

## BACTERIAL CONTAMINATION AT WHITELEG SHRIMP (*Litopenaeus vannamei*) IN AQUACULTURE

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**Abstract.** Indonesia has a very high biodiversity, which has later become one of the natural products of interest to the international community, including fishery products. One of the high-demand Indonesian fishery products is whiteleg shrimp *Litopenaeus vannamei*. However, safety food Exported whiteleg shrimp products must meet the criteria, including free from bacterial contamination such as *Escherichia coli*, *Salmonella*, *Vibrio cholerae*. This study attempted to analyze *E. coli*, *Salmonella*, *V. cholerae* contamination in 3 ponds in Bojo, Cilellang, and Palanro village in district Malusetasi, Barru Regency, South Sulawesi. Two samplings for each pond were conducted in the morning were pond water and fresh whiteleg shrimp. The *E. coli* test showed positive in Cilellang village (sample A) with 11 MPN/g, negative in Palanro village (sample B) and in Bojo village (sample C) with the value of <2 MPN/g. *Escherichia coli* test showed positive in sample D (Vannamei shrimp in Cilellang village) and sample E (Vannamei shrimp in Palanro village) with 2.0 MPN/g, 17 MPN/g, respectively. Only sample F (Vannamei shrimp in Bojo village) showed a negative result. As for the *Salmonella* test, positive results showed in sample A, while sample B and sample C showed negative results. The *Vibrio cholerae* test showed negative at all samples. This study concludes that Whiteleg shrimp from ponds in Mallusetasi district is classified as safe for consumption.

**Keywords:** bacterial, contamination, *Litopenaeus vannamei* shrimp, pathogenic, Sulawesi

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### INTRODUCTION

The demand of abroad consumers for Indonesian fishery products, especially shrimp in fresh and processed conditions increases. Some of Indonesia's shrimp export destinations, such as Japan, America, and Europe, have required strict quality requirements (Pramoda et al., 2017). Globalization of world trade places food security and quality

assurance as a prerequisite as the upgrading of production technology, handling and distribution of food, and awareness of the importance of safe and food quality (Panghal et al., 2018).

Microbiological conditions are one of the supporting parameters for the success of seawater (ponds) and freshwater cultivation (Sohel & Ullah, 2012; Rahman et al., 2013). The group of coli bacteria and pathogenic bacteria are used as indicators of marine

and terrestrial aquaculture (Sumampouw & Risjani, 2014; Mathew et al., 2019). The imported shrimp products must be guaranteed the requirement of safe food as follow: (a) free of heavy metals, (b) fresh and free of H<sub>2</sub>S, blackspot, and indole, (c) clean, free of bacterial contaminants (d) free of residue and antibiotics (Mathew et al., 2019).

Water ponds, water processing, and ice used in the production process are among the causes of shrimp contamination to pathogenic bacteria, besides sanitation and hygiene factors (Sohel & Ullah, 2012). Groups of microorganisms commonly used as indicators of water pollutants and have higher endurance than other pathogenic bacteria, also more easily isolated and grown are groups of coli bacteria (Sumampouw & Risjani, 2014; Mathew et al., 2019).

Inadequate water quality in shrimp farming activities causes massive seed loss and decline in post-harvest quality, indicated by the presence of pathogenic bacteria with high density (Armenta-Bojórquez et al., 2021). The regulation should take into account, *E. coli* bacteria found in density exceeded the threshold determined by the Ministry of Environment based on Government Regulation SNI 2728-2018 is 1000/100 ml (SNI, 2018). In the event of contamination, the number of *E. coli* bacteria becomes numerous and exceeds other pathogenic bacteria. These bacteria indicate the presence of pathogens in water other than viruses, protozoa, and parasites (De Brauwere et al., 2014; Mathew et al., 2019). WHO recommends three groups of marine and terrestrial pollution indicator bacteria, namely fecal coliform, fecal streptococcus, and pathogens (*Salmonella* sp, *Vibrio* sp, *E. coli*) (Sutiknowati, 2014).

One of Indonesia's shrimp-producing areas is Mallusetasi district located in Barru Regency, South Sulawesi. One of the shrimp

producers for export production. However, there is fear of harvest failure considering the last few years' drastic loss by mass deaths that occurred. Based on information from the farmers in Mallusetasi district, the marine waters of Makassar Strait are polluted by chemicals from the cultivation of pearl shells influencing shrimp aquaculture yield in Mallusetasi district. Other information from community leaders and direct observation in Mallusetasi district indicates that residents or communities of this coastal area do not have a permanent septic tank system, they only make a rectangular chamber for fecal stools in the house of each family. Besides, many people do not have a fixed stove (dispose of any place), and there are even coastal communities that make booths and floorboards by using poles and buffers above seawater so that the stool directly fell into the seawater.

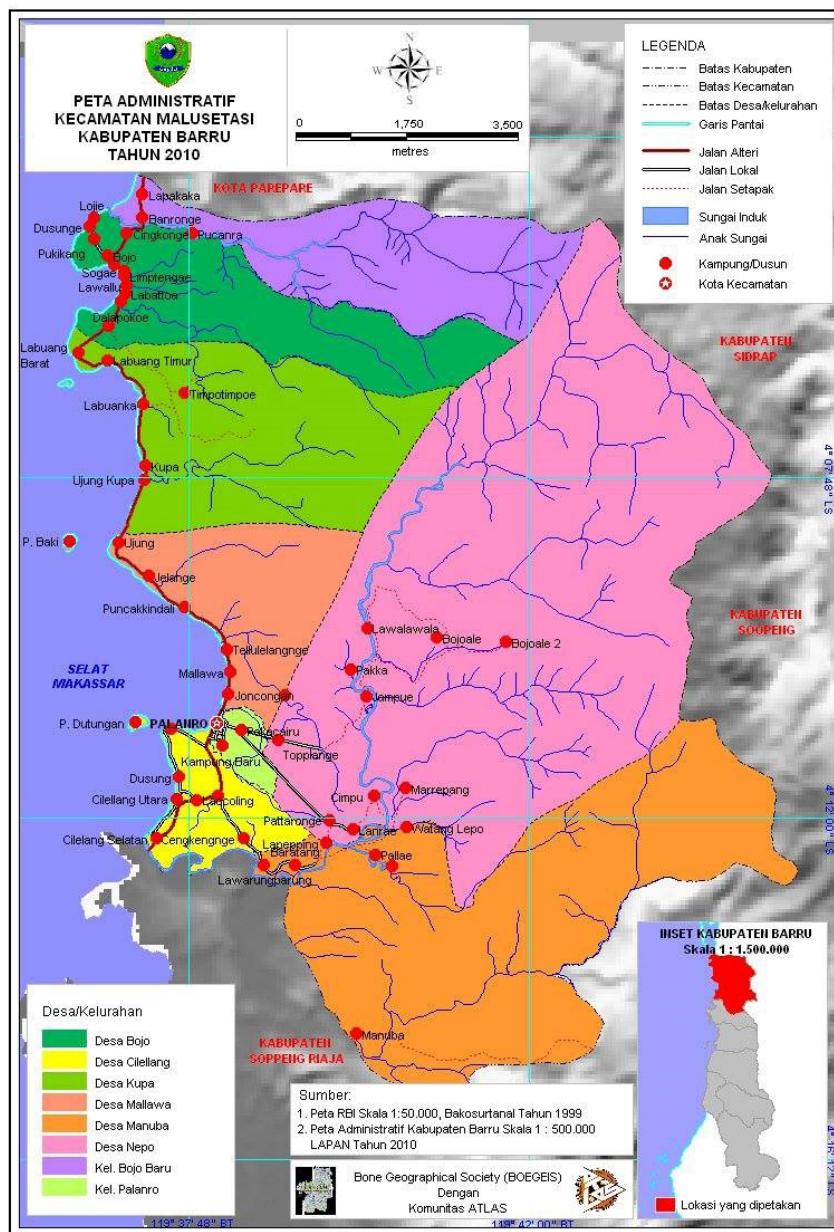
The coastal areas suffer from environmental degradation, which caused a decrease in shrimp cultivation (Rahman et al., 2013). The numerous contamination source encourages researchers to explore it, including this research, which tried to test *E. coli*, *Salmonella*, and *V. cholerae* contamination based on SNI 2728-2018, in 3 ponds in Bojo, Cilellang, and Palanro village in district Malusetasi, Barru Regency, South Sulawesi. The ponds in these 3 villages are far from one another but located very close to the coast and residential areas. The 3 ponds also have different pH and salinity, the Cilellang village pond has a pH of 7, 5-8, and 25-30% salinity, the Palanro village pond has a pH of 7-8 with a salinity of 20-25% while the Bojo village pond has a pH of 7-8 with a salinity of 30%.

## MATERIALS AND METHODS

Two samples which are fresh whiteleg shrimp and water ponds were taken using

nets in the morning in each village (Cillellang, Palanro, and Bojo) (Figure 1). All testing methods were carried out based on SNI 2728-2018 combined with Standard of Procedure

in The Makassar Fishery Product Quality Testing and Development Center (Balai Pembinaan dan Pengujian Mutu Hasil Perikanan (BPPMPH)) Makassar.



**Description:**

- Yellow : Cillellang Village
- Light Green : Palanro Village
- Dark Green : Bojo Village

Figure 1. Map of the sampling point

### Sample Preparation

Twenty-five (25) g samples of fresh whiteleg shrimp were prepared from the abdomen and the head of the shrimp, and put in a sterile icebox container. 225 ml of Butterfield's Phosphate Buffered solution were added and homogenized for 2 minutes. This homogenate was a solution with a dilution of  $10^{-1}$ . The Test was carried out right after collecting samples.

### *Escherichia coli* Test

#### Coliform Estimation Test

Dilution  $10^{-2}$  was prepared by dissolving 1 ml of  $10^{-1}$  solution into 9 ml of Butterfield's Phosphate Buffered. 1 ml of dilution of each dilution were then transferred by using a sterile pipette, into 3 series of lauryl tryptose broth (LTB) containing the Durham tube. The tubes were incubated for 48 hours at  $35^{\circ}\text{C}$ . The gas formed after 24 hours of incubation, and the negative tubes were reincubated for another 24 hours. The positive tube is marked by turbidity and gas in the Durham tube.

#### Coliform Affirmation Test

The positive LTB tubes were inoculated to the Brilliant Green Lactose Bile (BGLB) Broth tubes containing the Durham tube using the inoculation loop. The inoculated BGLB Broth tubes were incubated for 48 hours at  $35^{\circ}\text{C}$ . The BGLB Broth tubes that produce gas for 48 hours at  $35^{\circ}\text{C}$  were checked. The positive tube is characterized by turbidity and gas in the tube Durham. The most probable number (MPN) value was determined based on the number of positive BGLB Broth tubes. The value was expressed as "*MPN/g coliform*".

#### *Escherichia coli* Predicted Test

Each positive LTB tube was inoculated to the *E. coli* (EC) Broth tubes containing the Durham tube using the inoculation loop. EC Broth tubes were incubated in the water bath

for 48 hours at  $45^{\circ}\text{C}$ . The EC Broth tubes that generate gas for 24 hours were checked. If negative, the tubes were incubated again for up to 48 hours. The positive tube is characterized by turbidity and gas in the Durham tube. Water bath must be clean, water in it must be higher than the liquid's height in the incubated tube. MPN was determined based on the number of positive EC Broth. The value was denoted as "*MPN/g fecal coliform*".

#### *Escherichia coli* Affirmation Test

Samples from positive EC Broth tubes were inoculated by using a scratching inoculation loop to Levine's Eosin Methylene Blue (LEMB) agar and incubated for 24 hours at  $35^{\circ}\text{C}$ . The suspected colony of *E. coli* giving a distinctive feature of black on the middle with or without metallic green. More than one colony of *E. coli* in each LEMB agar plate was then scraped onto Plate Count Agar (PCA) media using the needle planting and incubated for 24 hours at  $35^{\circ}\text{C}$ .

#### Morphological Test

Unspecified (typical) colonies of *E. coli* were transferred to a tilted PCA medium.

#### Biochemical Test

Biochemical Tests include Indole, Methyl Red (MR), Voges Proskauer (VP), Citrate Test, and gas production.

Indole test was carried out to determine the ability of the bacteria to convert tryptophan into indole. Bacterial culture was made by taking 1 loop bacteria from PCA into the tryptone broth and incubating it for 24 hours at  $35^{\circ}\text{C}$ . The indole test was carried out by adding 0.2 ml–0.3 ml of Kovacs reagent into the culture, positive result was determined from the solution turns from yellow to cherry red

Voges proskauer test was carried out to determine if an organism produces acetyl-methyl carbinol from glucose fermentation. First, bacterial culture was made by inoculating 1 loop of inoculant from PCA into the



MRVP Broth and incubated for 48 hours at 35°C. 1 ml of each growing MRVP Broth was transferred to a sterile 13 mm x 100 mm test tube, 0.6 ml of alpha naphthol solution and 0.2 ml 40% KOH were then added. The mixture was shaken and added a small amount of creatine crystals to speed up the reaction. the mixture was then let stand for 2 hours. A pink-red color at the surface of the tube was considered to be a positive result. Methyl red test was carried out to detects the production of acid by bacteria during the fermentation of glucose. the MRVP Broth above was re-incubated for 48 hours at 35°C and 5 drops of Methyl red indicator were added to the MRVP Broth. A positive result was shown by a change in the color of the methyl red indicator from yellow to red. Citrate test was carried out to differentiate among the Gram-Negative Scratched 1 loop of PCA slanted onto the surface of simmon's citrate agar. Incubated for 96 hours at 35°C. Positive result showed by a color change from green to blue along the slant of media. Gas production in selective lactose media was also carried out. Bacterial culture was inoculated into LTB, and was incubated for 48 hours at 35°C (Standard of Procedure, 2018).

### **Salmonella Test**

#### **Isolation of *Salmonella***

The samples which previously inoculated Tetrathionate Broth (TTB) were incubated into Hectoen Enteric (HE), Xylose Lysine Desoxycholate (XLD), and Bismuth sulfite Agar (BSA) media for 24 hours at 35°C.

#### **The Urease Tests**

The urease test was carried out to determine the ability of an organism to split urea. 1 ose culture from each positive Triple Sugar Iron (TSI) presumptive test was moved into Rapid Urea Broth and incubated for 2 hours in the water bath at 37°C.

#### **Urease Negative Culture Test LDB (Lysine Decarboxylase Broth)**

One loop from TSI were transferred into LDB medium. The lid was loosened and incubated for 48 hours at 35°C, but it was observed after 24 hours. *Salmonella* gives an alkaline reaction characterized by a purple color on all media.

#### **Phenol Red Dulcitol or Purple Broth Base with 0.5% Dulcitol**

One loop from TSI was moved into dulcitol Broth medium. The lid was loosened and incubated for 48 hours at 35°C, but it was observed 24 hours. *Salmonella* gives positive results, characterized by gas formation in the Durham tube and acid pH (yellow) on media.

#### **Polyvalent Somatic (O) Serological Test**

One loop culture of TSI was taken that has been incubated for 24–48 hours, and it was placed on top of the preparatory glass, then 0.85% saline solution sterile was dropped and emulsified. 1 drop *Salmonella* Polyvalent Somatic (O) Antiserum was laid in addition to colony suspension. Colonies of Antiserum were Mixed gradually with colony suspension until well blended. The control was conducted using saline and Antiserum solution. The mixture was tilted to the left and right, and it was observed immediately on the dark background rear.

#### **Additional Biochemical Tests**

The additional biochemical Tests were: Phenol red lactose or purple Lactose Broth test, Phenol red sucrose or purple sucrose Broth test, Methyl Red – Voges-Proskauer (MR – VP) Broth test, and Simmons citrate Agar (Standard of Procedure, 2018).

#### ***Vibrio cholerae* Test**

##### **Isolation of *Vibrio cholerae* Test**

Without shaking the tube, 1 loop culture of each positive tube was taken at each dilution as much as 1 cm from the liquid surface,

and it was then scratched into the Thiosulfate-Citrate-BileSalts-Sucrose (TCBS) agar. TCBS agar was incubated at 36°C for 18–24 hours. The presence of *V. cholerae* on TCBS agar was observed. Colonies of *V. cholerae* are large, smooth surface, somewhat flat, the center is opaque, and the edges are bright, yellow (positive sucrose).

**Purification**

Three unexpected colonies or more were taken from each TCBS agar, and were incubated into T1N1 agar or TSA + 1.5% NaCl (total containing 2% NaCl) for 18–24 hours at 36°C ± 1°C.

**Preliminary Biochemical Test**

Preliminary biochemical tests include oxidase test, sensitivity to 0/129 vibriostat Test, TSI Agar and Kligger Iron Agar (KIA), ortho-nitrophenol test for beta-galactosidase production (ONPG) test, Oxidative fermentative test, and Gram staining.

**Advance Biochemical Test**

Advance biochemical Tests include urea hydrolysis, Arginine dihydrolase test, Lysine decarboxylase test, ornithine decarboxylase Test, salt-tolerant test, growth on 42°C Test, VP test, carbohydrate fermentation tests, serology test (Standard, 2018).

**RESULTS AND DISCUSSION**

Based on SNI 2728-2018 (SNI, 2018) about food quality and safety requirements for fresh shrimp, the presence of *E. coli* in fresh shrimp must be less than 2 MPN/g, while the presence of *Salmonella* and *Vibrio cholerae* must be negative/25 g. The result of pathogenic bacteria in pond water presented in Table 1.

Table 1. Pathogenic bacteria in pond water

| Test               | Unit     | Sample              |                       |          |
|--------------------|----------|---------------------|-----------------------|----------|
|                    |          | A                   | B                     | C        |
| <i>E. coli</i>     | MPN/g    | 11*                 | <2**                  | <2**     |
| <i>Salmonella</i>  | Per 25 g | Positive            | Negative              | Negative |
| <i>V. cholerae</i> | Per 25 g | Negative            | Negative              | Negative |
| SPC                | Kol/25 g | 1.6x10 <sup>4</sup> | 1.6 x 10 <sup>5</sup> | TNTC     |

Description:

- A : water pond of Cilellang village
- B : water pond of Palanro village
- C : water pond of Bojo village
- \* : Positive
- \*\* : Negative
- TNTC : too numerous to count

The analysis of some pond water samples in several villages in district Malusetasi, Barru Regency, South Sulawesi showed positive results that exceeded the maximum standard (table 1). Contaminated pond water is caused by the distance of ponds with adjacent residents' settlement, while some people lack awareness of environmental hygiene.

The results of the analysis of *E. coli* parameter test with a maximum threshold of microbial contamination on fresh shrimp based on SNI 2728-2018 (SNI, 2018), i.e., <2MPN / g, it can be seen that sample B (pond water of Palanro), sample C (pond water Bojo village), and sample F (shrimp Vannamei from Bojo village) have the same result of <2 MPN

/ g shows the negative result of *E. coli*. While sample A (water pond Cilellang village), sample D (shrimp Vannamei from Cilellang village), and sample E (shrimp Vannamei from

Palanro village) gave a result of 11 MPN / g, 2.0 MPN / g, and 17 MPN / g, respectively, meaning the samples are positive of *E. coli* (Table 1 & 2).

Table 2. Pathogenic bacteria on shrimp

| Test               | Unit      | Sample   |          |          |
|--------------------|-----------|----------|----------|----------|
|                    |           | D        | E        | F        |
| <i>E. coli</i>     | MPN/g     | 2,0*     | 17**     | <2**     |
| <i>Salmonella</i>  | Per 25 gr | Negative | Negative | Negative |
| <i>V. cholerae</i> | Per 25 gr | Negative | Negative | Negative |
| <i>SPC</i>         | Kol/25 gr | -        | -        | -        |

Description:

D : Vannamei Shrimp of Cilellang village

E : Vannamei Shrimp of Palanro village

F : Vannamei Shrimp of Bojo village

\* : Positive

\*\* : Negative

These results show the discontinuity especially between sample B and sample E, in which samples of shrimp contaminated with *E. coli* while the water sample negative *E. coli*. The possibility of these results because shrimp are contaminated with *E. coli* from pond sediment. *E. coli* bacteria can also come from mud or pond bottom sediment. According to Stocker et al. (2018) and Martin-Diaz et al. (2020), sedimentary particles from pond bottom attached to shrimp pleopods can be a source of coliform and *E. coli* contamination, while the discovery of *E. coli* in the water is possible because the pond water is sourced from seawater polluted by household wastes such as human feces and foodstuffs from the residential community (Stocker et al., 2018; Martín-Díaz et al., 2020). Chemical compounds, microorganisms, and hazardous physical contamination found in fishery products, caused by the environment where fish live, including the location of cultivation (Mathew et al., 2019). The ponds in these 3 villages are distant from one another but are found exceptionally near to the coast and residential areas. Residents of this coast-

al zone do not have a permanent septic tank framework, and as a replacement, they make a rectangular chamber with a hole in the bottom for fecal stools in the house of each family, the stool directly fell within the seawater.

From the analysis results, only sample A (water pond Cilellang village) showed positive results of *Salmonella* (Table 1) because it exceeds the maximum threshold of microbial contamination on fresh shrimp based on SNI 2728-2018 (SNI, 2018), i.e., negative/25 g. However, the test result in sample D (shrimp Cilellang village) shows this negative result because *Salmonella* bacteria do not contaminate shrimp sampling in the pond water (Table 2).

*Salmonella* contamination on shrimp causes the decrease of shrimp quality organoleptically, so consumers do not accept it. To reduce *Salmonella* contamination, *Salmonella* exposure should be decreased, which generally done by the use of antibiotics and synthetic preservatives by farmers and the shrimp processing industry (Done et al., 2015; Nair et al., 2018). Use of antibiotics in shrimp during cultivation and processing is prohibited by

Indonesia and export destination countries, thus detectable antibiotic components in shrimp export will be rejected (Thornber et al., 2020). The use of antibiotics at inappropriate doses, causing antibiotic resistance (Haddadin et al., 2019; Thornber et al., 2020), may also negatively affect food products consumed, reactivate sensitivity, or physiological disorders in humans (Lulijwa et al., 2020).

*E. coli* and *Salmonella* bacteria usually originate in animals and humans. If these types of bacteria are found in the waters, this indicates pollution occurs in the waters, so this bacteria can be used as an indicator of contamination (Frick et al., 2020; Wen et al., 2020). Cho et al. (2020) and Wen et al. (2020) stated *E. coli* and *Salmonella* are the indicator organisms of fecal contamination that are not derived from the aquatic environment (Cho et al., 2020; Wen et al., 2020).

According to Devane et al. (2020), *E. coli* and *Salmonella* are microorganisms indicating the occurrence of pollution that is not Indigenous in the aquatic environment (Devane et al., 2020). Besides, *E. coli* and *Salmonella* contamination in shrimp can become indications of low sanitation and hygiene conditions, this is supported by Hamilton et al. (2018), which says that the presence of harmful bacteria in food is an indicator of poor sanitation on handling and processing (Hamilton et al., 2018), and the contamination may originate from humans and pets (Cho et al., 2020).

From the result of the analysis on the shrimp sample of *V. cholerae* parameter test, it revealed that all samples showed the negative result of *V. cholerae* based on SNI 2728-2018 (SNI, 2018) (Table 2). The lack of pathogenic bacteria found in shrimp in ponds due to the treatment of probiotics in shrimp (Mathew et al., 2019). Following Knipe et al. (2021), the addition of probiotic bacteria to shrimp main-

tenance containers can serve as complementary sources of feed or contribution to the digestive system food and suppress the population of pathogenic bacteria (Knipe et al., 2021). Fish Farmers from 3 villages were used probiotic treatment in their ponds.

Transmission sources for *Vibrio* come from Food derived from fisheries, as one of the most common transmission sources (Mutiara et al., 2019; Helmi et al., 2020). Water with a high salt substance such as seawater may be a common living place of *Vibrio* sp that cause defilement (Mutiara et al., 2019), apart from some other factors such as temperature, hygiene, and concentration of Food that also affects the transmission (Baker-Austin et al., 2018). Taken together, it can be concluded that the whiteleg shrimp within the ponds in Mallusetasi district, Barru Regency, South Sulawesi considered secure for utilization based on SNI 2728-2018 with *E. coli*, *Salmonella*, and *V. cholerae* test.

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