Pathogenicity Profile of Indigenous Bacteria Isolated from Gastrointestinal Tracts and Fecal pellets of Nipah Worm (*Namalycastis rhodochorde*)

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**Abstract.** Screening and selecting of indigenous gastrointestinal bacteria and nipah worm fecal pellets are essential before being applied as probiotics. Previous studies have successfully isolated 10 bacterial isolates that having cellulolytic and proteolytic abilities from intestinal and fecal pellets of nipah worm. The purpose of this study was to determine the pathogenicity of all isolates against nipah worms in vitro and in vivo. Testing of pathogenicity in vitro was carried out on blood agar and DNAse agar, while in vivo testing was carried out by injecting 0.1 ml of bacterial suspension into the nipah worm body which was then cultured for 14 days. The results showed that only 10% (3 from 30 isolates) of all isolates were suspected having pathogenic activity. Isolates NrBF6, NrBF9, and NrBC4 had been indicated from hemolysis activity in blood agar and lysed DNA on DNAse agar medium. In vivo pathogenicity tests through injection into gastrointestinal cavity showed that isolates NrBF6, NrBF9 and NrBC4 had LD50 at the suspension dose of $10^3$ bacterial cells. LD50 reached for 5, 8 and 20 days, respectively. Symptoms of infection that appeared most dominantly in nipah worms were wounds on the surface of the body, broken body segments, and pale.

1. **Introduction**

The use of Polychaeta in the fisheries business has been carried out. For example the addition of Polychaeta in shrimp feed can improve the health of the digestive tract of shrimp. This can help in reducing the risk of shrimp being attacked by viruses, bacteria, and other parasites [1] [2]. Nipah worm (*Namalycastis rhodochorde*) is one of Polychaeta group in nipah mangrove waters in West Kalimantan. This worm is included in the group of sea worms that have the potential to be developed due to the high protein content, biomass, and the high selling value. Nipah worms with protein levels more than 58% are very beneficial for shrimp and fish farming [3].

One effort to solve the demand for nipah worms is intensive cultivation. Two components that influence the success of cultivation are nutrition and disease prevention [4]. According to Wiadnya *et al.* [5], physiological aspects of digestion and feed quality are important factors for increasing the growth of aquaculture animals. The growth of an organism is influenced by internal conditions, namely the level of digestibility and the use of feed to increase biomass; and external conditions, which are incomplete and inaccurate feed formulas. These things can inhibit the optimal growth of aquaculture animals [5]. The results of previous studies indicate that laboratory scale larvae growth is very slow and susceptible to disease due to parasites. The survival rate of nipah larvae to phase 3 is
only <20% and 20.77% in juvenile phase, furthermore to reach the adult level with 40 segments, it took around 3-4 months in cultivation. It never happens in their natural habitat [6].

One method that can be taken to improve the digestibility of Polychaeta of the genus Namalycastis is by utilizing living microorganisms (probiotics). Probiotic bacteria can be beneficial using enzymes that they produce in the digestive tract and fermenting nutrients that are easier to digest (prebiotics) [7]. Previous studies have found 50 bacterial isolates isolated from coelomic, gastrointestinal and nipah worm fecal pellets and their proteolytic and cellulolytic activity are known [8]. Cellulolytic and proteolytic activity test results show that these bacteria have great potential to be developed as probiotics as feed formulas. However, the potential of these bacteria must be tested for their pathogenicity to determine their pathogenicity and virulence. This is related to the safety of feed from pathogenic microbes that can cause diseases of the worm nipah worm at juvenile or adult level. What the researchers want to improve the quality of the feed is the main goal of getting probiotic bacteria but not harmful to aquaculture animals.

For this reason, it is necessary to test and select potential probiotic microbes based on their pathogenic character, so that these microbes can be used safely as feed ingredients and produced supplement feed products containing probiotics that can be an effective source of energy to support growth and as a strategy in aquaculture to improve biomass production. The purpose of this study was to obtain information on the pathogenicity profiles of probiotic candidate bacteria from the intestinal association of nipah worms (Polychaeta). This information can be developed as a basis for determining safe formulas of feed supplement products that contain probiotics. The ultimate goal is probiotic-fortified feed can support the growth of cultured nipah worms by the community.

2. Methods
2.1 Sterilization of tools and culture medium preparation
The equipments that used in the research, such as Petri dishes, measuring glass, test tubes and borosilicate glass are washed with detergent then rinsed with water until clean and dried. They then were sterilized in autoclave at 121°C at 0.15 Mpa for 15 minutes. The DNAase agar, nutrient agar, and nutrient broth powder were weighed 42 g, 20 g, and 8 g, respectively then dissolved into 1L distilled water. Agar medium solution was heated then sterilized in autoclave at 121°C at 0.15 Mpa for 15 minutes.

2.2 Pathogenicity Screening of Probiotic Bacterial Isolated from Coelom, Gastrointestinal and fecal pellets of Nipah Worms (Namalycastis rhodochordea) In-vitro
In vitro screening of bacterial pathogenic properties is conducted by the streak plate method in DNAse and blood agar. DNAse agar and blood agar that has been compacted in petri dishes were then streaked with bacteria continuously on the surface of agar. DNAse and blood agar were then incubated at 37°C for 48 hours. The pathogenic character of the test bacteria was known by looking at the presence of a clear zone around the bacterial colony after incubation for 48 hours and the surface of DNAse agar was flooded with 1N HCl solution. Visualization of clear zones in DNAse agar and the presence of greenish clear zones in blood media shows the potential for pathogenic bacteria for the host.

2.3 Pathogenicity Test of Probiotic Bacterial Candidates from Coelom, Gastrointestinal and Stools of Nipah Worms (Namalycastis rhodochorde) In vivo
The in vivo pathogenicity test was carried out on nipah worm (Namalycastis rhodochordea) using bacteria which had shown the pathogenicity character of DNAse screening. This test is to determine the pathogenicity and LD50 values of bacterial isolates. Bacterial isolates were cultured on NA medium at 37°C for 24 hours. Bacterial density was measured by the Mc Farland 0.5 method as standard. Then the bacteria are diluted to obtain an injection density of 10^1 to 10^8 cells/worms given by intramuscular injection. Before the injection is carried out, the worm is stunned by using clove oil at a dose of 1 mL / 20L. Observation of worm death was carried out for 20 days with an observation
interval of 1 day. LD50 values are determined using the modification method from Sarjito [9]. LD50 test was carried out in a 500 ml volume bucket. The density of bacteria that be given is as following:

a. Nipah infected by bacteria with a density of $10^6$ CFU/mL (PBS).
b. Nipah infected by bacteria with a density of $10^3$ CFU/mL.
c. Nipah infected by bacteria with a density of $10^5$ CFU/mL.
d. Nipah infected by bacteria with a density of $10^8$ CFU/mL.

2.4 Data analysis

This research is a descriptive research. Data from the research results are processed and analyzed descriptively by creating a table of pathogenicity and LD50 character descriptions.

3. Results

Previous research results showed the proteolytic and cellulolytic activity of 31 bacterial isolates that were successfully isolated from fecal pellets, coelom, and intestinal nipah (Namalycastis rhodochorde). Thirty-one isolates were characterized by the inoculation process on De Man Rogossa Sharp Agar (DMRSA) differential selective media to find out how many isolates were classified as lactic acid bacteria and non-lactic acid bacteria. The results of differentiation based on the ability to grow on the media showed that as many as 12 of 31 bacterial isolates were lactic acid bacteria.

All isolates, both lactic acid bacteria and non-lactic acid, had cellulolytic, proteolytic activity, and both. However, after preliminary in-vitro pathogenicity testing on the DNase Agar medium, it was suspected that three isolates had a tendency to be pathogenic (Table 1). Estimation of the nature of the pathogen is characterized by the formation of a clear zone around the colony.

![Figure 1](image-url)

**Figure 1.** Detection of bacterial pathogenicity through DNAsase activity and hemolysis on the DNAsase and blood agar medium. Clear zones around bacterial colonies indicated DNA decomposition activity by DNAsase (left), and greenish clear zone by hemolysis activity (right).
The results of qualitative detection on the DNAse medium showed that 10% of the total isolates that had cellulolytic and proteolytic potential had a tendency to be pathogenic (Figure 1). It can be seen that all isolates that have pathogenic tendency come from non-lactic acid bacteria group, while the lactic acid bacteria group did not show any pathogenic activity on DNAse agar. Isolates that have DNAse activity are NrBF6, NrBF9, and NrBC4. NrBF9 isolate (non lactic acid bacteria) had the highest index of DNAse activity (Table 1.).

Table 3. Values and categories of activity of blood hemolysis and DNA lysis of bacterial isolates

| Codes     | Haemolysis activity | DNAse activity (cm²) |
|-----------|---------------------|----------------------|
| LAB       |                     |                      |
| NrLtF1    | gamma               | 0                    |
| NrLtF2    | gamma               | 0                    |
| NrLtF4    | gamma               | 0                    |
| NrLtF5    | gamma               | 0                    |
| NrLtF6    | gamma               | 0                    |
| NrLtF7    | gamma               | 0                    |
| NrLtF8    | gamma               | 0                    |
| NrLtF9    | gamma               | 0                    |
| NrLtC2    | gamma               | 0                    |
| NrLtC4    | gamma               | 0                    |
| NrLtG2    | gamma               | 0                    |
| non-LAB   |                     |                      |
| NrBF1     | gamma               | 0                    |
| NrBF2     | gamma               | 0                    |
| NrBF6     | gamma               | 6                    |
| NrBF7     | gamma               | 0                    |
| NrBF8     | gamma               | 0                    |
| NrBF9     | beta                | 12                   |
| NrBC4     | alfa                | 6                    |
| NrBC6     | gamma               | 0                    |
| NrBC10    | gamma               | 0                    |
| NrBC13    | gamma               | 0                    |
| NrBC14    | gamma               | 0                    |
| NrBC19    | gamma               | 0                    |
| NrBG3     | gamma               | 0                    |
| NrBG4     | gamma               | 0                    |
| NrBG5     | gamma               | 0                    |
| NrBG6     | gamma               | 0                    |
| NrBG8     | gamma               | 0                    |
| NrBG9     | gamma               | 0                    |
| NrBG10    | gamma               | 0                    |

Pathogenicity testing was also carried out directly on nipah worms by injection into the worm's gastrovascular cavity. However, the injection was carried out using three isolates suspected to be pathogenic from previous DNAse activity tests, namely NrBF6, NrBF9 and NrBC4 isolates by treating bacterial cell concentrations of $10^3$, $10^5$, and $10^8$ cells/ml. Observations over the course of 20 days showed an increase in mortality of nipah worms that were injected with the three bacterial isolates. The percentage of deaths that occurred was equal by the three bacterial isolates, which was 66.7%,
while nipah worms that were only injected with sterile saline solution showed a smaller percentage of deaths (Figure 2).

![Graphs showing mortality rate and trend](image)

**Figure 2.** Percentage of total mortality of nipah worms (*Namalycastis rhodochorde*) and mortality trend after being treated with injection NrBC4, NrBF6, and NrBF9. Line charts showed the mortality trend of nipah worms caused by $10^3$, $10^5$ and $10^8$ sel/ml of bacteria.

Observation for 14 days also showed several symptoms appeared that caused by the infection process from suspected-pathogenic bacteria. The most prominent symptom of nipah after being injected with NrBF6, NrBF9, and NrBC4 bacterial isolate was a wound or lesion. Lesions had begun to appear on the ventral surface of the body on the third day after injection. White lesions appeared on several segments of the worm's body and then expanded on the fifth day, then caused worm death (Figure 3b). The expansion of the lesion was also followed by bleeding (some segments appear red) and the worm's body segment being cut into many parts. Broken worm’s segments that were previously attacked by lesions (Figures 3c and 3d). Not only lesions, but infected-worms also showed different symptoms. Although lesion did not appear, the worm's body turned pale (white). The color conditions differed from the color of healthy or untreated nipah worms by injecting suspected pathogen isolates. Healthy worm bodies appeared reddish in color (Figure 3f).
4. Discussion

The use of probiotic bacteria as an alternative antimicrobial has been widely recommended and applied in the field of aquaculture. This utilization is carried out to improve the immune system and optimize the digestive process of organic macromolecules of aquaculture animals. Caruffo et al. [10] recommend the use of probiotic bacteria isolated from aquaculture animals themselves, especially from the digestive tract. This is based on the fact that indigenous probiotic bacteria have been highly adapted to the environmental conditions of the host digestive tract, making it easier for the bacteria to grow optimally. Verschuere et al. [11] stated that the interaction of indigenous probiotic bacteria to the host has occurred during their growing. This results in a 'balanced relationship' for metabolic activity in both entities. The total number of pure bacterial cultures isolated from fecal pellets, coelomic fluid, and intestines from nipah worms in previous studies was 58 isolate cultures. 20 isolate cultures from total culture were lactic acid bacteria. Enzymatic activity screening conducted in previous studies showed 30 types of isolates that have the potential as cellulolytic, proteolytic or both. However, any potential risks that must be anticipated in the use of microbes, especially risks associated with pathogenicity.

To know the bacterial pathogenicity well, the use of test animal models is very important. The use of invertebrate animals such as worms has been widely recommended such as nematodes and silkworms [12]. Kaito and Sekimizu [12] reported that worms that have a length of 5 cm or more are easier to handle in terms of the injection process of pathogenic bacterial suspensions or drugs. Nipah worms have a larger body size with a length of up to 2 m, making it easier to process the injection of a bacterial suspension into the intestinal cavity.

The bacterial pathogenicity of 31 types of isolates that have the potential for an enzymatic activity must be checked as a safety requirement in addition to the feed. The results showed that 10% of the...
total number of potential bacteria have a tendency to be pathogenic. Clear zone area of DNase activity and bacterial hemolysis of NrBF6, NrBF9 and NrBC4 ranged from 6-12 cm$^2$ (Table 1). DNase activity and hemolysis indicate the tendency of these three types of potential bacteria to be pathogenic for the host. Zahid et al. [13] have conducted the same test on bacteria isolated from the digestive tract of chickens, the content of hemolysin causes hemolysis in the blood agar medium. Symptoms of the disease appeared several days after pathogenic bacteria have been injected into chicks. The results of direct pathogenicity testing for nipah worms showed that NrBF6, NrBF9 and NrBC4 isolates had the lethal effect of half the number of test worms started from the lowest dose ($10^3$ cells/ml).

LD50 generally occurs in all concentrations of test bacteria ranging from $10^3$ to $10^8$ cells/ ml. All three isolates (NrBF6, NrBF9 and NrBC4) were able to kill 50% of the total number of nipah test worms. The results of this study are almost the same as the results of research by Selim et al. [14] that investigated the virulence of ten Coxiella strains in G. mellonella at infectious doses ranging from $10^4$ to $10^7$ ml. From all isolates, there was a difference in the time needed to reach a total worm death of up to 50%. The bacterial suspension dose and the time needed to reach LD50 will be different for each bacterial isolate. According to Casadevall's statement [15], the LD50 measurement has the advantage that it allows comparisons across microbes, and the use of host death provides a unequivocal endpoint. However, the words "fulminant" and "aggressive" categories are used in the context of infectious diseases, they usually connect an element of rapidity or shortness of time between infection and disease.

Symptoms that first appeared, were lesions which then extended to almost the entire worm’s body. All three test bacterial isolates were detected to have proteolytic abilities in previous studies (Hepiyanti et al., 2017)[8]. Niu et al. [16] states that some proteases are capable of causing damage to parts of the body of the nematode because they are virulent. Virulent proteinase is derived from bacteria that can damage the protein present in nematode epithelial tissue. This process is thought to also affect the damage to nipah epithelial tissue when infected with these three pathogenic bacteria. Hemolysin protein is also considered one of the causes of bacterial pathogenicity through hemolysis activity. NrBF6 and NrBF9 bacteria were detected to be able to damage the blood (hemolysis) when grown on blood agar media. Worm's body becomes pale related to the blood flow that occurs in the worm's body. According to Riyandi [17], the reddish color of the worm is caused by blood flow inside. This shows that the pale worms caused by disruption or damage to blood cells due to hemolysis. Production of hemolysin is usually associated with the pathogenicity of bacteria, and especially responsible for more severe forms of infections [18].

The potential of probiotic bacteria added to the feed will have a good impact on increasing the production of worms aquaculture. But it is well known that there is a risk of pathogenic bacteria even though the bacteria are isolated from the digestive system of the worm itself. The proteolytic potential of bacteria can be beneficial or detrimental. Therefore further studies are needed on preventive techniques for proteases that harm host cells.

5. Conclusion
Three isolates of bacteria NrBF6, NrBF9, and NrBC3 which have proteolytic and cellulolytic activity, also have pathogenic properties through screening of hemolysis activity and DNA decomposition. Testing for the pathogenicity of worms causes 50% worm death at LD50 started from a bacterial suspension of $10^3$ cells/ml. The dominant symptoms that arise, such as lesions arisen on the surface of the body, the worm's body was cut into many parts, and the worm's body became pale.

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