Sensitivity of Vibrio parahaemolyticus, V. vulnificus and V. harveyi Against Chloroxylenol (4-Chloro-3,5-dimethylphenol, C8H9ClO) Antiseptic and Pine Oil Disinfectant

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Abstract

Vibrio spp. genus is known as a marine indigenous bacteria. Vibrio parahaemolyticus, V. vulnificus and V. harveyi are pathogenic Vibrio. This study aims to assess the sensitivity of three Vibrio species (V parahaemolyticus, V. vulnificus and V. harveyi) isolated from shrimp pond against two type of disinfectant with different active compound namely Chloroxylenol (4-Chloro-3,5-dimethylphenol, C8H9ClO) and pine oil. The assessment was done by Kirby-Bauer disk diffusion methods in Zobell agar media with two different concentration (10 and 100 ppm) and replicated in three times. Sensitivity of Vibrio spp. was analized based on the inhibition zone activity produced by disinfectant. Results showed that sensitivity of Vibrio spp. against disinfectant Chloroxylenol 4.8% at 100 ppm were higher than 10 ppm. The increment of V parahaemolyticus was 182 %, V. vulnificus was 47 % and V. harveyi was 43 %, respectively. Susceptibility of antiseptic with Chloroxylenol 4.8% at 100 ppm was arised to 152 % (V. parahaemolyticus), 43 % (V. vulnificus) and 31 % (V. harveyi) when compared to 2.5% pine oil disinfectant. It can be concluded that Chloroxylenol 4.8% active compound and pine oil were able to inhibit the Vibrio spp. growth.

Keywords: Vibrio spp.; antiseptic; disinfectant; chloroxylenol; pine oil

INTRODUCTION

Vibrio parahaemolyticus and Vibrio vulnificus are common bacteria from coastal and estuary zone. This bacteria are pathogenic to human (Huehn et al., 2014; Raszl et al., 2016). Vibrio parahaemolyticus infection is clinically sign by gastroenteretic and wound infection (Rezny et al., 2020), diarrhea, abdominal cramping, chills nausea, vomiting, and fever (Raszl et al., 2016), and in more severe cases this can cause sepsis (Rezny et al., 2020).

V. parahaemolyticus’ major virulence factor is Thermostable Direct Hemolysin (TDH) (Wang et al., 2015; Rezny et al., 2020) and TDH-related hemolysin (TRH) (Wang et al., 2015). On the other hand, V. vulnificus is an opportunistic pathogen to human (Gulig et al., 2005, Heng et al., 2017, Leng et al., 2019). V. vulnificus can cause primer sepsis, gastroenteretic and wound infection (Horseman & Surani, 2011; Al-Assafi et al., 2014; Raszl et al., 2016; Leng et al., 2019). The most important virulence factor of this bacteria are capsular polysaccharide (CPS), flagella and motility, acquisition of iron, hemolysin/cytolysin and metalloprotease as well as RtxA toxin (Gulig et al., 2005).

Vibrio harveyi is freely swim bacteria in tropical seawater and marine organism’s gut microflora. This bacteria is a pathogenic to marine organisms, eventhough, presumably is non pathogenic to human. Nevertheless, there are four articles reported the relation of V. harveyi infection to human (Pavia et al., 1989; Wilkins et al., 2008; Hundenborn et al., 2013; Gigia-Aguirre et al., 2017).

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There are some hygiene procedure to counteract this disease problem. Hygiene is an early effort or action to keep and enhance the cleanliness and health by personal maintenance and environmental factors. The purpose of this activity is to avoid from the disease problem. So, therefore, the antiseptic practical become more popular and beneficial. Furthermore, people was also use disinfectant for environmental purpose. Chloroxylenol is a well known active compound for antiseptic. While, pine oil is an active compound for disinfectant.

Chloroxylenol is also known as p-chloro-m-xylene, parachlorometatxylene, 4-chloro-3,5-xylene, 2-chloro-m-xylene, 2-chloro-5-hydroxy-m-xylene, 2-chloro-5-hydroxy-1,3-di methyl benzene, 4-chloro-1-hydroxy-3,5-di methylbenzene, PCMX (Final Report on the Safety Assessment of p-Hydroxyanisole, 1985). Due to the antibacterial and antifungi activity, Chloroxylenol is commonly used as disinfectant, preservative, topical antiseptic (Final Report on the Safety Assessment of p-Hydroxyanisole, 1985; Moore & Payne, 2013) and disinfectant (Moore & Payne, 2013). To improve the PCMX solubility, people used to diluted with soap and combined with terpineol (Moore & Payne, 2013). The assessment on antiseptic activity of chloroxylenol has been proven for Staphylococcus aureus, Klebsiella species, Salmonella typhi, Shigella dysenteriae, (Saha et al., 2009), Staphylococcus aureus, Micrococcus spp., Staphylococcus spp, Bacillus spp., Pseudomonas aeruginosa (Hassan & El Bagoury, 2018; Al-Talib et al., 2019), methicillin-resistant Staphylococcus aureus (MRSA), Acinetobacter baumannii, Klebsiella species (Al-Talib et al., 2019). Moreover, this antiseptic is able to decrease the total bacterial count for hand sanitizer (Riza et al., 2019).

Pine oil is an active compound for disinfectant purposes. Some researchers show that pinus oil have an antimicrobial activity. The essential pinus inhibited Staphylococcus aureus growth (Fit et al., 2009; Joshua et al., 2014; Leandro et al., 2014; Raho, 2014), Escherichia coli (Raho, 2014), Staphylococcus epidermidis, Staphylococcus aureus, Enterococcus faecium, Staphylococcus capitis, Enterococcus faecalis, Staphylococcus haemolyticus, and Klebsiella pneumonia (Leandro et al., 2014).

Up to now, the information concerning the activity of Chloroxylenol and pine oil against Vibrio spp., especially V. parahaemolyticus, V. vulnificus dan V. harveyi is unavailable. This research is providing this information to find out the sensitivity of these three Vibrio species against chloroxylenol. This data is useful as a basic information of chloroxylenol and pine oil application as an antiseptic as well as disinfectant to counter Vibrio spp.

MATERIALS AND METHODS

Vibrio spp. isolates

Vibrio parahaemolyticus, V. vulnificus and V. harveyi was obtained from microbiology collection of Biology Laboratory, Faculty of Fisheries and Marine Science, Diponegoro University. Sub-culture was applied, prior before used. Sub-culture was done in order to cultivate the microorganisms by inoculating one ose from stock bacteria to 5 mL nutrient broth media. This was then incubated for 24 hrs at 37 °C.

Antiseptic material

Antiseptic used in this research was commercial antiseptic with Chloroxylenol 4.8% w/v active compound and commercial disinfectant with pine oil 2.5% active compound.

Antiseptic Dilution

The concentration of antiseptic and disinfectant used in this trial was 10 and 100 ppm. Ten ppm concentration was prepared by diluting 0.01 mL to one L of aquadest. While antiseptic was prepared by diluting 0.1 mL antiseptic to the similar volume of aquadest.

Turbidity standard for inoculum preparation

Standard density of bacterial test for sensitivity assessment in this research was used BaSO₄ turbidity standard which is optically
equal to 0.5 McFarland. Standard BaSO₄ 0.5 McFarland was prepared by adding 0.5 ml aliquot 0.048 mol / L BaCl₂ (1.175% w / v BaCl₂. 2H₂O) into 99.5 ml 0.18 mol / L H₂SO₄ (1% v / v), and stirred well. The standard density was verified with 1 cm spectrophotometer light with suitable cuvette to determine the absorbance. The standard 0.5 McFarland (625 nm) has to be around 0.008-0.13. The standard was then placed in a covered tightly tube, kept in room temperature without any light. The barium sulfate standard was stirred well in the vortex before used and carefully checked to ensure the homogeneity (Hudzicki, 2009).

**RESULTS AND DISCUSSION**

**Sensitivity Test of Vibrio spp Against Antiseptic**

Sensitivity test of Vibrio spp. against antiseptic was done by Kirby-Bauer methods (Hudzicki, 2009). Subcultured Vibrio spp. was inoculated to nutrient broth medium, incubated in 24 hrs at 37°C. Bacterial density was adjusted to the standar 0.5 McFarland (1 - 2 x 10⁶ CFU). Sterile cotton was immerged into subcultured bacteria then spreaded gently at the surface of NA media in petridish. Leave it for 5 minutes, then sterile paper disk was placed at the surface of NA media-inoculated with Vibrio spp. Similar procedure was applied to the pine oil disinfectant. Incubation was administered at 24 hrs in room temperature. Each treatment was replicated in three times. Sensitivity of Vibrio spp. against antiseptic and disinfectant material was counted based on the production of inhibition zone.

Principles interpretation of inhibition zone for bacterial sensitivity against antiseptic was based on Wanja et al., (2020). Range of 0 – 5 mm from the periphery of petridish was categorized as weak inhibition. Range of 6 – 9 mm is categorized as moderate inhibition. Range of 10 - 14 mm is cathegorized as strong inhibition, whereas >15 mm is cathegorized as very strong inhibition.

**RESULTS AND DISCUSSION**

Chloroxylenol is a halophenol (Bednarek et al., 2020) which has the antimicrobial activity (USEPA, 1994). This due to the phenolic characters which interfere the microbial membrane (McDonnell & Russell, 1999), damaging the microbe cell wall (Bednarek et al., 2020), inactivated the cellular enzyme (USEPA, 1994; Bednarek et al., 2020) and also denatured the proteins (USEPA, 1994).

The explanation of mechanism is, the phenolic antiseptic of chloroxylenol contains –OH (hydroxyl) functional group. This hydroxyl group will bond to particular protein in the bacterial cell membrane resulting bacterial breakdown. Since there is a leakage of bacterial plasma cell, this will push chloroxylenol to enter into bacterial cell and continue to bond more proteins and enzymes. So, therefore, this will be inactivated the cell function. At highly chloroxylenol concentration, targeted bacterial protein and nucleic acid become coagulated and all the function has stopped. Finally, the rapid cell mortality was occurred (https://www.drugbank.ca/drugs/DB11121).

This present research was conducted by diluting the commercial antiseptic to 10 and 100 ppm (v/v). The stock concentration is 4.8 % (w/v). This can be calculated, that the chloroxylenol active compound from 10 ppm was 0.00048 mg/100 mL. While 100 ppm contained 0.0048 mg/100 mL. Results from Figure 1A and Table 1 showed that 10 ppm produced diameter inhibition zone of 8.53 mm for V. parahaemolyticus, 16.52 mm for V. vulnificus (Figure 1A) and 16.10 mm for V. harveyi (Table 1), respectively. Based on diameter inhibition zone produced, this showed that V. vulnificus dan V. harveyi were more sensitive to chloroxylenol, even at low concentration. Eventhough, at higher concentration (100 ppm), it showed the similar inhibition zone (Table 1) which is 24.07, 24.33 and 23.07 mm towards V. parahaemolyticus (Figure 1B), V. vulnificus (Figure 1C) and V. harveyi (Figure 1D), respectively. According to inhibition zones categorized by Wanja et al., (2020), the 10 ppm was categorized as weak inhibition, while 100 ppm was categorized as moderate inhibition (Table 1). In fact, this present study was also tested at the 1000 ppm though the results of inhibition zone was too wide and spreaded all over the petridish. This results proved that chloroxylenol have the powerful inhibition activity against Vibrio spp. In terms...
**Table 1. Diameter of Vibrio spp. Inhibition zone (mm) against antiseptic and disinfectant**

<table>
<thead>
<tr>
<th>Active compound</th>
<th>Concentration (ppm)</th>
<th>Bacteria</th>
<th>Diameter of inhibition zone (mm)</th>
<th>Percentage enhancement (%)</th>
<th>Inhibition zone of paper disc peripheral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroxylenol</td>
<td>10</td>
<td>V. parahaemolyticus</td>
<td>8.53±1.81</td>
<td>1.27(R)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>V. vulnificus</td>
<td>16.52±1.51</td>
<td>5.26(R)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>V. harveyi</td>
<td>16.10±1.89</td>
<td>5.05(R)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>V. parahaemolyticus</td>
<td>24.07±1.89</td>
<td>182</td>
<td>9.04(R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V. vulnificus</td>
<td>24.33±1.21</td>
<td>47</td>
<td>9.17(R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V. harveyi</td>
<td>23.07±0.85</td>
<td>43</td>
<td>8.54(R)</td>
</tr>
<tr>
<td>Pine oil</td>
<td>10</td>
<td>V. parahaemolyticus</td>
<td>6.17±0.29</td>
<td>0.09(R)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>V. vulnificus</td>
<td>6.95±0.47</td>
<td>0.48(R)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>V. harveyi</td>
<td>13.43±2.98</td>
<td>3.72(R)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>V. parahaemolyticus</td>
<td>9.52±0.69</td>
<td>54</td>
<td>1.76(R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V. vulnificus</td>
<td>17.02±2.61</td>
<td>144</td>
<td>5.51(R)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V. harveyi</td>
<td>17.5±1.96</td>
<td>30</td>
<td>5.75(R)</td>
</tr>
</tbody>
</table>

Denoted : value above is average and standard deviation (n=3). Percentage of inhibition zone enhancement from 10 to 100 ppm was counted based on the measurements of inhibition zone produced from the peripheral of paper disk outward. R : weak inhibition, S : moderate inhibition

**Figure 1.** Paper disc inhibition zone A). chloroxylenol active compound at 10 ppm (V. harveyi); B) pine oil active compound at 100 ppm (V. harveyi); C) pine oil active compound at 100 ppm (V. vulnificus); D) chloroxylenol active compound at 100 ppm (V. parahaemolyticus)

of pine oil, people used to treat pine oil for disinfection agent. According to this results, pine oil produced less inhibition zone, compared to chloroxylenol (Table 1).

Based on the power of disinfectant categorized by Wanja et al., (2020), it can be clearly shown that pine oil have the less inhibition one against Vibrio spp. at 100 ppm dilution. The major component of pine oil is α-terpineol and terpinolene (Tadtong et al., 2015). Oyedemi et al. (2009) confirmed that α-terpineol gave the strong effect to the leakage of gram negative and gram positive
bacteria which leads to the disturbance of the outer membrane. Furthermore, a-terpineol was also damaged the cell wall which will stimulate the lipid leakage.

This is confirming that pine oil has the mechanism as disinfectant by damaging the membrane and cell wall against gram positive and gram negative bacteria. Li et al., (2014) reported some research concerning to the application of a-terpineol 0.78 μL/mL against bacteria Escherichia coli (CMCC (B) 44102). Their research showed some evidence that the size cell was reduced and became irregular, the wall and cell membrane were broken, cytoplasmic nucleus was reduced and nucleus area was aggregated.

**CONCLUSION**

This research confirmed that V. parahaemolyticus, V. vulnificus and V. harveyi were sensitive against antiseptic chloroxylenol active compound. This has proven by the production of inhibition zone at low concentration (10 ppm). The sensitivity of Vibrio spp. against antiseptic chloroxylenol was even higher compared to disinfectant contains pine oil antiseptic. Based on the results above, it can be concluded that antiseptic contains chloroxylenol active compound can be applied to combat or inhibit Vibrio spp. growth. Moreover, disinfectant contains pine oil active compound is suitable for the application of Vibrio spp. disinfection.

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