Antimicrobial susceptibility and minimum inhibition concentration of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* isolated from a white shrimp (*Litopenaeus vannamei*) pond

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Abstract. Shrimp infection by *Vibrio* spp. has led to loss of production. To control this bacterial epidemic, people have applied antibiotics. Uncontrolled antibiotic treatments have led to *Vibrio* spp. pathogenic-resistance. This study aimed to determine the antimicrobial susceptibility and minimum inhibition concentration (MIC) of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* towards certain antibiotics. The trials used 10 and 100 ppm concentrations of 10 antibiotics: Chloramphenicol, Gentamicin, Amoxicillin, Co-Amoxiclay, Ciprofloxacin HCL, Azithromycin, Doxycycline, Tetracyclin, Erythromycin stearate and Ampicillin. Paper disc diffusion followed standard methods with incubation for 24 hrs. The inhibition zone was then measured. The results showed that at 100 ppm all antibiotics had activity toward all three *Vibrio* spp. (100 ppm). At 10 ppm, Amoxicillin and Ampicillin did not produce an inhibition zone in the *V. parahaemolyticus* culture. Antibacterial activity at 100 ppm resulted in inhibition zone diameters ranging from 6.93±0.34 mm (Ampicillin/*V. parahaemolyticus*) to 31.85±2.5 mm (Gentamicin/*V. harveyi*). The reduction in bacterial activity ranged from 3.89% (Doxycycline/*V. harveyi*) to 75.30% (Gentamicin/*V. parahaemolyticus*). The MIC was determined for six antibiotics. The lowest MIC was ≤0.625 ppm (Gentamicin/*Vibrio* spp.), and the highest was 10 ppm (Doxycycline/*V. vulnificus*, *V. harveyi* and Ampicillin/*V. vulnificus*). This research revealed that some antibiotics, especially Amoxicillin and Ampicillin, were ineffective against Vibriosis, conforming that the *Vibrio* spp. isolates used in this research were resistant to these antibiotics.

1. Introduction

[1] reported that shrimp as Indonesian main commodity had been produced 547,934 metric tonnes, year⁻¹ and 67% from pond culture. Globally, it has been recorded that shrimp, either frozen or processed product have an important role in market demand [2]. It has been noted, that volume and export value of Indonesian shrimp product has been increased. In 2002-2011 was 124,765 tonnes (836,563 USD), in 2011 was increased up to 158,062 tonnes (1,309,674 USD) [3]. Despite of the success on production, in fact, serious problem in disease, is still present particularly Vibriosis. In some countries, shrimp production was diminished by 75% in two years [4].
Vibriosis affected by 14 species of Vibrio, which are: *Vibrio harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. cincinnatiensis*, *V. fluvialis*, *V. furnissii*, *V. methcnikovii*, *V. vulnificus*, *V. ordalii*, *V. carchariae*, *V. azureus*, *V. mimicus*, *V. anguillarum* dan *V. damsela* [5,6]. The clinical signs of Vibriosis are internal organ liquefaction, appendages and skin necrosis, weariness, slow growth, body malformation, muscle opacity, blindness and mortality [7].

Since 35 years ago, [8] has reported the distribution of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in Jakarta, Indonesia. Another researchers in Kendal, Central Java has denoted five Vibrio isolates, namely *Vibrio vulnificus*, *V. mimicus*, *V. damsella*, *V. parahaemolyticus* dan *V. fluvialis* [9]. Moreover, in 2017 [10] informed the presence of three Vibrio species in *Litopenaeus vannamei* which are *V. parahaemolyticus*, *V. harveyi* dan *V. alginolyticus*. While [11] described two Vibrio species (*Vibrio alginolyticus* dan *V. harveyi*) from the same species. [12,13] informed that imported Indonesian seafood have also been contaminated by *Vibrio parahaemolyticus* and its’ virulence strain. This need several effort to counteract this problem such as preventing from infection, boosting the host immune system with natural products or probiotics, controlling the expanse of infection as well as vaccine/vaccination [7].

In fact, up to now, people is still using antiobiotics to control the bacterial infection due to the low cost and simple technique of application. It has been examined that Chloramphenicol, Erythromycin, Gentamycin and Oxytetracycline were still effective to be a therapeutic agent of *Vibrio alginolyticus*, *V. vulnificus*, *V. fluvialis*, *V. pelagius* dan *V. anguillarum* [14]. *V. parahaemolyticus* was also still sensitive to Azitromicin and Chloramphenicol [15]. The unwise application of antibacerial agent in shrimp culture leads to create the susceptible and antibiotic resistance gene of bacteria. [16] have been reported that Vibrio possess gen resistance to β-lactamase, Tetracycline. In addition, chloramphenicol (*catA2*) and kanamycin (*aphA-3*) was reported by [17]. This present research is aimed to determine the antimicrobial susceptibility and minimum inhibition concentration (MIC) of pathogenic *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* from *L. vannamei* shrimp pond towards 10 types of antibiotics.

2. Materials and methods

2.1. Bacterial isolates

*Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* have taken from the collection of Marine Tropical Biotechnology Laboratory, Department of Marine Science, Diponegoro University. This *Vibrio* spp. was isolated directly from *L. vannamei* pond culture.

2.2. Antimicrobial susceptibility

Bacterial susceptibility to antibiotics were determined by Kirby-Bauer disc diffusion test with Whatman no. 3 paper disc (diameter 6 mm) [18]. Ten type of different commercial antibiotics were applied for the test (Chloramphenicol, Gentamicin, Amoxicillin, Co-Amoxiclav, Ciprofloxacin HCL, Azithromycin, Doxycycline, Tetracyclin, Erythomycin strearate and Ampicilin). Antibiotics were prepared in the liquid at concentration of 100 ppm and standard concentration. The culture medium was using Nutrient Broth (Merck, USA) and test medium was using Nutrient Agar (Merck, USA). Bacteria were cultured at liquid media for 18 hrs at 0.5 McFarland [19].
*Vibrio* spp. have grown in liquid media was swabbed using sterile cotton to be cultured in nutrient agar in the petridish. Paper disk was immersed into antibiotics and placed into sterile petridish. Leave to dry at room temperature. This then followed by dropped the paper disc into bacterial cultured petridish. These all were repeated in three times. The petridishes were then incubated for 24 hrs (38°C). The appearance of inhibition zone was measured by digital callipers. A shown in Table 1, performance of inhibition zone are categorized by resistance, intermediate and and sensitive based on Clinical and Laboratory Standards Institute m45 [20]. On the other hand, Doxycycline and Azithromycin was interpreted to Enterobacteria ceae based on CLSI m100 [21] and Erythromycin Strearate based on Erythromycin [18].

**Table 1. Interpretive Criteria for Disk Diffusion Susceptibility Testing *Vibrio* spp.**

| Antibiotics                  | Zone Diameter (mm) | Interpretive Criteria |
|------------------------------|--------------------|-----------------------|
|                              | resistance | Intermediate | sensitive |
| Chloramphenicol (30ppm)      | ≤ 12       | 13–17        | ≥ 18       |
| Gentamicin (10ppm)           | ≤ 12       | 13–14        | ≥ 15       |
| Co-Amoxiclav (10ppm)         | ≤ 13       | 14–17        | ≥ 18       |
| Ciprofloxacin HCL (5ppm)     | ≤ 15       | 16–20        | ≥ 21       |
| Ampicilin (10ppm)            | ≤ 13       | 14–16        | ≥ 17       |
| Tetracyclin (30ppm)          | ≤ 11       | 12–14        | ≥ 15       |
| Azithromycin (15ppm)         | ≤ 12       | –            | ≥ 13       |
| Doxycycline (30ppm)          | ≤ 10       | 11–13        | ≥ 14       |
| Erythromycin (15ppm)         | ≤ 13       | 14–17        | ≥ 18       |

2.3. *Minimum inhibitory concentration (MIC)*

Minimum inhibitory concentrations (MIC) was assessed to selected eight type of antibiotics, using 96-wells microplate and nutrient broth. The assessment were using six doubled concentration, start from 20 ppm to 0.625 ppm. Antibiotics were diluted at concentration of 64 ppm, followed by pipetting 50 μl and put into wells. This was done in triplicates. Additionally, the 50 μl cultures bacterial (1 x 10⁸) were than added, so, therefore, the concentration of antibiotics were 32 ppm. The microplate was then incubated in 15 hrs (35 °C). 30 μl Resazurin (0,18%) was then added and followed by two hrs incubation. Discolorization of resazurin from purple into pink was recorded as negative results. Blue and/or purple colour indicated positive results [22,19,23]. MIC was chategorized as Resistant, Intermediate and Sensitive based on Clinical and Laboratory Standards Institute m45 [20]. Azithromycin was interpreted from Enterobacteriaceae referred to CLSI m100 [21].

**Table 2. Interpretive Criteria for Broth Microdilution Testing *Vibrio* spp.**

| Antibiotics                  | MIC (ppm) | Interpretive Criteria |
|------------------------------|-----------|-----------------------|
|                              | Resistant | Intermediate | Sensitive |
| Chloramphenicol              | ≥ 32      | 16         | ≤ 8       |
| Gentamicin                   | ≥ 16      | 8          | ≤ 4       |
| Co-Amoxiclav                 | ≥ 16      | 8          | ≤ 4       |
| Ciprofloxacin HCL            | ≥ 4       | 2          | ≤ 1       |
| Ampicilin                    | ≥ 32      | 16         | ≤ 8       |
| Tetracyclin                  | ≥ 16      | 8          | ≤ 4       |
| Azithromycin                 | ≥ 32      | –          | ≤ 16      |
| Doxycycline                  | 16        | 8 ≥        | ≤ 4       |
3. Results and Discussion

3.1. Antimicrobial susceptibility

It has been reported that 10 antibiotics used in this research were used to treated the Vibrio spp. infection, including Chloramphenicol, Gentamicin, Co-Amoxiclav, Ciprofloxacin HCL, Ampicilin, Doxycycline, Tetracyclin [24], as well as Penicilin (Amoxicilin), Erythomycin strearate and Azithromycin [25].

Based on Table 3, it is shown that there is susceptibility of Vibrio parahaemolyticus, Vibrio vulnificus and Vibrio harveyi towards 10 types of antibiotics. At 100 ppm concentration, all antibiotics show the antibacterial activity. The highest inhibition zone was exhibited by Vibrio parahaemolyticus (30.17±1.76), Vibrio vulnificus (30.65±1.65) dan Vibrio harveyi to Gentamicin and the lowest inhibition zone was from amoxicillin. Another results was also shown the diverse inhibition zone 6.93±0.34 (Ampicilin/V. parahaemolyticus) -31.85±2.5 (Gentamicin/V. harveyi).

It has been noticed that Vibrio parahaemolyticus, Vibrio vulnificus [26] and Vibrio harveyi [27] as the main caused of Vibriosis. Seven out to 10 antibiotics in this present research have been recommended by CLSI to counteract disease caused by Vibrio spp. Chloramphenicol, Gentamicin, Co-Amoxiclav, Ciprofloxacin HCL, Tetracyclin, Azithromycin, Doxycycline dan Ampicilin [15, 20, 28,29]. Similar to this present research, Ampicilin, apparently were not able to combat Vibrio sp. Similar to previous research reported by [30-33].

Based on Table 4, the tested antibiotics resulting several categories. Results from V. parahaemolyticus and V. harveyi show the resistant of Ampicilin, Ciprofloxacin HCL, Co-Amoxiclav, Erythomycin strearate, while intermediate categoris referred to Tetracyclin, while the sensitive categories referred to Doxycycline, Kloramfenikol, Gentamicin and Azithromycin, respectively. Other similar class of Ampicillin (B-lactam) such as Amoxicillin, has already resistance. This is approving the earlier data from some researchers [29,30,34-37]. Analogous to Co-Amoxiclav, Vibri sp. in this study tend to be categorized as resistant, instead of moderate, in agreement to the study of [38-40]. Trial on Short Mackerels showed the effectiveness of V. parahaemolyticus was still high (91.04%) [29]. Nevertheless, some trials were still cited that Vibrio parahaemolyticus, Vibrio vulnificus dari ready-to-eat shrimp were still sensitive (42-100%) to Co-Amoxiclav [41]. Moreover, according to Co-Amoxiclav, Ciprofloxacin HCL exhibit the similar results to the study. On diffusion disk test, Erythromycyn in this study was also sensitive to three Vibrio spp., analogous to research by [32,42,43].

Analogous to this research, Gentamicin and Chloramphenicol have been reported has the high sensitivity to three species of Vibrio as examined by [34-36,41,42,44]. Antibiotics from Tetracyclines class such as Tetracyclin and Doxycycline have a comparable results towards three Vibrio spp. which are Intermediate-Sensitive, except to V. parahaemolyticus. This study indicates that those two antibiotics were still effective to counteract this disease as reported earlier [41]. Azithromycin was resistance towards V. parahaemolyticus and V. harveyi though it was sensitive towards V. vulnificus.

3.2. Minimum inhibitory concentrations (MIC)

Table 5 shows the diverse results of MIC from eight selected antibiotics. Resistant was developed from V. parahaemolyticus to Ciprofloxacin HCL and Azithromycin. Cathegorized intermediate to Doxycycline, Tetracyclin, Ampicilin,Co-Amoxiclav while sensitive from Chloramphenicol and Gentamicin, respectively. Furthermore, V. vulnificus have two classification ie. intermediate (Ampicilin, Doxycycline, Ciprofloxacin HCL, Co- Amoxiclav) and sensitive (Tetracycin, Chloramphenicol, Gentamicin and Azithromycin). V. harveyi developed two classification which is resistance (Co-Amoxiclav, Ciprofloxacin HCL, Azithromycin), intermediate (Doxycycline, Ampicilin) and sensitive (Tetracyclin, Chloramphenicol and Gentamicin).

Antibiotics have the specific characters in terms of inhibiting or killing the targetted microorganisms. Chloramphenicol was bacteriostatic type, basically from phenolic class and have the menchanism to prevent protein biosynthesis [45-48]. The activity of Gentamicin from aminoglicoside
class is by interrupting the ability of bacteria to synthesize protein [49,50]. Antibiotics from B-lactam (Ampicilin, Amoxicilin dan Co-Amoxiclav) kill the bacteria by inhibiting the synthesis of bacteria cell walls [51,52]. Furthermore, antibiotics from Tetracyclines class (Tetracyclin and Doxycycline) inhibit the protein synthesis of bacteria by interfering the function of subunit 30S ribosom [53]. Ciprofloxacin from fluoroquinolone class is a strong broad spectrum antibiotics which fastly blocked the replication of bacterial DNA by inhibiting the DNA gyrase. DNA gyrase is the essensial procaryotik enzyme that catalyse supercoiling chromosomes of DNA [54-56]. Moreover, the activity of Erythromycin from macrolid group is by inhibiting the protein synthesis by reversibly attached to ribosom subunit 50S [57].

This study exhibit that some bacteria had resistence to certain type of antibiotics which relates to some inhibition mechanism. According to the response of bacteria to B-laktam (Ampicilin, Amoxicilin dan Co-Amoxiclav) groups, there was three resistance mechanisms which are synthesizing the beta-lactamase compound to destruct the antibiotics, decrease the penetration of antibiotics by bonding the protein transpeptidase and reduce the affinity from bonded protein with antibiotics compound [58]; [59]; [60]; [61]. Some finding has shown that Vibrio parahaemolyticus isolated from sea water has B lactamase gene [62]. It has been reported in southern and norhtern Java Sea that there are two types of B-lactamase from plasmid pVHA1 dan pVHA 4 in Vibrio harveyi [63].
Table 3. Susceptibility of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* towards 10 types of antibiotics at 100 ppm

| Bacteria | Do* | Tc* | Kf* | Gm* | Amo* | Amp* | Cfx* | C-A* | Azm* | ES* |
|----------|-----|-----|-----|-----|------|------|------|------|------|-----|
| Vp*      | 16.05±1.3 | 19.43±1.47 | 22.32±2.34 | 30.17±1.76 | 10.07±1.94 | 6.93±0.34 | 20.17±2.26 | 10.27±1.82 | 12.83±1.02 | 10.13±1.16 |
| Vv*      | 21.15±0.7 | 20.28±0.5 | 26.03±1.51 | 30.65±1.65 | 13.55±0.7 | 18.60±2.33 | 25.03±1.34 | 9.83±0.82 | 27.03±1.29 | 21.50±3.1 |
| Vh*      | 18.00±1.46 | 15.05±1.77 | 28.45±2.79 | 31.85±2.5 | 11.05±1.32 | 7.50±0.43 | 20.23±2.41 | 12.00±1.49 | 14.50±0.45 | 18.33±6.8 |

Table 4. Susceptibility of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* towards 9 types of antibiotics

| Bacteria | Do* (30 ppm) | Tc* (30 ppm) | Kf* (10 ppm) | Gm* (10 ppm) | Amp* (10 ppm) | Cfx* (5 ppm) | C-A* (10 ppm) | Azm* (15 ppm) | ES* (15 ppm) |
|----------|--------------|--------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Vp*      | Sensitive     | Intermediate | Sensitive   | Sensitive   | Resistance  | Resistance  | Resistance  | Sensitive   | Sensitive   |
| Vv*      | Sensitive     | Sensitive    | Sensitive   | Sensitive   | Resistance  | Resistance  | Resistance  | Sensitive   | Sensitive   |
| Vh*      | Sensitive     | Intermediate | Sensitive   | Sensitive   | Resistance  | Resistance  | Resistance  | Sensitive   | Sensitive   |

Table 5. Minimum Inhibition Concentration of *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio harveyi* towards 8 types of antibiotics

| Bacteria | Do* | Tc* | Kf* | Gm* | Amp* | Cfx* | C-A* | Azm* |
|----------|-----|-----|-----|-----|------|------|------|------|
| Vp*      | Intermediate | Intermediate | Sensitive | Sensitive | Intermediate | Resistance | Intermediate | Resistance |
| Vv*      | Intermediate | Sensitive | Sensitive | Sensitive | Intermediate | Intermediate | Intermediate | Sensitive |
| Vh*      | Intermediate | Sensitive | Sensitive | Sensitive | Intermediate | Intermediate | Resistance | Resistance |

Denoted:

*V. parahaemolyticus* (Vp); *V. vulnificus* (Vv); *V. harveyi* (Vh); Doxycycline (Do); Tetracyclin (Tc); Kloramfenikol (Kf); Gentamicin (Gm); Amoxicilin (Amo); Ampicilin (Amp); Ciprofloxacin HCL (Cfx); Co-Amoxiclav (C-A); Erythomycin strearate (ES); Azithromycin (Azm).
The particular clinical parameter from sea water and foods from Java sea confirmed that there are 156 to 160 *Vibrio parahaemolyticus* isolate was able to produce B-lactamase [64]. On the other hand, this study reveals that Ciprofloxacin from fluoroquinolone class was resistance to three tested *Vibrio* spp. The mechanism of resistance were by two systems which is efflux activity to pump out the compound, so, therefore reduce the quinolon concentration [65]. Secondly, the intracellular resistant gene mediated by plasmid has produced protein bonded DNA gyrase [66]; [67]; [68]. Specifically, Azithromycin had resistant to *V. parahaemolyticus* and *V. harveyi*. Based on trials from[69], they managed to gain two species of *V. parahaemolyticus* which keep oqxB from 12 isolates which evidently produced OqxB (Efflux pump membrane transporter).

Azithromycin resistance can be obtained by intercellular plasmid which can synthesize compound and change the bonding target through the mutation of ribosome compound or methylation rRNA nucleotide as well as by efflux sytem [70].

The method to avoid the *Vibrio* spp. resistance is by antivirulency agent which basically destroying the virulence factor of pathogen which facilitates the disease [71]. The application of polysaccharide in shrimp have solved the problem [72]. Antibiotics can be combined to meet the benefits of the drug specific interaction, so, therefore, the resistance mutation of particular drugs can be modulated the interaction. Furthermore, this mechanism was also expectantly enhanced the sensitivity of bacteria to other compounds [73]. In addition, treatment by using another bacteria might be useful [74]; [75]; [76]; [77]. There are several techniques to avoid the drug resistance. The strategy of monitoring, risk assessments and mitigation need to develop [78].

**Conclusion**

It has revealed that some type of antibiotic was failed to counteract *Vibrio* spp due to their drug resistance. The type of antibiotics were beta lactam (Ampicilin, Amoxicilin dan Co-Amoxiclav), Ciprofloxacin HCL and Azithromycin (*V. parahaemolyticus* dan *V. harveyi*). *Vibrio* spp. isolates applied in this research was sensitive to Gentamycin dan Chloramphenicol, intermediate-sensitive to Doxycycline and Tetracyclin. There are some strategis proposed to manage this resistance incident.

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