic pathogen in \( L. \text{vannamei} \) because
bacteria are less capable to adapt with external environment that affect on certain pressure to bacteria growth. Therefore, *V. parahaemolyticus* will become opportunistic pathogen in *L. vannamei* when achieve such optimal condition in host organisms to obtain nutrient for bacteria metabolism purpose.

Maximum standard of *V. parahaemolyticus* exposure in *L. vannamei* is negative per 25 gram (ISO: SNI 7388-2009). If *V. parahaemolyticus* are found exceeding maximum standard of microbe exposure in food, then *L. vannamei* are not supposed to be consumed because it have high risk that *V. parahaemolyticus* to become opportunistic pathogen in human host. According to Letchumananan [26], consuming raw or undercooked *L. vannamei* can become one of a major cause of *V. parahaemolyticus* infection in human host. *tdh* gene confirmation test with *tdh* negative result showed optimal water quality, in which *tdh* gene that encode virulence factor thermostable direct hemolysin were not found in *V. parahaemolyticus* bacteria from *L. vannamei* aquaculture water in Rembang regency. This result affirmed that *V. parahaemolyticus* as natural inhabitant in *L. vannamei* aquaculture environment in Rembang regency were detected to have no virulence factor to initiate colonization and begin infection in host organisms.

5. Conclusion

Bacteria isolation from *L. vannamei* aquaculture water in Compact Dry VP media showed the growth of green-blueish bacteria colonies which were specifically identified as *Vibrio parahaemolyticus* bacteria. *V. parahaemolyticus* bacteria colonies in thiosulfate citrate bile salts sucrose (TCBS) media grew to be green-blueish bacteria colonies. Result of molecular identification based on *tlh* gene amplification showed that bacteria isolates from *L. vannamei* aquaculture water in Rembang regency were specifically identified as *Vibrio parahaemolyticus*. *V. parahaemolyticus* bacteria isolates from *L. vannamei* aquaculture water were detected as *tdh* negative in which indicated that *V. parahaemolyticus* isolates identified to have no *tdh* gene to encode thermostable direct hemolysin to determine virulence factors. *tdh* gene confirmation test for *Vibrio parahaemolyticus* with wagatsuma agar media resulted Kanagawa negative in which indicated that the bacteria isolates did not produce thermostable direct hemolysin to determine one of virulence factors to initiate colonization in host organisms.

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