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# Penemuan *Vibrio harveyi* pada *Litopenaeus vannamei* Terinfeksi *White Feces Disease* di Situbondo Jawa Timur

# The Discovery of *Vibrio harveyi* on *Litopenaeus vannamei* Infected White Feces Disease in Situbondo, East Java

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**Abstract** Tujuan penelitian ini untuk mengetahui keberadaan *Vibrio harveyi* pada udang vanname yang terinfeksi WFD (*White Feces Disease)* di Situbondo, Provinsi Jawa Timur. Penelitian ini dilakukan bulan November 2017 hingga September 2018. Bakteri *Vibrio* diisolasi dari udang yang menunjukkan gejala klinis terkena penyakit WFD kemudian diisolasi pada media TCBS. Isolat tunggal bakteri selanjutnya dimurnikan di media TSA. Semua isolat *Vibrio* selanjutnya diidentifikasi dengan tes biokimia, *Analytical Profile Index* / API 20NE (BioMeriuex), dan PCR dengan primer spesifik untuk *V. harveyi*. Parameter tambahan yang digunakan adalah kualitas air, kelimpahan plankton air tambak, dan uji resistensi antibiotik. Hasil penelitian ini ditemukan adanya *V. harveyi* pada semua sampel baik dari udang terinfeksi WFD dan di lingkungan (hepatopankreas, usus, feses dan air tambak). Total isolat yang diidentifikasi adalah sebanyak tujuh belas isolat yaitu sepuluh isolat *V. harveyi* (58,82%), tiga isolat *V. alginolyticus* (17,65%), satu isolat *V. fluvialis* (5,88%), satu isolat *V. parahaemolyticus* (5,88%), dan dua isolat bukan *Vibrio* yang diidentifikasi sebagai *Shewanella putrefaciens* (11,76%). Kesimpulan penelitian ini adalah ditemukan *V. harveyi* pada udang vanname yang terkena WFD dengan kualitas air yang buruk dan dominasi kelimpahan plankton tertentu dalam suatu tambak.

**Kata Kunci:** API 20NE; *Litopenaeus vannamei;* PCR, uji biokimia; *Vibrio harveyi;* WFD

**Abstract** This research was conducted to discover *Vibrio harveyi* infected vannamei shrimp with White Feces Disease (WFD) in Situbondo, East Java Province. This research was conducted in November 2017 until September 2018. All *Vibrio* isolates from shrimp infected WFD were identified with biochemical tests, Analytical Profile Index/ API 20NE (BioMeriuex), and PCR with specific primers for *V. harveyi*. Additional parameters were the water quality, plankton brackishwater pond abundance, and antibiotic resistance test. Result showed that from 17 bacterial isolates identified, 10 isolates were *V. harveyi* (58.82%), three isolates were *V. alginolyticus* (17.65%), one *V. fluvialis* isolate (5.88%), one *V. parahaemolyticus* isolate (5.88%), and two non *Vibrio* isolates which were identified as *Shewanella putrefaciens* (11.76%). All isolates of *V. harveyi* also showed resistance activity on more than one antibiotic. Poor water quality had been identified as abnormal result of pH, alkalinity, salinity, ammonia levels and total organic matter level. Plankton abundance observation showed that Chloropyceae, Diatom, and Dinoflagellata dominated all sampled brackishwater ponds. This research concluded that *V. harveyi* were discovered on vannamei shrimp with poor water quality and plankton abundance in the pond samples.

**Key words:** API 20NE; *Litopenaeus vannamei;* PCR, Biochemical Test; *Vibrio harveyi;* WFD

## INTRODUCTION

White feces disease (WFD) is an emerging disease in vannamei shrimp (*Litopenaeus vannamei*). WFD symptoms were characterized by a discoloration of pale hepatopancreas and white feces presented, floating on the water pond surface (Somboon *et al*., 2016), as well as the discoloration of intestine that became white (Rajendran *et al*., 2016). WFD had been reported to occur in Malaysia, Thailand, Vietnam, China and India (Inthusai, 2006; Limsuwan, 2010; Somboon *et al*., 2012; Cao *et al*., 2015; Mastan, 2015; Hou *et al*., 2018). WFD attack in Indonesia has been reported on some locations of vannamei shrimp culture in Central Java, East Java, Bali, Lombok, Sumbawa (Fuadi, 2016), West Java, North Sumatera, Lampung, and Sulawesi (Tang *et al*., 2016). WFD attack could cause a production decrease until 10-12% in Thailand (Sriurairatana *et al*., 2014), due to less Average Daily Weight Gain (ADG) than 0.1 g/day and feed conversion ratio (FCR) 1.7 to 2.5 (ADG normal was 0.2 g/day with FCR should be less than 1.5) (Tang *et al*., 2016). Vibriosis symptom is also found on shrimp suffered WFD called as systemic or enteric vibriosis/ septic hepatopancreatic necrosis (SHPN) (Aranguen *et al*., 2017). Identification of *Vibrio* on shrimp infected WFD in Indonesia has been reported to emerge on Central Java Province with several species found, such as V.

*parahaemolyticus*, *V. vulnificus*, *V. cholera*, and *V. anguillarum* (Jayadi *et al*., 2016). *Vibrio harveyi* was a *Vibrio* species that became the most dangerous species, leading to a cau of death on shrimp during the larval and adult stadia. *V. harveyi* is a main cause of luminescene vibriosis disease occurrence on shrimp culture in the brackishwater pond. *V. harveyi* also caused 100% mass mortality of vannamei shrimp on one cycle shrimp culture (Chythanya *et al*., 2002). *V. harveyi* domination in the water can cause this bacteria to penetrate through the shrimp immune system easily until reaching tubulus cell of hepatopancreas, disrupting the epithelial cells. This results in tubulus epithelial cells suffer from sloughing, making the intestine become white and white color feces (WFD) (Sriurairatana *et al*., 2014; Somboon *et al*., 2012). This research aimed to observed the infection of *V. harveyi* on vannamei shrimp culture in Situbondo, East Java Province, as it had never done before on this region.

## MATERIALS AND METHODS

### *Materials*

Equipments used in this research were binocular microscope (*Olympus, Japan*), Incubator (*Incucell MMM, German*), autoclave (*Hirayama, Japan*), analitical scale (*Pioneer, OHAUS, USA*), reaction tubes, Petridish, object glass, heating b k (*Benchmark, USA*), centrifuge (*Thermo Scientific, USA*), thermal cycler (*Veriti 96-Well Thermal Cycler Applied Biosystems, USA*), electrophoresis set (*Mupid 2 Plus, Takara, Japan*). Materials used in this research were vannamei shrimp (*Litopenaeus vannamei*) infected WFD, *Promega* GoTaq® Green Master Mix, Specific primer of V. *harveyi* VHARF forward primer (5 ,-CCG CAT ACC AAT TAC GGG TC-3 ,), VHARR primer reverse (5 ,-GGA AGT CGA GCT ACC AAA CA-3 ,) Agarose (*Promega*), *peqGreen* DNA RNA *Dye* (Peqlab), Biochemical test, Analytical Profile Index/ API 20NE (BioMeriuex). All isolates were tested using PCR with *V. harveyi* specific primer, biochemical test, and API 20NE kit. Biochemical test comprised Gram (KOH 3%), ca lase, oxidase, motility, O/F, TCBS, TSIA, $H_2S$ production, methyl red, Voges-Proskauer, Simmons citrate, indole production, lysine decarboxylase, arginine d rolase, ornithine decraboxylase, urea hydrolysis, 10µg O/129 sensitivity, 150 µg O/129 sensitivity, nitrate reduction, 0% NaCl, 1% NaCl, 6% NaCl, 8% NaCl, 10% NaCl, gelatin hydrolysis, esculin rolysis, hemolysis, 5% SBA, 30°C growth, 35°C growth, D-glucose acid, D-glucose gas, L-arabinose acid, cellobiose acid, D-galactose acid, myoinositol acid, lactic acid, D-Mannitol acid, n-Mannose acid, Salicylic acid, sucrose acid, trehalose acid, swarming, and MacConkey Agar.

### *Sampling (Shrimp infected WFD, white feces, and pond w7ater)*

This research used a survey method sampling, which was done with quota sampling instead of non random/ probability sampling method. Sampling location is shown on Fig. 1. Samples of vannamei shrimp should be infected by WFD, which could be identified as the shrimp floated on the pond surface ponds at the three different locations of shrimp culture in Situbondo. Water pond samples were taken to isolate some bacteria, measure the water quality control (pH, salinity, alkalinity, nitrite, ammonia and organic materials), and count the plankton abundance for determining whether the environmental conditions were the cause of WFD. 10 Vannamei shrimps in each sampling site were taken for identification (Pusat Karantina Ikan, 2013). Bacteria found in the samples were identified specifically in Brackish Water Aquaculture Development Center Situbondo. This research was conducted in November 2017 until September 2018. Vannamei shrimp samples that were infected WFD had clinical symptoms, such as pale hepatopancreas and white colored intestines. White feces that were found floating on the pond surface of ponds and water pond were taken and put in the coolbox. All samples were brought to Fish and Environmental Health Laboratory, Brackish Water Aquaculture Development Center Situbondo for bacterial testing.

### *Bacterial isolate*

Vannamei shrimp samples were measured based on their weight. Bacteria samples were isolated from hepatopancreas, intestine, and feces using streak method, while bacteria in pond water samples were observed by by spread plate isolation method. Spread plate is tion was done by culturing 100 µl isolate suspension on *Thiosulfate Citrate Bile alts Sucrose Agar* (TCBS) (Merck) selective media dan 37°C incubation for 24 hours. Single bacterial colony was chosen based on the different macroscopic morphology (size, shape, and pigmentation) after 24 hours of incubation and purified on *Tryptic Soy Agar* (TSA) (Merck) media (Somboon *et al*., 2012; Mastan, 2015).

### *Phenotipe and biochemical characteristics*

All bacterial isolates retrieved were observed based on the colony and cell morphology, Gram coloration, as well as cytochrome oxydase, catalase, motility, oxidative/fermentative (OF), and TCBS presence test. Biochemical test result was then identified by comparing the similar biochemical characteristic isolate with literatures (MacFaddin, 1980; Holt *et al*., 1994; Austin & Austin, 1999; Buller, 2004; SNI, 2006). API 20NE test procedure was done based on the manual instruction on (BioMeriuex, 4 03). Assays included in this test were $NO_3$ production, indole production, glucose acidification, arginine dihydrolase, Urease, esculin hydrolysis (β-glucosidase), gelatine hydrolysis (protease), β-galactosidase, glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate, phenyl-acetate, oxidase test. API 20NE biochemical test result was observed by determining the changing coloration on every biochemical test media used which was based on the instruction procedure on API 20NE kit. The interpretation result was performed by inserting the test result (positive or negative reaction on API 20NE one strip) using APIweb™ versi 7.0. software.

### *DNA isolation and molecular identification*

*Vibrio harveyi* gene detection using Polymerase Chain Reactions (PCR) with the specific primary gene of *V. harveyi* based on (Fukui & Sawabe, 2007) with 16S ribosomal RNA (16S rDNA) gene target, VHARF forward primer sequence (5 '-CCG CAT ACC AAT TAC GGG TC-3 '), VHARR primary reverse sequence (5 '-GGA AGT CGA GCT ACC AAA CA-3 '). DNA extraction was done to retrieve the bacterial DNA by taking one bacterial colony aged 24 hours from TSA medium using loop ose and putting it on microtube filled with 200 µl Nuclease Free Water/ NFW (Promega). Microtube was heated at 95°C for 5 minutes (Espinosa *et al*., 2013; Raja

et al., 2017). Amplification was done with GoTaq® Green Master Mix (Promega) based on the instructional kit. 12.5 µl Go Taq Green (Promega) master mix was added with 2 µl (10 pmol/µl) specific primer (forward and reverse) of *V. harveyi*, 2 µl of DNA template sample, and 8.5 µl of NFW until the total of reaction volume was 25 µl (Promega) on each reaction using 95°C 5 minutes setting from denaturation amplification setting, 20 cycle 94°C 45 seconds, 55°C 45 seconds, and 72°C 30 seconds. DNA Amplicon were electrophorized on 1.5% of agarose gel. DNA with positive *V. harveyi* would exist on 967 bp band.

### *Antibiotic sensitivity test*

Isolates containing positive *V. harveyi* were tested using antibiotic sensitivity test. 24 hours bacteria on Nutrient Broth/ NB (*Merck*) media were cultured on Mueller Hinton Agar/ MHA (*Merck*) with $10^7$ CFU/ml using spread method and were given the antimicrobial sensitivity discs. Antibiotic discs used were 5 µg/ml Enrofloxacin, 15 µg/ml Erythromycin, and 30 µg/ml Oxytetracycline. Plates containing antibiotic and bacteria were incubated for 18-24 hours at 35°C. The diameter of inhibition zone was measured and compared with the Clinical and Laboratory Standards Institute (CLSI) zone diameter interpretative chart to determine the isolate sensitivity to the antibiotics (Clinical and Laboratory Standards Institute/ CLSI, 2012).

## RESULT AND DISCUSSION

### *Result*

Sampling location is the vannamei farm infected WFD that located in in Situbondo, East Java Province ( Fig. 1). Samples were obtained from vannamei shrimp culture location site in Situbondo, East Java. The first and second location were semi-intensive brackishwater pond. Samples were obtained consecutively on 50 and 63 DOC. The culture stocking density on two locations were 42 shrimps/$m^2$ with 3000-4000 $m^2$ area. The first location was reported to suffer from WFD since 30 Days Of Culture (DOC), while the second location suffered from WFD on 60 DOC. The third location was intensive brackishwater pond with 150 shrimps/$m^2$ stocking density on 2000 $m^2$ area. WFD clinical symptoms were reported on 68 day shrimp. Samples were obtained on 70 DOC.


Figure 1. WFD sampling location in Situbondo, East Java (arrow sign shows sampling area).

Vannamei shrimp infected WFD and water sample were obtained from the brackishwater pond indicated white color feces floating on the pond surface. Morphological observation was also performed on the shrimp with softened and loosened carapace unattaced to the body. Health vannamei shrimp will show clear and transparent body color. On the other hand, external symptoms occured were dark color followed with body and caudal melanization. Clinical symptomps of vannamei shrimp infected WSD are presented on Fig. 2.

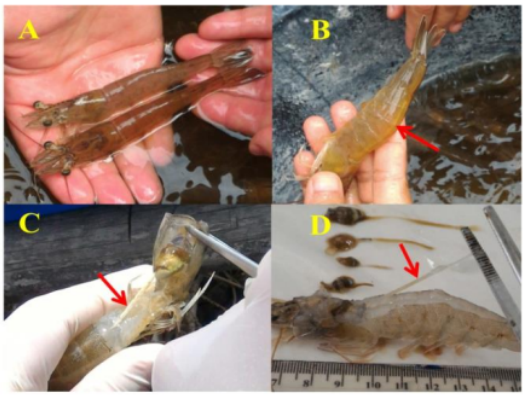


Figure 2. Vannamei shrimp (L. *vannamei*) infected WFD showed white intestine with vibriosis disease symptomps, note: A, discoloration/ shrimp melanization; B,C,D, white intestine of shrimp infected WFD (arrow sign).

Vannamei shrimp infected WFD were taken on DOC 50, 63 dan 70. The shrimp weight measurement is shown on Fig. 3. 50, 60, and 70 DOC shrimp had average weight of 7.6 g, 10 g, and 16 g respectively, while the average weight of shrimp infected WFD were 5.5 g, 7.3 g, and 7.7 g respectively. Feed consumed on the normal shrimp was utilized for growth (moulting) and activity energy, while the remaining was exploited as feces. Hepatopancreatic epithelial tubular cell disruption was occured on the shrimp infected WFD, therefore the main process of absorption and digestion was disrupted. Hepatopancreas was the main organ for feed absorption, transportation, digestive enzyme secretion, as well as lipid, glycogen, and some minerals deposition. Hepatopancreas disruption caused disrupted nutrient and low shrimp growth. This condition caused decreased ADG level. Decreased ADG level caused high FCR level as the accummulation of feed given during the culture was undigested by shrimps.

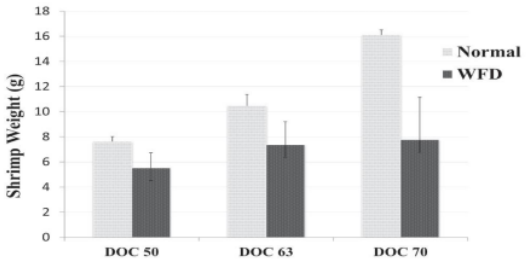


Figure 3. Vannamei shrimp weight with WFD infection.

The result of this research showed that there was no uniformity between WFD-infected and healthy shrimp. WFD- infected shrimp show higher standard deviation (1.20-3.38) than normal shrimp (0.4-0.9). High standard

deviation was followed with increased culture period. This condition happened as slow growing shrimp had lost on competing with bigger shrimp. The uniformity level also influences on choosing the appropriate pellet size difficulties and canibalism occured. Canibalism on shrimp occurs as the shrimp cultured in the pond has various size. Smaller shrimp is vulnerable against bigger shrimp.

Test result on alkalinity, nitrite, ammonia, and organic matter contents showed higher content than the preferred limited range content (SNI, 2014) dan is shown on Table 1. Water quality measurement on the pond was performed by taking the water sample from three locations suffering WFD. Alkalinity, nitrite, ammonia, and total organic material level showed high value result. The organic matter level in the pond suffered from WFD had more than 90 ppm value (113-140 ppm). Total alkalinity level was also high with more than 200 ppm. Nitrogen cycle in the pond was thought to be abnormal as presented on high anorganic nitrogen level, namely ammonia ($NH_3$) (3.6 ppm) and nitrite ($NO_2^-$) (8.12 ppm).

Table 1. Shrimp pond water quality infected WFD.

| Parameter | Location | | | Optimum Range (SNI, 2014) |
|---|---|---|---|---|
| | Pond A | Pond B | Pond C | |
| pH | 8.385 | 8.375 | 7.76 | 7.5-8.5 |
| Alkalinity Total (mg/l) | | | | |
| $CO_3^{2-}$ | 0 | 0 | 0 | 100-150 |
| $HCO_3^-$ | 120 | 168 | 236 | |
| Salinity (ppt) | 31 | 32 | 21 | 10-32 |
| $NO_2$- (mg/l) | <0.001 | <0.001 | 8.12 | <1 |
| $NH_3$ (mg/l) | 0.068 | 2.790 | 3.62 | <0.1 |
| Organic matter total (mg/l) | 140.30 | 113.76 | 117.55 | <90 |

Plankton abundance calculation result on vannamei shrimp infected WFD ponds with *Green alga* Chloropyceae, Diatom, and Dinoflagellata dominated the ponds. Plankton identification assay resulted green algae chlorophyceae domination, namely genus *Oocystis* (97.6%), and diatom genus *Coscinodiscus* (81.54%), while blue green algae Cyanophyceae comprised 1.44-18.41% containing genus *Ocylatoria* and *Oocystis*. Blue green algae domination causes the water pond color become dark green or bluish green. Genus *Coscinodiscus* is diatom type found as plankton that dominated on the pond infected WFD (81.54%). The other pond infected WFD was also discovered blue green algae, dinoflagellata, and chlorophyceae composition in balanced level (30% respectively) (Table 2.)

Table 2. Plankton abundance in *L. vannamei* infected WFD pond.

| Plankton Cumulative Abundance | Result (%) | | |
|---|---|---|---|
| | Pond A | Pond B | Pond C |
| Cyanophyceae (BGA) | 1.44 | 18.41 | 31.65 |
| Dinoflagellata | None | None | 32.91 |
| Chloropyceae | 97.6 | None | 30.38 |
| Diatom | 0.82 | 81.54 | 5.06 |
| Zooplankton | 0.02 | 0.03 | None |

17 bacterial isolates were obtained from the isolation method. All isolates were tested using biochemical test, API 20NE, and confirmed with PCR with *V. harveyi* specific primer. All isolates obtained were bacteria grown on TCBS media. Most colonies grown on TCBS presented as 1-3 mm circular shape with convex elevation, green color (2 samples), yellow (13 samples), and dark green (2 samples). Biochemical test indicated that all isolates were Gram negative, rod-shaped, capable of growing at 30°C and 35°C bacteria with inability of fermenting lactose. All isolates were characterized as different *Vibrio* with other genus in Vibrionaceae family, which requires NaCl on the media (NaCl 1-10%), besides capable of producing positive oxidase and catalase, nitrate reduction, and glucose fermentation without producing gas. *Vibrio* is generally resistant against 150µg O129 and Y hemolytic bacteria on 5% SBA, however some isolates discovered were sensitive against 150µg O129 antibiotic and capable of lysing blood cells (α and β hemolysis). Two isolates showed biochemical test result similarities and were identified as *V. alginolyticus*/*V. harveyi* due to capable of swarming as the main characteristic of *V. alginolyticus*, nevertheless other tests pointed similarities with *V. harveyi* (Table 3), therefore advanced test using API 20NE kit and PCR were performed. Based on the characterization test performed, spesies discovered were *V. harveyi*, *V. alginolyticus*, *V. fluvialis*, *V. parahaemolyticus*, whereas two isolates were identified as *Shewanella putrefaciens*. This condition happened as incapable of fermenting glucose and sucrose, besides producing $H_2S$. This result was different from *Vibrio* characteristics as capable of fermenting glucose and sucrose without producing $H_2S$. Previous study conducted by Korun *et al.* (2009) succeded to identify *S. putrefaciens* on european seabass *Dicentrarchus labrax* in Turkey.

API 20 NE kit assay result the same identification discovery. Spesies discovered were V. *alginolyticus*, V. *vulnificus*, V. *cholerae*, V. *parahaemolyticus*, S. *putrefaciens* (Table 4). The analysis result of V. *cholerae* were unaccepted as the biochemical test showed that this isolate could only grow on the media containing NaCl until 10%. Based on these results, the advanced identification was performed using PCR (Fig. 4) with V. *harveyi* specific primer. *V. harveyi* specific primer was utilized to differ V. *alginolyticus*, V. *vulnificus* and V. *harveyi*, which having the biochemical morphology characteristic similarities based on the biochemical and API 20NE kit assay.

Table 3. Biochemical characteristics of bacteria isolated from WFD infected shrimp L. vannamei using biochemical test.

| Character | BL1U2 | BL1W1 | BL1W3 | KL2U1 | KL2F1 | KL2W1 | AL3H3 | AL3H6 | AL3U2 | AL3F1 | BL1H4 | KL2H1 | KL2H2 | BL1H3 | KL2W7 | KL2F2 | BL1F1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gram (KOH 3%) | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Katalase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Oxidase | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Motility | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| OF | F | F | F | F | F | F | F | F | F | F | F | F | F | F | F | - | - |
| TCBS | K | K | K | K | K | K | K | K | K | K | K | K | K | K | H | H | H |
| Slant/Stand TSIA | A/A | A/A | A/A | A/A | A/A | A/A | A/A | A/A | A/A | A/A | A/A | A/A | A/A | A/A | K/A | K/K | K/K |
| $H_2S$ production | - | - | - | - | - | - | (+) | - | - | - | (+) | - | - | - | - | + | + |
| Hemolysis, 5% SBA | γ | γ | γ | γ | γ | β | γ | γ | γ | γ | β | γ | γ | γ | γ | γ | γ |
| Methyl red | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | ND |
| Voges-Proskauer | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | ND |
| Simmons citrate | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | - | ND |
| Indole production | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| Lysine decarboxylase | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + |
| Arginine dihydrolase | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Ornithine decarboxylase | - | - | - | - | - | - | - | - | - | + | - | - | + | - | - | + | + |
| Urea hydrolysis | - | - | + | - | - | - | - | - | - | - | + | - | - | - | - | - | - |
| 10 µg O/129 sensitivity | ND | ND | ND | S | R | ND | ND | S | S | ND | S | S | ND | S | S | ND | R |
| 150 µg O/129 sensitivity | ND | ND | ND | S | ND | ND | ND | S | S | ND | S | S | ND | S | S | ND | S |
| Nitrate reduction | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ND |
| 0% NaCl | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| 1% NaCl | - | + | + | - | + | + | + | + | + | + | - | - | + | - | + | + | + |
| 6% NaCl | - | + | + | + | + | + | + | + | - | + | - | - | + | + | + | + | + |
| 8% NaCl | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + |
| 10% NaCl | + | - | - | + | - | - | + | + | - | + | + | + | + | - | + | - | + |
| Gelatine hydrolysis | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | ND |
| Esculin hydrolysis | - | + | + | + | + | + | - | (+) | - | - | - | - | + | - | + | - | - |
| Grown at 30°C | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Grown at 35°C | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| D-Glucose, acidic | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| D-Glucose, gas | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| L-Arabinose, acidic | - | - | - | - | - | - | - | - | - | + | - | - | ND | + | + | - | - |
| Cellobiose, acidic | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - |
| D-Galactose, acidic | + | + | + | + | + | + | + | + | + | - | + | + | + | - | + | - | - |
| Myoinositol | - | - | - | - | - | - | - | - | - | - | + | - | ND | - | - | - | - |
| Lactose, acidic | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| D-Mannitol, acidic | + | + | + | + | + | + | + | + | + | + | + | + | ND | + | + | - | - |
| n-Mannose, acidic | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + |
| Salicin, acidic | + | + | + | - | + | + | + | + | + | - | + | + | + | + | + | - | - |
| Sucrose, acidic | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - |
| Trehalose, acidic | + | + | + | + | + | + | + | + | + | + | + | ND | ND | + | + | + | - |
| Swarming | ND | ND | ND | + | ND | ND | ND | + | + | ND | + | ND | + | ND | ND | ND | ND |
| MacConkey agar | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Identifcation result | 1 | 1 | 1 | 1 | 1 | 1 | 1/2 | 1/2 | 1 | 2 | 2 | 2 | 2 | 3 | 4 | 5 | 5 |

Note:

1, *V. harveyi*; 2, *V. alginolyticus*; 3, *V. fluvialis*; 4, *V. parahaemolyticus*; 5, *S. putrefaciens*; +, positive; -, negative; F, Fermentative; K, Yellow; H, Green; A/A, Acidic / Acidic; γ, no hemolysis; α, alpha hemolysis; β, Beta hemolysis; R, Resistant; S, Sensitive; ND, No data presented

Table 4. Biochemical characteristics of bacteria isolated from WFD infected shrimp, L. vannamei using analytical profile index/ API 20NE (BioMeriuex).

| Isolate code | Bacteria | % | Identification result |
|---|---|---|---|
| BL1H3 | - | | Unacceptable profile |
| BL1H4 | V. *alginolyticus* | 3.8 | Good identification |
| BL1U2 | V. *vulnificus* | 99.8 | Very good identification |
| BL1F1 | S. *putrefaciens* | 99.8 | Good identification |
| BL1W1 | V. *alginolyticus* | 13.2 | Good identification to the genus |
| BL1W3 | V. *vulnificus* | 99.8 | Very good identification |
| KL2H1 | V. *vulnificus* | 99.6 | Very good identification |
| KL2H2 | V. *alginolyticus* | 99.9 | Excellent identification |
| KL2U1 | V. *cholerae* | 86.1 | Presumptive identification |
| KL2F1 | V. *vulnificus* | 99.6 | Very good identification |
| KL2F2 | S. *putrefaciens* | 99.9 | Excellent identification |
| KL2W1 | V. *alginolyticus* | 53.5 | Good identification to the genus |
| KL2W7 | V. *parahaemolyticus* | 8.2 | Low discrimination |
| AL3H3 | V. *alginolyticus* | 99.8 | Very good identification |
| AL3H6 | V. *vulnificus* | 99.8 | Very good identification |
| AL3U2 | V. *alginolyticus* | 99.9 | Excellent identification |
| AL3F1 | V. *alginolyticus* | 98.1 | Good identification |

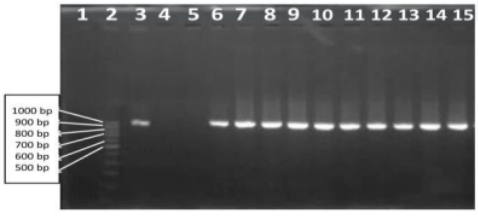


Figure 4. a

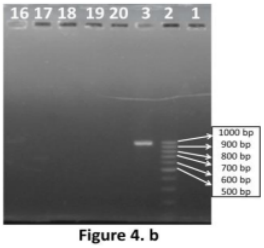


Figure 4. b

Figure 4. PCR result on 17 bacterial isolates. Line : 1, Negative control; 2, 100 bp marker; 3, positive control, 4, KL2W7 isolate; 5, BL1H3 isolate; 6, BL1U2 isolate; 7, BL1W1 isolate; 8, BL1W3 isolate; 9, KL2U1 isolate; 10, KL2F1 isolate; 11, KL2W1 isolate; 12, AL3H3 isolate; 13, AL3H6 isolate; 14, AL3U2 isolate; 15, AL3F1 ; 16,BL1H4 isolate; 17, BL1F1 isolate, 18, KL2H1 isolate; 19, KL2H2 isolate; 20, KL2F2 isolate.

Identification result using three methods (biochemical test, API 20NE, and PCR) showed an unknown isolate profile on API 20 NE that could not be identified. Isolates assay using biochemical test were identified as *V. fluvialis* and confirmed V. *harveyi* negative with PCR. This indicated that there was a kit limitation in reading and identifying the assay result. In addition, API 20NE test was unable to differ the isolate profile between *Vibrio* bacteria species, such as *V. alginolyticus*, V. *vulnificus* and *V. harveyi* which had similar biochemical characteristics, therefore V. *harveyi* assay using API 20NE kit requires additional parameters based on the biochemical test and genetic detection with PCR (Oakey *et al.*, 2003; Buller, 2004). *Vibrio* bacteria identification on species and strain level almost presented identical gene sequences in several loci. Thompson *et al.* (2007) pointed that atpA gene sequence utilization to construct phylogenetic tree showed *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* were on the same cluster with *V. champbelli* at Harveyi Clade.

Result showed that from 17 bacterial isolates identified, 10 isolates were *V. harveyi* (58.82%), three isolates were *V. alginolyticus* (17.65%), one *V. fluvialis* isolate (5.88%), one *V. parahaemolyticus* isolate (5.88%), and two non *Vibrio* isolates which were identified as *Shewanella putrefaciens* (11.76%). Based on target isolation of *Vibrio* bacteria, there were three isolates identified from intestine (all *V. harveyi*), six isolates from hepatopancreas (two isolates were *V. harveyi*, three isolates were *V. alginolyticus*, and *V. fluvialis* isolate), four isolates from feces (two isolates were *V. harveyi* and the other were non *Vibrio* species, which were S. *putrefaciens*), and four isolates were identified from water pond sample (three isolates were *V. harveyi* and *V. parahaemolyticus* isolate). Analysis result percentage of *Vibrio* bacteria isolated from every target sample is presented on Figure 5.

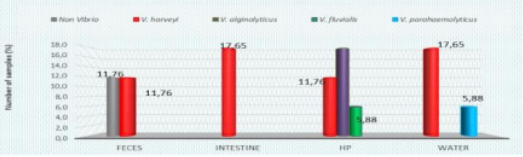


Figure 5. *Vibrio* species percentage isolated from targeted sample on vannamei shrimp infected WFD.

### *Antibiotic resistance*

*V. harveyi* bacteria that were isolated from three different culture location showed 100% Tetracyclin (Oxytetracyclin) resistance, 80% Fluoroquinolon (Enrofloxacin) resistance, and 100% Macrolide (Erythromycin) intermediate resistance. This showed that *V. harveyi* observed on WFD represented resistance level for more than one antibiotics (Figure 6.)

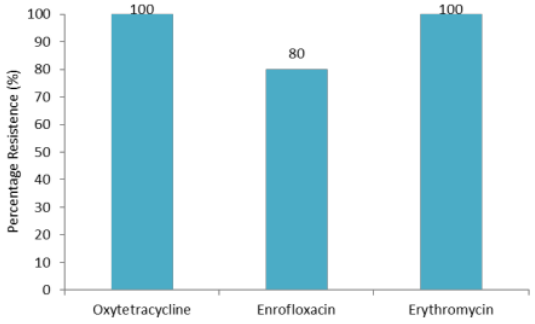


Figure 6. Antibiotic resistance of V. *harveyi* isolates.

## DISCUSSION

Sriurairatana *et al.* (2014) stated that WFD on vannamei shrimp would make the vannamei shrimp experience a decrease in Average Daily Gain (ADG) and varied weight level. High content of organic matter would improve the *Vibrio* density. Types of *Vibrio* bacteria is capable of proliferating quickly, as the organic material in the pond water increased (Kharisma & Manan, 2012). Malaroli and Gopalakrishnan, 2016 stated that the WFD occured in high level of salinity and alkalinity conditions on the brackishwater pond. *Vibrio* tended to like alkaline water, thus making the increased proliferation of *Vibrio* (Percival & Williams, 2014). Total *Vibrio* count (TVC) level on the brackishwater pond possessed with pathogenic *Vibrio* was ranged between 0 until 5 x $10^3$ CFU/ml, (SNI, 2014; Heenatigala & Fernando, 2016).

The plankton abundance is an overview of water quality control in aquaculture (Case *et al.*, 2008). The result of plankton abundance on one of the observed pond was dominated by Chloropyceae with 97.6% population. The presence of plankton was profitable in the brackwishwater culture pond, as it could be used as the natural shrimp feed (Boyd, 2014), however this would greatly harm the shrimp, as the phytoplankton was in excess populations that would become competitors for shrimp to use dissolved oxygen at night, leading to a drastic decreased of dissolved oxygen content (Rodriguez & Osuna, 2003). Other observed ponds were mostly dominated by diatoms and dinoflagellate genera. Dinoflagellate and diatom became the dangerous algal groups (harmful algal blooms/HABs) due to the blooming existence from those two species (*Coscinodiscus*, *Nitzschia*, *Rizosolenia*) (California Department of Public Health, 2014).

*Vibrio* bacteria found on vannamei shrimp infected WFD vannamei shrimp were *V. harveyi*, *V. alginolyticus*, *V. fluvialis*, *V. parahaemolyticus*, and non *Vibrio* bacteria genus (*S. putrefaciens*) with the predominance level was observed in *V. harveyi*. This was in accordance with (Inthusai, 2006; Limsuwan, 2010; Mastan, 2015), who mentioned that *Vibrio* bacteria were discovered in the vannamei and tiger shrimp (*Penaeus monodon*) infected WFD in Thailand and India. *Vibrio* species found were *V. vulnificus*, *V. fluvialis*,*V. parahaemolyticus*, *V. alginolyticus*, *V. cholerae* (non01), *V. mimicus*, *V. damselae*, and *Vibrio* sp., although *V. cholerae* species was not found in this research. According to (Somboon *et al.*, 2016), *V. cholerae* was found in vannamei shrimp infected WFD in Thailand, as well as Cao *et al.* (2015) also reported in China. There has been no publication of *V. harveyi* dominance level in WFD case. This could be caused due to the bacterial taxonomy in Harveyi Clade had high phenotype similarities (Austin & Austin, 1999; Oakey *et al.*, 2003; Buller, 2004). API 20NE test in this research could not distinguish *V. alginolyticus* with *V. harveyi* without additional biochemical tests (Oakey *et al.*, 2003; Buller, 2004). The result of this research on bacterial identification was in accordance with Hou *et al.* (2018), who also mentioned that bacteria found in shrimp intestine infected WFD in China were identified with a DNA sequencing method with 16S rRNA gene target, resulting *Vibrio* (5.89%) and *Shewanella* (1.17%) discovery.

Major *V. harveyi* isolated in this research had developed their ability to resist any antibiotics used. All *V. harveyi* isolates observed in this research were tested to determine the antibiotic activity using Phluoroquinolon (Enrophloxacin), Macrolide (Erithromycin), and Tetracyclin (Oxytetracyclin). Result showed that all *V. harveyi* isolated were resistant against Tetracyclin and Fluoroquinolon antibiotics, while showing intermediate resistant against Macrolide antibiotic. This result showed that these *V. harveyi* species were resistant to more than one antibiotics. Antibiotic resistance obtained on the bacteria w[13] generally distributed by extra plasmid chromosome and transmitted to the next

generation (vertical gene transfer), as well as being exchanged between different bacterial population (horizontal gene transfer) (Manjusha *et al*., 2005) thus making the resistance gene was distributed to the next generation bacteria.

## CONCLUSION AND RECOMMENDATION

### *Conclusion*

*Vibrio harveyi* was discovered from vannamei shrimp infected WFD in Situbondo, East Java. WFD was presented infecting vannamei shrimp during the poor water quality and planton abundance in the brackishwater pond samples.

### *Recommendation*

It is necessary to conduct a further research dealing with *V. harveyi* virulence gene detection to observe the pathogenicity capability and toxin or enzyme potential production which cause *V. harveyi* become pathogen as an effort to prevent WFD on the vannamei shrimp brackishwater pond.

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