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DETERMINATION OF ANTI- INFECTIOUS MYONECROSIS VIRUS (IMNV) PROPERTIES OF CYNODON DACTYLON IN PENAEUS VANNAMEI

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Abstract

Infectious myonecrosis virus (IMNV) is one of the most pathogenic viruses causing severe mortality in *Penaeus vannamei* in many countries. Several strategies have been implemented to inhibit the presence of IMNV disease. The present study was carried out to examine the anti-IMNV activity of *Cynodon dactylon* powdered extract on shrimp by *in vivo* testing in three systems: indoor tank and cages in raceways and cages in the commercial ponds. The *C. dactylon* plant powder mixed with shrimp feed in four different concentration which was named as RAV-S A (10 %), RAV-S B (15%), RAV-S C (20 %), and RAV-S D (25%). All the four doses were tested in the tank trial, then the best two doses RAV-S C (20 %), and RAV-S D (25%) from tank trial were tested in cage trials. The best-selected dose RAV-S C (20 %) from cage trial was tested in the cage-pond trial. The shrimp were infected using sub-lethal dose of IMNV using *per os* method of challenge in tank and cage trial. No artificial IMNV infection made for the cage-pond test. The efficacy rate of treatment groups against IMNV in tank level trial was as followed, 20% survival in RAV-S A (10 %), 12 % survival in RAV-S B (15%), 30 % survival in RAV-S C (20 %), and 30% survival in RAV-S D (25%) and 0 (zero) % survival in Positive control. The efficacy rate of treatment groups against IMNV in cage trial was as followed, 75.2 % survival in RAV-S B (15 %), and 91.1 % survival in RAV-S C (20 %) and 79.6 % survival in Positive control and 99.17 % survival in the negative control. The typical gross sign of IMNV were also monitored which was 28.90 % in RAV-S B (15 %), and 2.55 % in RAV-S C (20 %) and 50 % in Positive control and 0 (zero) % in the negative control. The efficacy rate of treatment groups against IMNV in the cage-pond trial for RAV-S C (20 %) was 69% survival and 53% survival for the control. The typical gross percentage of treatment RAV-S C (20 %) was 10% whereas control was 46%. The trial showed that *Cynodon dactylon* contains anti-IMNV properties. The limitation is in finding the right dose and appropriate way for inoculation.

Keywords: IMNV, *Cynodon dactylon* plant extract, survival rate, Penaeus vannamei, tank trial, cage trial, cage-pond trial.

Introduction:

The shrimp, *Penaeus vannamei*, is one of the main species for aquaculture in many countries. It Indonesia alone, it has been cultured in at least 17 provinces. IMNV infection in *P. vannamei* in Indonesia was confirmed after sequencing the PCR fragment of IMNV from shrimp sample originated from East Java in 2006 (Senapin *et al.*, 2007). Subsequent analysis revealed that the Indonesian IMNV sample had 99.6% nucleic acid sequence identity than the Brazilian IMNV reported at GenBank (Nur, 2007; Senapin *et al.*, 2007). This disease affects the juvenile and grows out shrimp (DOC 30 - 90) with 10-30 % mortalities. Similar symptoms also found in several countries like north-eastern Brazil as well as in other South-East Asian countries (Lightner *et al.* 2004 a, 2004 b, Nunes *et al.*, 2004; Poulos *et al.*, 2006, Andrade *et al.*, 2007; Prasad *et al.*, 2017). Infectious myonecrosis virus (IMNV) will present focal to extensive white necrotic areas in striated (skeletal) muscles, especially in the distal abdominal segments and tail fan, which can become necrotic and reddened in some individual shrimp. Severely affected shrimp become moribund, and mortalities can be instantaneously high and continue for several days (Jha et al., 2021 a, b, c and Babikian *et al.* 2017).

Several strategies have been carried out to prevent the spread of IMNV infection, among the good management practice of shrimp farming, specific pathogen-free broodstock, and disinfection of eggs and larvae. However, to control the viral spread, the limited knowledge of the mode of action and various agents' application restricts the success. One of the most promising methods of preventing diseases is strengthening the shrimp defence mechanisms through immunostimulant administration (Balasubramanian *et al.* 2008 a, 2008 b , Kaleeswaran *et al.* 2011). Immunostimulants are enhancing both specific and nonspecific immunity against infectious diseases. Various studies have been carried out to obtain immunostimulants' performance to improve immune response and reduce disease impacts. Jha et al. 2016, has successfully developed a formulation of essential oil blend extracted from ten plants as a feed additive and determine the efficacy against WSSV.

The development of antiviral plant extract has been done against shrimp virus, like, WSSV to control this disease's spreading and protect cultured shrimp from this virus. *Cynodon dactylon* is widely available and distributed in almost all parts of the world. *C. dactylon* used for long as ancient medicine in Indian culture (Kaleeswaran *et al.*, 2011). The aqueous and ethanolic extracts of *C. dactylon* have shown antiviral properties against WSSV for *Penaeus monodon* (Balasubramanian *et al.* 2008 a, 2008 b). In our previous work, the application of *Cynodon dactylon* extract has anti-WSSV properties that protect shrimp from WSSV infections (Howlader et al. 2020, Jha et al. 2021 b and 2021 c). In the present study, an attempt was made to explore the efficacy of the administration of *C. dactylon* (RAV-S) to protect shrimp against Infectious Myonecrosis Virus (IMNV) in tank, cage and at cage-pond level trials.

Materials and Methods:

1. **Study Area**

The presented study was performed at the Disease Research Centre, Bandar Lampung of P.T. Central Pertiwi Bahari. The bioassay and cage trials were conducted in a biosecure facility, whereas the pond trial was performed in a commercial level research farm.

2. **Preparation of Plant Extract from *C. dactylon***

Cynodon dactylon (leaf and shoot) was collected from P.T. Central Pertiwi Bahari, Lampung. The clean and fresh specimens were dried in the shade for 7-10 days. The dried grass was carefully screened to discard mould and other abnormalities. The dried material was finally ground and powdered using an electrical blender. The powder was sieved, weighed, packed in sealed bags and stored in a cool place for further use. The powdered form of *C.*

dactylon was used to conduct the trials on the cage and commercial pond levels. The method as previously described in detail by Jha et al. 2021b, and 2021 c.

3. **Preparation of *C. dactylon*-Supplemented Feed**

The trial's shrimp feed was produced in a feed mill, P.T. Central Proteina Prima, Surabaya. Three doses of the powdered extract, including 100 g/kg of feed or 10%, 150 g/kg of feed or 15%, 200 g/kg of feed or 20%, and 250 g/kg of feed or 25%, were mixed. The treatment feed was named RAVs A (10%), RAVs B (15%), RAVs C (20%) and RAVs D (25 %). The extracted powder's required concentration was mixed with other feed materials, and all components were combined in a feed mill. The shrimp feed was produced at a lower temperature (80°C) to avoid degeneration of the active ingredients of *C. dactylon* (Jha et al. 2021b, and 2021 c).

4. **Investigation of anti-IMNV activity of *C. dactylon***

4.1. IMN Virus Preparation for *per os* challenge

The IMNV-infected shrimp were collected and checked for the presence of the virus. The offal parts, like, head, shell, and gut were removed, and the muscle was first to cut into small pieces with the help of scissors and then minced into fine pieces by using a sterilized manual blender. The obtained semi-solid gel-like muscle was filtered through an 80-micron filter and recovered. The acquired minced muscle carrying IMNV was homogenized and checked for the presence and quantification by RT-PCR. The minced tissue containing IMNV was stored in small blocks at -200C before use. At the time of using were thawed slowly shifting to cooler temperature (-200C to 40C and finally to 18-20- 25^{0}C. The complete procedure took 4-5 hours.

4.2. **Laboratory Experiment of IMNV-challenged Shrimp**

Shrimps were collected from Marine Research Center, Lampung. The treatment tanks were distributed into four groups of 20 shrimp per tank. The trial duration was 27 days, including three days of acclimatization, 14 days of feeding; on day 15, shrimp were challenged with IMNV. From day 16 onwards, it continues feeding and observation for ten days. The method in detail was as followed:

4.3. **Tank and water preparation**

The transparent plastic tanks with 125 L capacity are used for the lab-scale trial. The chlorinated saline water was obtained from CPB Hatchery. The water temperature was maintained at 25±10C. The water salinity was maintained at 20 ppt, pH 7.6-8, Dissolved Oxygen 5.1-6 ppm, the water exchange was done at the rate of 20% daily. Siphoning was done once a day.

4.4. **Trial groups**

Altogether six trial groups were used, four treatment groups (RAVs A (10%), RAVs B (15%), RAVs C (20%) and RAVs D (25 %), negative control and positive control. In the negative control group, shrimp were fed with standard pellet feed on regular CP-3 feed throughout the experimental period. In the positive control group, the shrimp were fed with standard pellet feed and challenged with 1 % IMNV muscle on day 15 and continued regularly feeding on day 16. In each treatment group, shrimp were fed with *C. dactylon* extract supplemented feed (RAV-S) for the first 14 days and challenged with IMNV at day 15 and feeding continued throughout the experimental period.

4.5. **Challenge method**

Shrimps was challenged per *os* with 1 % IMNV-infected muscle.

4.6. **Post challenge observation**

The challenged shrimp were under intense observation for their behaviour and feeding rate and cumulative mortality during the trial. The feeding rate and tank bottom siphoning schedule and maintained the same for both control and treatment group throughout the experiment. Remaining food and waste matter were taken out before the next day feeding. The experimental animals were examined twice a day for gross signs of disease, and the number of deaths was recorded. The dead and

the moribund shrimp were taken out and confirmed by PCR.

5. **Cage Trial of IMNV-challenged Shrimp**

The shrimp were divided into five groups of 40 shrimp per cage, and each group was conducted in three replicates. The trial duration was of 35 days, including ten days of acclimatization, 14 days of feeding, at day 15 shrimp were challenged with IMNV and from day 16 onwards continues feeding and observation for ten days. The method in detail was as followed,

5.1. **Cage and water preparation**

The water preparation was done by following the standard procedure of culture pond preparation. The three cages in each group (1 m3 size and 4 mm mesh size) were set in 150 m2 raceways. The water depth maintained at least at 70 cm. The saline disinfected water was obtained from CPB Hatchery. The water temperature was maintained at 25±10C. The water salinity was maintained at 20 ppt, pH 7.6-8, Dissolved Oxygen 5.1-6 ppm, no water exchange during the experimental period.

5.2. **Treatment groups**

Altogether four trial groups, two treatment groups (RAVs B (15 %) and RAVs C (20 %)), negative control and positive control. Feeding treatment in the cage scale experiment was carried out the same as on the lab scale. Specific Pathogen Free (SPF) shrimp were stocked with a stocking density of 80 pieces /m2 with an average body weight of 4 g. Acclimatization was carried out for 2-3 days and started feeding.

5.3. **Challenge method**

Shrimps was challenged *per os* with 2 % IMNV-infected muscle. From day 16, onwards continued feeding and mortality and behaviour were observed.

5.4. **Post challenge observation**

Observation on the challenged shrimp were done as the lab scale experiment.

6. **Cage-Pond Scale Trial**

6.1. Pond and Cage Preparation

Cage-Pond trial was conducted in commercial ponds in IMNV prone area. The 3x1 m cage were prepared following the standard and water preparation adopted from CPB's standard operation procedure (SOP). Water for the experiment were treated with one ppm of CuSO4, one ppm of Pondfos (Dichlorvos), and 20 ppm of Chlorine. The SPF shrimp were stocked with the same density of 80 pieces/m2 in each cage. Shrimp were cultured in an autotrophic system with less water exchange and high aeration. Water was maintained at the maximum level (120 cm) during the culture and was added only to replace the open water by siphoning, leaking and evaporation.

6.2. Treatment groups

Altogether three trial groups were used, one treatment group (RAVs C (20%)), negative control and positive control. Feeding treatment in the cage scale experiment was carried out the same as on the lab scale. Specific Pathogen Free (SPF) shrimp were stocked with a stocking density of 80 pieces /m2 with an average body weight of 4 g. Acclimatization was carried out for 2-3 days and started feeding. The treatment group were fed with *C. dactylon*-supplemented feed (RAV-S) that were prepared in CP Feed mill in Surabaya, whereas the control group were fed with CP regular feed.

6.3. **Challenge method**

No artificial challenge was performed in the commercial pond.

6.4. Observation

Observation was performed on daily basis. IMNV gross sign and mortality was estimated during weekly sampling.

Results and Discussions:

The anti-IMNV activity of *C. dactylon* was determined by observing the survival of shrimp through an *in vivo* challenge test both in a lab-scale experiment and in a cage scale experiment.

1. **Bioassay challenge in tank**

From the lab-scale experiment, total mortality was observed in positive control within four days after infection. Ten days after infection, the cumulative mortality was significantly higher in the positive control (100%). Mortality in the treatment group started from day four post-infection. The highest protection, i.e., 30 %

survival against IMNV recorded in two groups, RAV-S C (20%) and RAV-S D (25%) (Figure 1).

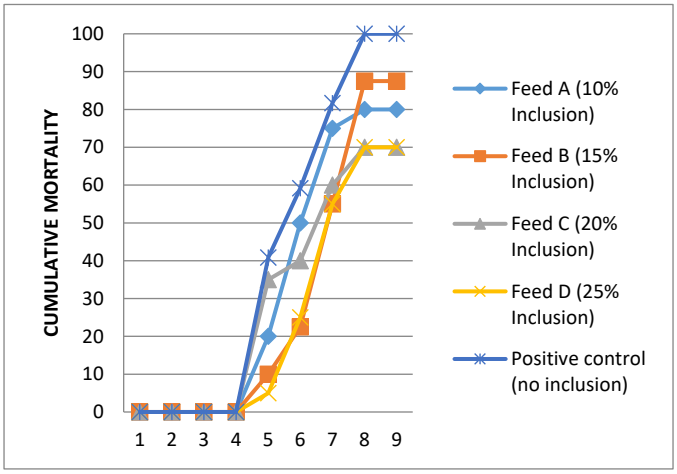

Figure 1 Percentage of cumulative mortality of L.vannamei in the in vivo challenge test with IMNV and four different concentrations (10, 15, 20 and 25 mg/kg) of C. dactylon crude extract

2. **Cage Trial**

The cage level experiment was conducted to re-confirm the efficacy of *Cynodon dactylon* (RAV-S feed) to control IMNV infection in shrimp culture. Effect of *Cynodon dactylon*-coated feed on shrimp survival rate found satisfactory. The survival difference was clear from each treatment (Table 1), with the highest protection provided by feed RAV-S C (SR 91.1%) after 60 days post-infection and followed by feed RAV-S B with SR 75.2%. The positive control showed high survival (79.6%) too. The major difference between survived positive control and treatment groups fed *C. dactylon* feed was observed in clinical sign presence. In the positive control group, the percentage of shrimp with IMNV clinical signs was 50%, whereas Feed RAV-S B was 28.9%) and the lowest in Feed RAV-S C (2.55%). This could be concluded that *C. dactylon* can protect shrimp from IMNV and reduce the appearance of disease symptoms.

Treatment	Cage	shrimps (n)	Mortality	Healthy Shrimps	%SR		Total Gross sign											
					SR/cage	Ave	G-01		G-02		G-03		G-04		All		Ave
Feed B	1	86	24	62	72.09	75.2	4	5.90%	10	14.70%	2	2.90%	1	1.50%	17	25%	28.90%
	2	86	23	63	73.26		6	8.50%	15	21.10%	1	1.40%	0	0%	22	30.90%	
	3	86	17	69	80.23		4	6.20%	15	23.10%	1	1.50%	0	0%	20	30.80%	
Feed C	1	45	3	42	93.33	91.1	1	2.60%	0	0%	0	0%	0	0%	1	2.60%	2.55%
	2	45	5	40	88.89		0	0%	1	2.50%	0	0%	0	0%	1	2.50%	
Positive Control	1	79	21	58	73.42	79.6	4	7.40%	23	42.60%	0	0%	0	0%	27	50%	50%
	2	77	16	61	79.22		5	12.50%	18	45%	0	0%	0	0%	23	57%	
	3	80	11	69	86.25		2	3.50%	23	40.40%	0	0%	0	0%	25	43%	
Negative control	1	80	0	80	100	99.17	No Gross Sign											
	2	80	0	80	100													
	3	80	2	78	97.5													

Table 1 Percentage of Shrimp Survival rate and gross sign appearance of Cage trial

4. Cage-Pond trial

The cage-pond experiment was carried out to reassure the efficacy of *Cynodon dactylon* (RAV-S C feed) to control IMNV infection in shrimp culture. The experiment results showed that shrimp given *C. dactylon* crude extract meal had a higher survival rate than the control. Lower gross sign data also supported this compared to the control group.

Cage (RAV-S C)	Pond (reguler feed)
Survival Rate : 69 %	Survival Rate : 53 %
Gross sign: 10%	Gross sign: 46%

Table 2 Percentage of Shrimp Survival rate and gross sign appearance of Cage-Pond trial

The study demonstrated that IMNV-challenged from all the experiments. We observed that shrimp fed with RAV-S-C (20% inclusion) provides 69 % protection of *L. vannamei* with 10 % of the clinical or gross signs of IMNV during the culture (Table 2). The results indicate that *C. dactylon* extract contains anti-IMNV properties either independently or in combination.

Conclusions:

The results of this study show that *C. dactylon* contains antiviral properties with many phenolic compounds. The *in vivo* challenge test showed that *C. dactylon* has anti-IMNV properties that protect shrimp from IMNV infections. Application of 20% of *C. dactylon* (Feed RAV-S C) has potent activity against IMNV in *P. vannamei*. Further research on isolation, characterization, and purification of this plant's active compounds will help find the suitable and appropriate dose against IMNV infection.

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