Effect Of Extracellular Proteins (ECPS) Edwardsiella Tarda On The Innate Immune Response In Nile Tilapia (Oreochromis Niloticus)

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Abstract: E. tarda infects Tilapia (O. niloticus) that it has extracellular products (ECP) and immunogenic competent so it has the ability to more quickly activate the host's immune response. The first defense is carried out by the innate immune response for example the blood cells such as phagocytes, macrophages, lymphocytes and neutrophils. The purpose of research was to analyze the effect of ECP E. tarda on the innate immune response of Tilapia (O. niloticus). The methods used were ECP E. tarda isolation, vaccination, total white blood cells, differential leukocytes and phagocytic activity. The ECP treatment by peritoneal injection is 0.1 ml tail^{-1} for 9 days. Results showed total white blood cells differed significantly (P <0.05), the highest and lowest mean values were 29 x 10^3 cells ± 2.64 (Days-3 and 6) and 11 x 10^3 cells ± 2.25 (Days-9). The differential leukocytes showed differed significantly (P<0.05) that lymphocytes has >50%, monocytes between 23-32% and neutrophil ≤ 20%. The results of phagocytosis activity showed differed significantly (P<0.05) compared the control, the highest mean value 84.18% ± 1.81 on the 6th day. ECP causes cytokinesis which characterized by two dividing cells. ECP has a positive effect on phagocytic activity, total white blood cells, differential leukocytes and cytokinesis cell in Tilapia (O. niloticus).

Keywords: Differential Leukocytes, Edwardsiella tarda, ECP, Phagocytosis, White Blood Cell.

1 Introduction

Tilapia is one of the host pathogens of E. tarda bacteria that are dangerous and can cause economic losses in aquaculture. Clinical symptoms observed in fish are characterized by discoloration, excessive mucus production, hemorrhagic and necrotic caudal parts of the body [1]. In addition, it causes fluid in the abdominal cavity with enlarged liver, kidneys and lymph [2]. The mortality rate of E. tarda infection in tilapia after three days was 57.5% and reached 60% when the bacterial density was 104 cfu ml^{-1} [3,4]. E. tarda as a Gram-Negative bacterium (2-3 µm) produces hemosiline, dermatoxin, exotoxins of extracellular products and endotoxins from intracellular components [5,2]. Condroitinase, effector protein, EseB, EseC, and EseD as a component of extracellular proteins are secreted through a secretion system type III [6,7]. These bacterial products are immunogenic in that they can activate innate and adaptive immune responses to the host's body. Health evaluation of fish can be analyzed through the system immune both innate and adaptive. Innate immunity as the first barrier acts faster in eliminating pathogens such as skin, antimicrobial enzymes, cytokines, granulocytes, monocytes and macrophages [8]. Blood is one component of the defense of infection by looking at changes in the number of patterns and division of blood cells [9]. The process of elimination is carried out by phagocytic cells such as monocytes, macrophages and polynuclear cells (lymphocytes and neutrophils) [10]. The purpose of research was to analyze the effect of ECP E. tarda in the innate immune response in tilapia (O. niloticus).

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2 Material and Method

2.1 Extracellular Protein E. tarda Isolation

E. tarda 10^7 cfu ml^{-1} culture on Luria-Bertani (LB) 500 ml at 26^{o}C for 18 hours (OD 600 nm). Sentrifuse 3000 g x 30 minutes and the supernatant filtered a 0.22 µm miliphore filter [11]. Then, ethanol 100% was precipitated and overnight incubated at 0^{o}C. After that, the centrifuge is 10,000 x g, 4^{o}C for 15 minutes. Pellets are resuspended with phosphate buffer pH 7 [12]. The total ECP protein is measured by nanodrop spectrophotometer [10].

2.2 ECP Vaccination

Tilapia (size 7-9 cm) is spread as much as 10 aquariums-1 and acclimatization for 3 days [13]. ECP with peritoneal injection is 0.1 ml tail^{-1} fish and booster 1 time. The treatment was differentiated based on maintenance days namley 1 day, 3 days, 6 days and 9 days (A, B, C, D) and NaFis controls (K).

2.3 Total White Blood Cell

A blood sample of 20 µl and added 4 ml of Natt-Herrick solution. Then incubate at room temperature for 5 minutes. Samples are included in the haemocytometer and can be observed [14]. White blood cell calculation formula:

$$\text{Total White Blood Cells} = \frac{\text{Total White Blood Cells}}{4} \; x \; 2000$$

2.4 Differential Leukocytes

Blood samples are dripped and removed in the direction of the glass object. After drying, fix methanol for 10 minutes and dry. Preparations were stained with Giemsa for 3-5 minutes and rinsed with distilled water [14]. Differential leukocytes formula:

$$\% \text{ Limphocytes} = \frac{L}{100} \; x \; 100\%$$

$$\% \text{ Monocytes} = \frac{M}{100} \; x \; 100\%$$

$$\% \text{ Neutrophil} = \frac{N}{100} \; x \; 100\%$$

Note:
L + M + N = 100 cells
L% + M% + N% = 100%

2.5 Phagocytosis Activity

A blood sample of 0.1 ml in the tube was added with 1 ml of E. tarda 10^7 cfu ml^{-1} and 1 ml PBS (1: 1) and incubated at 30°C for 45 minutes. Then, the sample was dropped and dismissed on a glass object. After drying, fix with alcohol 95% for 3 minutes. Preparations were stained with Giemsa for 20 minutes and rinsed with distilled water [14]. Calculation formula for phagocytosis activity:

$$\text{Phagocytosis} = \frac{\sum phagocytes\ cell}{\sum phagocytes\ cell\ identified}\ x\ 100\%$$

3 Result and Discussion

3.1 Total White Blood Cells

White blood cells play a role in the innate immune system by localizing and eliminating pathogens [15]. The role was positively correlated with the results of the study where there was a significant increase compared to controls after ECP vaccination (Table 1). Total white blood cells showed significantly different between treatments ($P < 0.05$). The increase in suspected tilapia as a host recognizes the ECP as a foreign pathogen so that the host's body with the role of white blood el is defending the pathogen. The immunogenic properties of the ECP can increase the total leukocytes count [16]. This increase is thought to be the value of effectiveness in developing cellular immune responses [17,8]. When an infection occurs, white blood cells will phagocytes pathogens so that they do not develop and spread widely in the host's body [10].

Table 1
Data of Total White Blood Cells

No	Treatment	Results (10^3 sel mm^{-3})	Total (sel mm^{-3})	Average (sel mm^{-3})
1	K1	12.5 ± 1.32	96 x 10^3 ± 1.32	32 x 10^3 ± 1.32
	K2	10.50 ± 1.32		
	K3	10.50 ± 1.32		
2	A1	29.00 ± 3.00	86 x 10^3 ± 3.00	28.66 x 10^3 ± 3.00
	A2	35.00 ± 3.00		
	A3	32.00 ± 3.00		
3	B1	31.50 ± 2.92	87 x 10^3 ± 2.92	29 x 10^3 ± 2.92
	B2	30.50 ± 2.92		
	B3	26.00 ± 2.92		
4	C1	28.00 ± 2.64	87 x 10^3 ± 2.64	29 x 10^3 ± 2.64
	C2	32.00 ± 2.64		
	C3	27.00 ± 2.64		
5	D1	31.50 ± 2.25	33 x 10^3 ± 2.25	11 x 10^3 ± 2.25
	D2	27.00 ± 2.25		
	D3	29.00 ± 2.25		

K: control, A: Treatment Day-1, B: Treatment Day-3, C: Treatment Day-6, A: Treatment Day-9

The number of leukocytes increases and then decreases until the 9th day of maintenance period (Fig. 1). Increased leukocytes are a positive response that ECP recognizes as an immunogen body so that it is effective in initiating immune function. In general, leukocyte are 20 - 150 x 10^3 mm^{-3} cells [19,20,21]. The function of white blood cells is a marker of infection in the host's body by production of white blood cells in large quantities and as a host cellular defense process [10,22]. After vaccination, the number of white blood cells increases as an effort to mediate the immune response in the host's body. Then a decrease occurs which is thought to be the process of leukocyte migration at the site of infection. The number of white blood cells depends on factors of infection and inflammation of leukocytes in several organs [23].

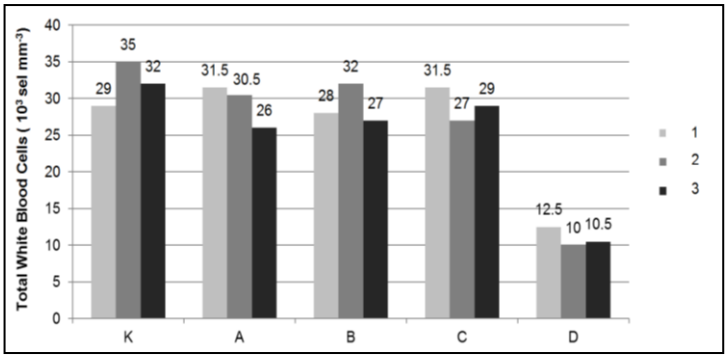

Fig. 1 *Presentation of Total White Blood Cells*

3.2 Differential Leukocytes

The results of differential leukocytes after ECP vaccination showed a sequential increase in treatment A and decreased in treatment D (Table 2). It is suspected that the ECP given effectively increases the differential activity of leukocytes to eliminate pathogens. The percentage of lymphocytes reaches 95%, other leukocytes are 5% in the blood [24].

Table 2
Data of Differential Leukocytes

No	Treatment		Ulangan (%) ± STD			Rerata (%) ± STD
			1	2	3	
		L	47 ± 2.31	51 ± 2.31	51 ± 2.31	50 ± 2.31
1	K	M	38 ± 1.53	35 ± 1.53	37 ± 1.53	37 ± 1.53
		N	15 ± 1.53	14 ± 1.53	12 ± 1.53	14 ± 1.53
		L	52 ± 1.00	51 ± 1.00	50 ± 1.00	51 ± 1.00
2	A	M	31 ± 0.58	31 ± 0.58	32 ± 0.58	31.3 ± 0.58
		N	17 ± 0.58	18 ± 0.58	18 ± 0.58	17.7 ± 0.58
		L	57 ± 0.58	58 ± 0.58	57 ± 0.58	57.3 ± 0.58
3	B	M	24 ± 1.00	22 ± 1.00	23 ± 1.00	23 ± 1.00
		N	19 ± 0.58	20 ± 0.58	20 ± 0.58	19.7 ± 0.58
		L	54 ± 2.00	56 ± 2.00	58 ± 2.00	56 ± 2.00
4	C	M	30 ± 2.00	28 ± 2.00	26 ± 2.00	28 ± 2.00
		N	16 ± 0.00	16 ± 0.00	16 ± 0.00	16 ± 0.00
		L	40 ± 1.53	38 ± 1.53	37 ± 1.53	38.3 ± 1.53
5	D	M	34 ± 1.53	33 ± 1.53	36 ± 1.53	34.3 ± 1.53
		N	26 ± 1.53	29 ± 1.53	27 ± 1.53	27.3 ± 1.53

K: control, A: Treatment Day-1, B: Treatment Day-3, C: Treatment Day-6, A: Treatment Day-9, L: Lymphocytes, Monocytes, N: Neuthrophil

The results of lymphocyte, monocyte and neutrophil cells showed significantly different between treatments. Lymphocytes reach more than 50%, monocytes between 23-32% and neutrophils with a value of ≤ 20%. Differential leukocytes consisting of lymphocytes, moocytes and neutrophils have their respective roles. Lymphocytes act as immunocompetent cells in immune response activation and defense, mediating cellular and humoral immune responses and evaluating immunomodulation in tilapia [14]. An increase in the number of monocytes post vaccination is the success of recognizing materials to be eliminated in the host immune system [25]. Whereas neutrophils are granulocytes that are activated to digest pathogens and mediate the acute inflammatory response in fish [26].

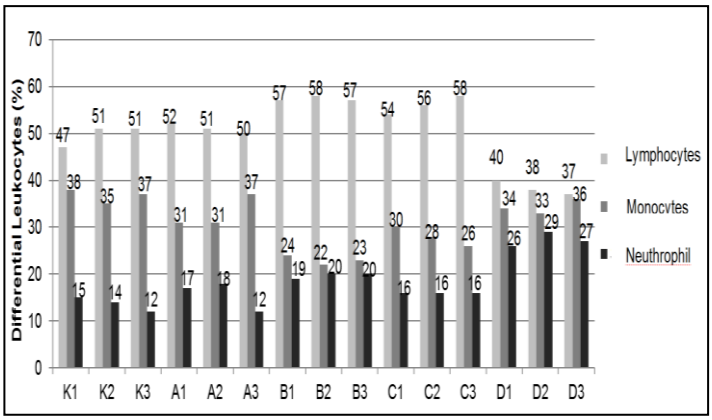

Fig. 2 *Presentation of Differential Leukocytes*

The differential leukocyte percentage in Fig. 2 shows increased fluctuations from the first day (A) to the 6th day (C) and decreased on the 9th day (D) after 0.1 ml tail^{-1} ECP vaccination. The cellular response in fish is faster in 1-5 days after treatment. Increased leukocytes occur on day 4, wherein the invasion antigen is first and and there is a humoral response on days 6-7. While the decline occurred on days 7 and 14 as an effort to get to normal conditions after exposure and can stop producing leukocytes [27]. Intraperitoneal vaccination shows an effective response faster and better characterized by a relatively higher survival rate [28].

3.3 Phagocytosis Activity

Phagocytosis plays an important role in one mechanism of the immune system, that it evaluate the health status of fish and can promote immunoglobulin production [29,30]. ECP vaccination showed a significant mean of> 81% versus controls 70.57% (Table 3) and differed significantly (P <0.05). Phagocytic activity increase as an indicator of an increase in the immune system and continues to increase until the 21st day [27,31].

Table 1
Data of Phagocytosis Activity

No	Treatment	Results (%)	Total	Average
1	K1	70.77 ± 0.59	211.72 ± 0.59	70.57 ± 0.59
	K2	69.90 ± 0.59		
	K3	71.05 ± 0.59		
2	A1	81.35 ± 1.46	248.66 ± 1.46	82.89 ± 1.46
	A2	84.26 ± 1.46		
	A3	83.06 ± 1.46		
3	B1	85.56 ± 1.86	251.42 ± 1.86	83.89 ± 1.86
	B2	84.01 ± 1.86		
	B3	81.85 ± 1.86		
4	C1	83.31 ± 1.81	252.54 ± 1.81	84.18 ± 1.81
	C2	82.97 ± 1.81		
	C3	86.26 ± 1.81		
5	D1	80.99 ± 1.10	244.55 ± 1.10	81.52 ± 1.10
	D2	80.77 ± 1.10		
	D3	82.78 ± 1.10		

K: control, A: Treatment Day-1, B: Treatment Day-3, C: Treatment Day-6, A: Treatment Day-9

Graph presentation results (Fig. 3) shows that during the 9-day maintenance period it has the same ability in the phagocytosis process. Smaller aggregate sizes can be easier to phagocytes to eliminate pathogens in cells [32]. Phagocytosis is carried out by macrophages by producing antibacterial substances to kill and destroy pathogens in phagolysosomes [26]. The particle pulverization is carried out by the lysosome enzyme and residual expenditure occurs [17]. The components released by phagocytes are antimicrobials such as degradative enzymes (proteases, phosphatases, esterases and lipases) and antimicrobial peptides that help destroy ingested pathogens [33,34,35].

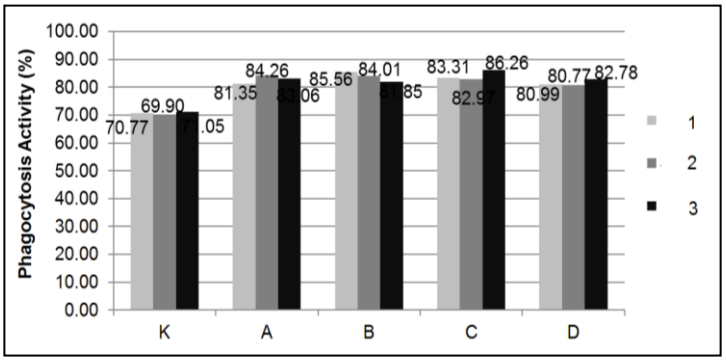

Fig. 3 *Presentation of Phagocytosis Activity*

3.4 Cytokinesis

The hematopoietic system plays a role in constant cell alteration to maintain a population of leukocytes, platelets and erythrocytes [36]. The cell cycle process consists of doubling genetic material, cell division and interactions between proteins and enzymes [37]. The results of the study with ECP vaccine treatment on Tilapia (O. niloticus) showed cell differentiation characterized by cell division into two new cells (Fig. 4). This is thought to be a form of self-defense from

antigens that the immune system responds to. The process is cytokinesis as the last process in cell division characterized by one cell into two daughter cells [38].

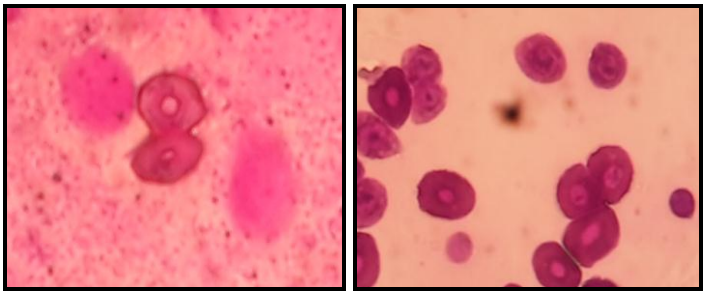

Fig. 4 *Cytokinesis with Midbody*

Two cells that are close together and consist of 2 nuclei t are still connected to the central axis of the microtubule and cytoplasm (midbody). During abscess, the new cells complete the midbody and dissociate with the secretion of midbody vesicles, specialization of the plasma membrane domain, reformation of the microtubule bundle and plasma membrane fission [39]. The protein needed in reducing the midbody is centralspindilin which includes kinesin ZEN-4, CYK-4, GAP from GTPase and B kinase and AIR-2 homologous proteins [38]. The mechanism of mitosis in general is with the formation of spindle and chromosome threads that are parallel to the metaphase. Then when the initial anaphase, the chromosome moves towards the pole with a spindle thread that extends. After that, the spindle has elongated and cylindrical cells. Cell division is characterized by the formation of a central shaft (contractile ring) and the formation of the midbody mediates two identical cells that will separate into new cells. The midbody stage is separated with proteins and produces two new daughter cells [40].

4 Conclusion

The treatment of intrapertonial vaccination of ECP 0.1 ml spesies^{-1} gave a positive influence on the innate immune response in total white blood cells, differential leukocytes and phagocytic activity and cell cytokinesis for 9 days after treatment.

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