Original article DOI: 10.19027/jai.17.1.94-103

Characterization of pathogenic bacteria in eel *Anguilla bicolor bicolor*

Karakterisasi bakteri patogen pada ikan sidat *Anguilla bicolor bicolor*

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(Received April 4, 2017; Accepted May 31, 2018)

ABSTRACT

This research aimed to characterize bacteria caused disease in eel *Anguilla bicolor bicolor*. The research was conducted in two steps. The first step included the isolation and identification of bacteria from the disease infected glass eel (average body length: 5.0 ± 0.5 cm, average weight: 0.5 ± 0.1 g). The observation were colony and cell morphology, physiology, and biochemical characterization of bacteria, hemolysis test, and bacteria identification performed by KIT API 20 E, KIT API 20 Strep, and KIT API 20 Listeria. The second step was Koch's postulate, tested on healthy elver with an average length of 15.00 ± 0.65 cm and weight of 3.00 ± 0.75 g. The results showed three dominant species of bacteria suspected as a causative agent in eel, namely: *Aeromonas hydrophila*, *Streptococcus agalactiae*, and *Listeria grayi*. Koch's postulates test proved that the *Aeromonas hydrophila* and *Streptococcus agalactiae* were virulent to *Anguilla bicolor bicolor*. Thus, *A. hydrophila* and *S. agalactiae* were disease-causing agent bacteria in eel.

Keywords: *Anguilla bicolor bicolor*, bacteria, *A. hydrophila*, *S. agalactiae.*

ABSTRAK

Penelitian ini bertujuan untuk mengkarakterisasi bakteri penyebab penyakit pada ikan sidat *Anguilla bicolor bicolor*. Penelitian ini dilakukan dalam dua tahap. Tahap pertama meliputi isolasi dan identifikasi bakteri dari ikan sidat kondisi sakit pada stadia *glass eel*. Ukuran panjang ikan sidat rata-rata 5 ± 0,5 cm dan bobot rata-rata 0,5 ± 0,08 g, pengamatan bentuk morfologi koloni dan morfologi sel, karakterisasi fisiologi, dan biokimia bakteri, serta uji hemolisis, dan identifikasi jenis bakteri dengan KIT API 20 E, KIT API 20 Strep, dan KIT API 20 Listeria. Tahap kedua yaitu uji postulat Koch pada ikan sidat kondisi sehat stadia elver yang berukuran panjang rata-rata 15 ± 0,65 cm dan bobot rata-rata 3 ± 0,75 g. Hasil penelitian diperoleh tiga jenis bakteri dominan yaitu *Aeromonas hydrophila*, *Streptococcus agalactiae*, dan *Listeria grayi*. Uji postulat Koch membuktikan bahwa bakteri *A. hydrophila* dan *S. agalactiae* bersifat virulen pada ikan sidat *Anguilla bicolor bicolor.* Dengan demikian maka bakteri *A. hydrophila* dan *S. agalactiae* sebagai bakteri penyebab penyakit pada ikan sidat.

Kata kunci: *Anguilla bicolor bicolor*, bakteri, *A. hydrophila, S. agalactiae*

INTRODUCTION

The eel *Anguilla bicolor bicolor* is a potential fish that has a very good prospect to be reared, because it has high economic value, both in the domestic and international markets. The countries such as Japan, South Korea, China, and Taiwan are the main eel market in Asia. The largest consumer of eel in the world is Japan, needs 150,000 tons from 250,000 tons of the world needs. Based on data from the FAO (2016), the global market demand for eel was 285,342 tons/year with a price range between Rp 150,000/kg–Rp 255.000/kg. This high demand of eel due to its advantages including the flesh is soft, tastes good, and contains important nutrients like DHA, EPA, and vitamin A (Bae *et al*., 2010).

The development production of eel can trigger the disease attack due to unbalanced interactions between the host, the pathogen, and the environment. The unbalanced interaction resulted in stress on the fish thus weakening the mechanism of self-defense and the disease attack the fish. Some previous study about the disease that usually attacks the eel is bacteria.

Joh *et al.* (2010) have reported the characteristics of *Yersinia ruckeri* bacteria isolated from a rearing pool of *Anguilla japonica* in Korea. In addition to *Yersinia ruckeri*, other pathogenic bacteria that attacked the *A.japonica* in Korea was *Edwardsiella tarda*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Aeromonas veronii*, *Streptococcus iniae*, *Citrobacter friend*, and *Vibrio alginolyticus* (Joh *et al*., 2013). Yi *et al*. (2013) have been doing a molecular characterization of Aeromonas sp strains that suspected as the agent of the disease in *A.japonica*. Seven potential virulence genes that tested were cytotoxic enterotoxin (act), two cytotonic enterotoxins (alt and ast) were glycerophospholipid acyltransferase, cholesterol: (gcaT), DNase (exu), lipase (lip), and flagellin (fla).

All of these genes were detected in all strains of *Aeromonas* sp. that being tested. Nakase *et al*. (2015) have reported another type of bacteria that isolated and characterized from *A. japonica* were *Lacinutrix algicola*, *Crocinitomix catalasitica* and *Pseudoalteromonas rubra* as the cause of death in the *A. japonica* glass eel stadia in Japan. This indicates that pathogenic bacteria is potential to cause disease in eel. Study of the bacterial species into a disease-causing agent in *A. japonica* in Korea and Japan have been many reported, but specifically in *A. bicolor bicolor* that is reared in Indonesia has never been reported.

Therefore, it needs a further study on disease-causing agent bacteria in *A. bicolor bicolor,* expects to be used as preliminary information to determine the proper method to control the disease in eel. This study aimed to characterize the disease-causing agent bacteria in eel *Anguilla bicolor bicolor*.

MATERIALS AND METHODS

The experimental fish

The eel with the symptoms of infected clinical disease, such as tend to dwell at the bottom of the container, not actively move, has a slow response, bleeding in pectoral fin, pelvic fin, and dorsal fin, weak, missing body balance, decreased appetite, has the white patches around the body, and the body becomes not slippery. Then the eel put in a plastic bag with oxygen, it carried to the Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, to be identified.

Bacteria isolation

The bacteria was isolated from sick eel *A. bicolor bicolor* derived from PT. Laju Banyu Semesta, Jalan Cikampak-Segog Km. 8, Kampung Cipicung, Desa Cibening, Kecamatan Pamijahan, Kabupaten Bogor, Jawa Barat. The sample was 10 fishes with an average length of 5 ± 0.5 cm and weight of 0.5 ± 0.08 g.

The experimental fish crushed in Eppendorf tube by using grinder until subtle. Then it weighed up to 0.1 g and added the sterile phosphate buffer saline (PBS) solutions 0.9 mL, homogenized it with the vortex. Furthermore, for the experimental water was used 0.1 mL to dissolve in 0.9 in sterile PBS. The dissolved sample was diluted in serial (Madigan *et al*., 2011) with dilution 10^{-1}–10^{-7}. Each of serial dilution was plating two times with sampling of 0.05 mL of every inoculated sequence in tryptone soy agar (TSA) plate, then incubated for 24 hours in 28°C.

Bacterial identification carried out by separating the colony that grows based on the color, the shape, the elevation, consistency, and the size of each colony. Different bacteria that grew on each dilution was refined through the scratch quadrants on TSA plate by taking all the

different bacteria colonies at each dilution. When in a plate was already obtained homogeneous colonies (pure colonies), then it inoculated again into TSA in the tube as a stock and incubated at a temperature of 28 °C for 24 hours.

The identification of bacterial isolates

The growth of bacteria colony observed based on the morphology characteristics of the colony with the scratch method and the cell morphology with the Gram staining. Each different morphology of colony further tested through the biochemical and the physiological. Biochemical tests used include oxidase test, catalase test, oxidative/fermentative (O/F) test, and motility test. If the bacterial colony looked homogeneous, then the bacteria colony separated and tested further by using KIT API 20 E, KIT API 20 Strep, and KIT API 20 Listeria (Biomeriaux, France). The bacteria that tested in KIT API was the result of a biochemical test and Gram staining, it was *Aeromonas* sp., *Streptococcus* sp., and *Listeria* sp.

Hemolysis test

Hemolysis test refers to Sharma & Gupta (2014) aims to analyze the ability of the isolates of bacteria to lysis the blood cells characterized by the formation of a clear zone around the colony. There are three types of hemolysis, i.e., β-hemolysis that is capable to lysis the blood cells in total characterized by a clear zone; α-hemolysis that is capable to lysis most of the blood cells formed the green color around the colony; γ-hemolysis that is incapable to lysis the blood cells formed purple color around the colony. The medium that used was TSA added with 5% of sheep blood. The bacterial isolates from the Gram staining test results and the biochemical test grew on blood agar media, and then it incubated at a temperature of 28 °C for 24 hours.

Koch's postulate

The experimental fish for Koch's postulates was the healthy eel derived from Production Engineering and Management of Aquaculture Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University. The fish had an average length of 15.00 ± 0.65 cm and an average weight of 3.00 ± 0.75 g. Fish were reared in the aquarium sizing 30×28×30 cm^3. There were five fishes with two replications for each aquarium. For rearing the fishes, the water precipitated for 48 hours, after that the aquarium filled with that water as much as 20 liters. Then the 0.0005 mL/L of chlorine added as antiseptic for killing the pathogen and the water aerated for 24 hours. All the treatment water then disposed and the aquarium filled again with the water to rear the fishes. The fishes acclimatized for 30 minutes before rearing. The fishes for Koch's postulate reared for seven days. The fish fed three times a day (at 08.00 a.m, 13.00 p.m, and 20.00 p.m) by at satiation. The feed that used was commercial feed sizing 2 mm with 50% of protein. The water treatment has done by uptake the uneaten feed and the fish's feces before and after giving the feed. The water changed every day as much as 30%.

The Koch's postulate has done by injecting the chosen isolate through intramuscular injection (Peyghan et al., 2010). The chosen isolates for this study were *A. hydrophila, S. agalactiae,* and *L. grayi. A. hydrophila* and *L. grayi* cultured in 10 mL of trypticase soy broth (TSB) media then incubated in water bath shaker at 28 °C for 24 hours. Beside of that, the *S. agalactiae* cultured in 10 mL of brain heart infusion broth (BHIB) media at 29 °C for 48 hours. The Koch's postulate was done by using five different doses of bacterial density (10^1, 10^2, 10^3, 10^4, and 10^5 CFU/fishes) and a control (using PBS) to inject the fish as much as 0.05 mL, respectively. Every treatment used two replications. The fishes reared for seven days, then the clinical symptoms and the mortality rate are observed.

Parameters

The dominance of bacteria

The determination of the dominance of bacteria referred to Thomas *et al.* (2015), the sample diluted through serial dilution as in the procedure by Madigan et al. (2011) by plating in TSA media. The bacterial density for the initial dilution was 30–300 colonies; it used to find the dominance group of bacteria. The highest dilution used to determine three bacteria that most dominate the colonies.

Lethal doses $_{50}$ (LD_{50}) test

The LD_{50} test used to determine the dose of bacteria density as the result of chosen isolates in eel that cause 50% of mortality from the total observed fishes referred to Reed and Muench (1938).

The changes of swimming behavior

The changes of swimming behavior in fish referred to Hardi *et al.* (2011), i.e. the change

Table 1. The parameter of water quality in the rearing of eel *Anguilla bicolor bicolor* for seven days

Parameters	Units	The average values	The average optimal values	Measurement tools
Water temperature	°C	27–30	28–33 (Harianto *et al*., 2014)	Thermometer
pH	-	6.80–7.50	6.0–8.0 (Harianto *et al*., 2014)	pH-meter
Dissolved oxygen	mg/L	4.50–6.35	>3 (Harianto *et al.*, 2014)	DO-meter
Ammonia	mg/L	0.03–0.05	<0.1 (Tesch, 2003)	Spectrofotometer

of movement in the water column (swim on the surface, drifting, or at the bottom of the aquarium), the body movement (weak or aggressive), and swimming behavior (recurring, whirling, and irregular swim). The observations conducted every day at the same time, at 13.00 p.m. The number of the experimental fish was five fishes of each aquarium in each treatment with two replications. The swimming behavior of every fish observed.

The clinical symptoms and the changes of fish's organs

The observed clinical symptoms were the eye condition (exophthalmia), the body color (pigmentation), hemorrhage, lesions, dropsy, and discoloration of the operculum (Yardimci & Aydin, 2011). The observations conducted every day at the same time, at 13.00 p.m. The number of the experimental fish was five fishes of each aquarium in each treatment with two replications. When fish has experienced one of the clinical symptoms, the clinical symptoms observed. The observation parameters were the discoloration and the condition of the liver and intestinal organ.

Survival rate

The survival rate is the percentage of the ratio of survived fish at the end of the study with the initial number of fish stocked. The following formula:

$$SR\ (\%) = \frac{Nt}{No} \times 100$$

Notes:
SR : Survival rate (%)
Nt : The number of fish at the end of the study
No : The number of fish at the initiation of study

The cumulative mortality could be known from observing the total mortality of eel during 15 days of rearing. Every replication in every treatment is averaged by using the SR formula above.

Water quality measurement

The water quality measurements carried out for seven days of rearing. The water quality includes water temperature, dissolved oxygen, and pH were done twice, in the morning and evening. The ammonia measurement was done twice during the rearing period, on the first day and the seventh day of rearing (Table 1).

Data analysis

The descriptive data includes the changes in swimming behavior, the clinical symptoms, the changes of fish's organs, and the water quality. The table formed data carried out with Microsoft Excel 2013 and Adobe Photoshop CS6 for picture formed data. Analysis of survival rate data presented with an analysis of variance (ANOVA), when the result considered significantly different then the analysis continued with post hoc test by using SPSS 22.

Table 2. The morphology of bacteria colony in eel *Anguilla bicolor bicolor*

Isolate code	Morphology				
	Color	Shape	Edge	Elevation	Size (cm)
A1	White murky	Round	Flat	Convex	0.2–0.3
A2	Yellow bluish	Round	Flat	Convex	0.1
A3	Milky white	Round	Wavy	Convex	0.2
A4	White transparent	Round	Wavy	Datar	0.2
A5	Tawny	Round	Flat	Datar	0.3
A6	Milky white	Filament	Stringy	Datar	0.4
A7	Yellow transparent	Round	Flat	Convex	0.2

Tabel 3. The bacteria identification results in eel *Anguilla bicolor bicolor*

Isolates code	Identification assay							Bacteria
	Gram	Shape	SIM	O/F	Catalase	Oxidation	Hemolysis	
A1	Negative	Rod	(+)	(+)	(+)	(+)	β- hemolysis	*Aeromonas* sp.
A2	Positive	Round	(-)	(+)	(-)	(-)	β- hemolysis	*Streptococcus* sp.
A3	Positive	Rod	(-)	(-)	(+)	(-)	α- hemolysis	*Listeria* sp.

RESULTS AND DISCUSSION

Results

The colony of bacteria in TSA medium

The observation result on the colony of bacteria from isolating sample in eel was seven different kind of bacteria grew in TSA medium for 24 hours in 28 °C in 10^{-2} of serial dilution (Table 2).

From seven different colonies, has chosen most three dominant colonies of bacteria. This was referred to the ability of bacteria to grow in the highest dilution serial. The dominant colony at the initial platting has shown off and decreased intensively at the next serial dilution so that it has chosen the most three dominant colonies in 10^{-7} of dilution, they were A1, A2, and A3.

Bacteria verification

All three different bacteria isolates were stained by Gram staining (Figure 1), analyzed the biochemist characteristic, the hemolysis characteristic, and the bacteria verification was used KIT API 20 E, KIT API 20 Strep, and KIT API 20 Listeria (Biomeriaux, France).

The isolate verification result by using KIT API 20 E showed 99.0% of similarity was A. hydrophila (Figure 1a, then the isolate verification result by using KIT API 20 Strep showed 99.3% of similarity was *S. agalactiae* (Figure 1b, and by using KIT API Listeria showed 93.4% of similarity was *L. grayi* (Figure 1c).

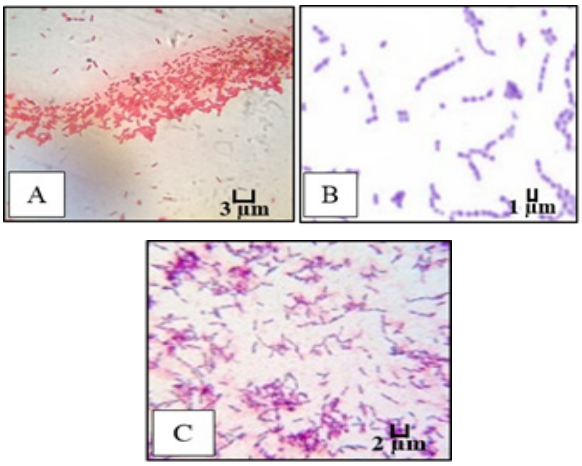

Figure 1. Cell morphology and Gram characteristic; (A) *Aeromonas* sp. (B) *Streptococcus* sp. (C) *Listeria* sp.

Lethal dose 50 (LD_{50}) test

This test used to prove the virulence of three bacteria isolated from an eel. The chosen isolates were *A. hydrophila, S. agalactiae,* and *L. grayi*. The results of the LD_{50} test for *A. hydrophila* was 10^4 CFU/mL and for *S. agalactiae* was 10^5 CFU/mL (Table 4), whereas the result of LD_{50} for L. grayi was none because all of the experimental bacteria concentration caused less than 50% of fish mortality.

The changes of swimming

The changes of swimming behavior after *A. hydrophila* and *S. agalactiae* injection has happened step by step in a different time, whereas the changes of swimming behavior after *L. Grayi* injection has not happened yet during seven days of observation after injection (Table 5). The eel showed the changes of swimming behavior symptoms started at 48 hours after injection (Figure 2).

Clinical symptoms

After injection of *A. hydrophila* and *S. agalactiae,* the eel showed some clinical symptoms, whereas after injection of *L. grayi* did

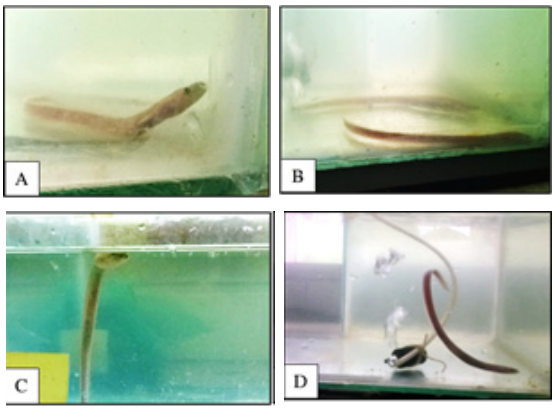

Figure 2. The changes of swimming behavior in eel: (A) weak response, not aggressive; (B) the fish stayed in the bottom of the aquarium; (C) the fish swamp close to the water surface (gasping); (D) whirling

Table 4. The LD_{50} calculation for *A. hydrophila, S. agalactiae,* and *L. grayi*.

Bacteria	Density dose	Dead	Alive	Accumulation value		Percentage (%)	Log LD_{50}	LD_{50} (CFU/mL)
				Dead	Alive			
A. hydrophila	10^5	10	0	24	0	100	4.429	10^4
	10^4	8	2	14	2	87.5		
	10^3	6	4	6	6	50		
	10^2	0	10	0	16	0		
	10^1	0	10	0	26	0		
S. agalactiae	10^5	10	0	22	0	100,0	5.482	10^5
	10^4	8	2	12	2	85.7		
	10^3	4	6	4	8	33.3		
	10^2	0	10	0	18	0.0		
	10^1	0	10	0	28	0		
L. grayi	10^5	2	8	2	8	20.0	-	-
	10^4	0	10	0	18	0.0		
	10^3	0	10	0	28	0.0		
	10^2	0	10	0	38	0.0		
	10^1	0	10	0	48	0.0		
Control	PBS	0	10	0	10	0.0	-	-

not show any clinical symptoms. The time when clinical symptom in eel occur showed in Table 6. The redness in eel's body occurred within 24 hours after injection. Moreover, the lesions and the redness in eels' gill occurred in 48 hours after injection (Figure 3).

The changes of organs after the injection of *A. hydrophila* and *S. agalactiae* experienced with unhealthy clinical symptoms (Figure 4), whereas the changes of organs after the injection of L. grayi showed none (Table 6).

The survival rate

The survival rate of eel after injection with *A. hydrophila*, *S. agalactiae*, *L. grayi,* and control showed significantly different among treatments ($P<0.05$) (Figure 5).

According to seven days of observation showed that the eel started to experience mortality

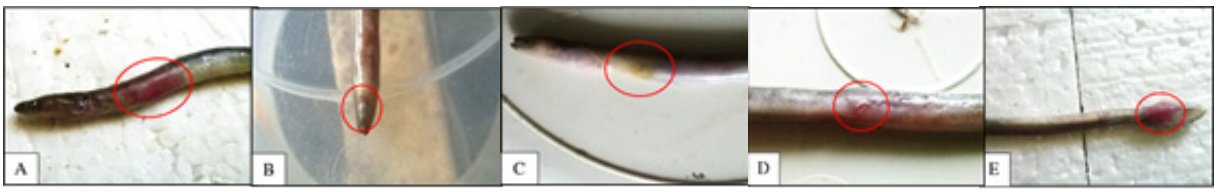

Figure 3. The clinical symptoms in eel: (A) redness; (B) exophthalmia; (C) green patches in abdomen; (D) lesions; (E) redness in gill

Table 5. The changes of swimming behavior in eel after *A. hydrophila, S. agalactiae*, and *L. Grayi* injection

SB	The first time of swimming behavior changes after injection (hour)															
	A. hydrophila					*S. agalactiae*					*L. grayi*					Control
	10^1	10^2	10^3	10^4	10^5	10^1	10^2	10^3	10^4	10^5	10^1	10^2	10^3	10^4	10^5	PBS
A	120	120	96	48	48	120	120	120	48	48	TG	TG	TG	144	168	TG
B	TG	144	72	48	48	TG	144	120	72	48	TG	TG	TG	TG	TG	TG
C	TG	TG	TG	96	96	TG	TG	TG	TG	96	TG	TG	TG	TG	TG	TG
D	TG	TG	144	48	48	TG	TG	120	72	72	TG	TG	TG	TG	T G	TG

Notes: SB= swimming behavior; A= weak response, not aggressive; B= the fish stayed in the bottom of aquarium; C= the fish swamp close to the water surface (gasping); D= whirling; TG= the fish swamp normally

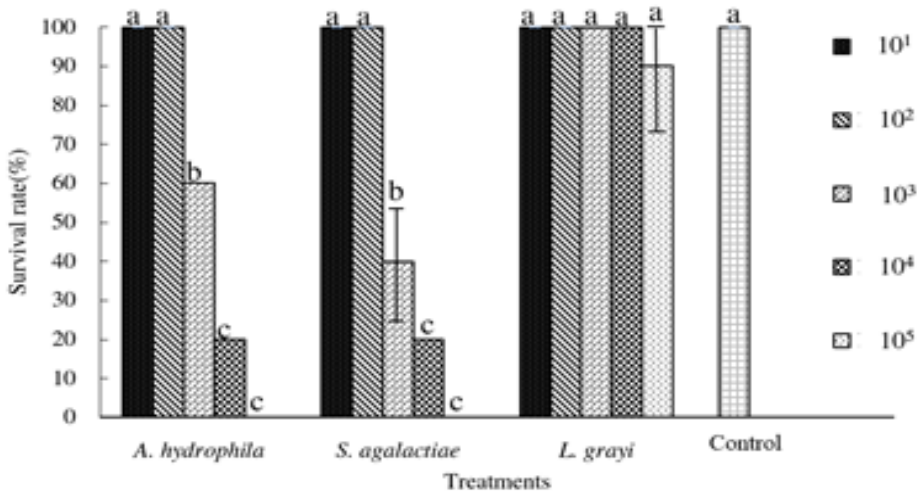

Figure 5. The survival rate of eel after injection with *A. hydrophila*, *S. agalactiae*, *L. grayi*, and control. The different superscript in every group of bacteria showed significantly different (P<0.05) in Duncan's test.

in day 2 after injection. The survival rate in eel injected with *A. hydrophila* and *S. agalactiae* in 10^4 CFU/mL of the density of each was 20%, respectively. Whereas the eel injected with *L. grayi* and control in 10^4 CFU/mL of the density of each reached 100%. The survival rate of eel injected with *A. hydrophila* and *S. agalactiae* was significantly different with *L. grayi* injection (P<0.05). The differences of cumulative mortality after *A. hydrophila* and *S. agalactiae* injection with 10^4 CFU/mL of density that showed in day 5 were 60% and 40%. The peaks of mortality in eel that injected with *A. hydrophila* and *S. agalactiae* has happened on day 6 of each were 80% and 60%, respectively. This showed that cumulative mortality of eel injected with *A. hydrophila* was higher than *S. agalactiae* injection. Whereas, the PBS injection was not experienced a mortality until the end of 15 days of rearing (Figure 6).

Discussions

The disease is an abnormal condition caused by a harmful microorganism. The microorganism occurred because of the unbalanced interaction between the environment, the host, and the pathogen. This unbalanced interaction caused a stressful condition for fish thereby the immunity mechanism becomes terrible (Nakase *et al.*, 2015). One of microorganism that caused disease in eel is bacteria. Bacteria can cause disease in fish through some attacks in both external and internal organs. Tesch (2003) stated that clinical symptoms in eel *Anguilla* sp. that caused by the bacteria are an infection in the skin, hemorrhage in fish's body, lesions (ulcer), exophthalmia, white patches, and redness in fish's body. The observation result in experimental eel that has been indicated experience some clinical symptoms were the weakness movements, tend to

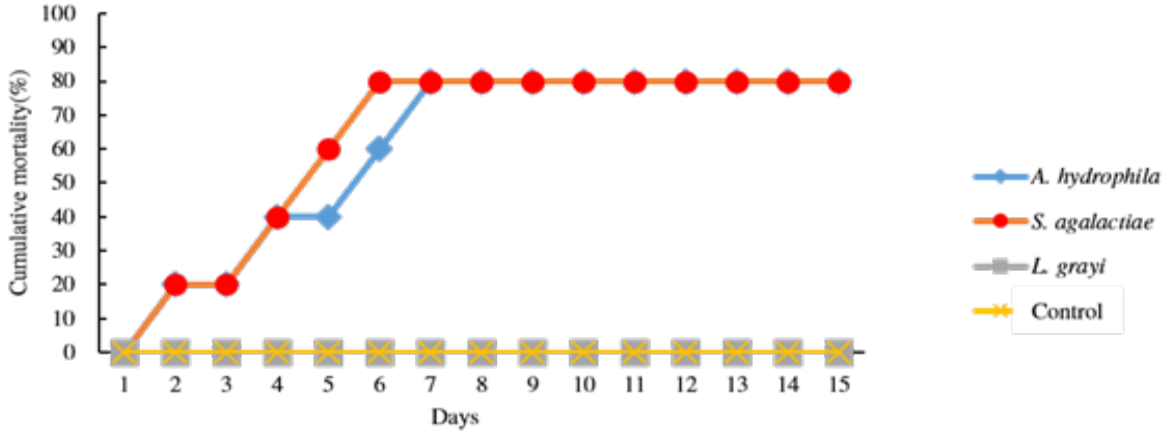

Figure 6. The cumulative mortality in eel after the challenge with *A. hydrophila*, *S. agalactiae*, *L. grayi* at 10^4 CFU/mL

stay at the bottom of the aquarium, white patches in fish's body, and loss of appetite.

The isolated bacteria of eel is a pathogen kind bacteria, it was in line with Joh *et al*. (2013). *A. hydrophila* is a negative Gram bacteria, short rod-shaped bacteria, motil, fermentative, and β-hemolytic (Table 3). The β-hemolytic bacteria is able to lysis the erythrocyte perfectly, proved with the existence of clear zone in blood agar medium (Sharma & Gupta, 2014). *A. hydrophila* is a common bacteria in fresh water fish. Yi et al. (2013) have proved that some strains of *Aeromonas* are completely pathogen in eel.

S. agalactiae is a positive Gram bacteria, coccus pairs-shaped or coccus chain-shaped bacteria, non-motile, fermentative, and β-hemolytic (Table 3). Sheehan *et al*. (2009) mentioned that *S. agalactiae* could be β-hemolytic and non-hemolytic bacteria. *S. agalactiae* is one of the cause streptococcosis disease in tilapia. While *Streptococcus iniae* caused disease in eel *A. japonica* (Joh *et al*., 2013). This showed that *Streptococcus* bacteria is a pathogen in eel.

L. grayi is a positive Gram bacteria, rod-shaped, non-motile, oxidative, and α- hemolytic (Table 3). The α- hemolytic bacteria is not able to lysis the erythrocyte proved with the green-scratch in the blood-agar medium. There are six species of Listeria, they are Listeria diantaranya *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri*, and *Listeria grayi*. *L. monocytogenes* is a saprophytic bacteria in the soil and is a pathogenic bacteria in the animal and in the sensitive human (Freitag *et al*., 2009).

From the explanation above, *A. hydrophila* and *S.agalactiae* are pathogenic bacteria in eel. It has proved with the virulency of both bacteria, furthermore, it needs Koch's postulate test to find the causing-agent disease in eel *A. bicolor bicolor.*

According to the result of Koch's postulate, the LD_{50} value of *A. hydrophila, S. agalactiae, L. grayi*, dan the control was different from each other. The LD_{50} value of *A. hydrophila* was 10^4 CFU/mL (virulence bacteria). Lailler & Daigneault (1984) classified that *A. hydrophila* with 10^3-10^4 CFU/mL of LD_{50} value was very virulence strain bacteria, 10^4-10^5 CFU/mL of LD_{50} value was virulence strain bacteria, 10^5-10^6 CFU/mL of LD_{50} value was weak virulence strain bacteria, and more than 10^7 CFU/mL of LD_{50} value was non-virulence strain bacteria.

The LD_{50} value of *S. agalactiae* was 10^5 CFU/mL so that the *S. agalactiae* is a virulence bacteria. This was in line with Delannoy *et al.* (2014) that stated *S. agalactiae* with 10^2 to 10^5 CFU/mL of density dose classified as virulence strain bacteria. It showed that there was no significantly different between two bacteria isolates used for Koch's postulate test, but it was significantly different with *L. grayi* or with the control.

Koch's postulate test that has done through intramuscular injection method for each bacteria isolate gave an impact on the swimming behavior of eel. The occured changes of swimming behavior were fish swamp near the water surface (gasping), showed weak the response and not aggressive, the fish stayed at the bottom of the aquarium, and whirling. The changes occurred within 48 hours after injection. The behavioral changes in infected eel caused by the endotoxin virulence enzymes that has produced by *A. hydrophila*, i.e hemolysin, protease, and elastase (Samal *et al*., 2014).

The virulence of bacteria associated with the ability of bacteria to the invasion, to replicate, and to survive towards the host's immunity system, and the ability to cause cells damaged during the growth of disease (Khajanchi *et al*., 2009). This also happened in the changes of swimming behavior after *S. agalactiae* injection in 48 hours. *A. hydrophila* and *S. agalactiae* are biological stressors that can interfere the physiological condition of fish (Lin *et al*., 2014).

The infection affected an increase in respiration and blood pressure. The erythrocyte cells spare will be release during circulation process. In this condition, the erythrocyte cells tend to rudimentary, thereby the ability of hemoglobin to bind the oxygen is not been optimized yet. It causes oxygen deficiency in fish. The fish would adapt to the condition of the environment as swimming near the water surface to ease taking up the oxygen. But, instead of the lower the bacteria density dose so that the more normal the swimming behavior of eel. This was significantly different with the condition after *L. grayi* and control injection that has not shown the significant swimming behavior during seven days of rearing.

All of the clinical symptoms that occur were in line with Joh *et al.* (2013) stated that the clinical symptoms of eel infected by *A. hydrophila* were weak moving and swimming, anorexia, darker body, and less of appetite.

After injection with *A. hydrophila* and *S. agalactiae*, the eel showed the same clinical symptoms, as red patches in the body, lesions, and hemorrhage, the gill became red exophthalmia, and red patches in the abdomen. The clinical symptoms occurred in 24 hours were red patches

in the body and exophthalmia, whereas the lesions occurred within 48 hours after injection. This indicated that the virulence of *A. hydrophila* and *S. agalactiae* occurred in the density dose of 10^4–10^5 CFU/mL, meanwhile the density dose of 10^1–10^2 CFU/mL had lower virulence because the clinical symptoms occurred in 144 hours 50 168 hours after injection. The clinical symptoms occurred in 72 hours and 120 hours after *L. grayi* injection were red patches and lesions.

After the eel injected with PBS as control was not showing any abnormalities in the fish body since the first day to seven days of rearing. Figures *et al.* (2007) mentioned that *Aeromonas* strain is a pathogenic bacteria in freshwater fish. Meanwhile, the *S. agalactiae* characterized as pathogenic bacteria with septicemia and meningoencephalitis as initial symptoms (Mian *et al*., 2009).

Hemorrhage in the fish body caused by hemolysin toxic through destroying the erythrocyte cells, thereby the cells out from the blood vessels evoked the red patches in the skin. The toxin power associated with the specific receptor cells. The interaction between the receptor cells and hemolysin occurs lesions in the body of fish (Mangunwardoyo *et al*., 2010). The extracellular toxin has two determining virulence marker, i.e inherent area is an area for the toxin to attach to specific receptor cells and active area as the main cause of infection in cells.

Hardi *et al.* (2011) mentioned that the symptoms occurred in the eye of fish infected by *S. agalactiae* are opacity and purulent moreover, exophthalmia and hemorrhage can occur. Exotoxin (ECP) of *S. agalactiae* spreading out to the eye that causes hypertrophy, this condition cause exophthalmia and some changes in fish. It indicated that ECP of *S. agalactiae* is a causing agent of the change of the eel's eye.

The fish experienced clinical symptoms after injected with *A. hydrophila* and *S. agalactiae* and experienced none (normal) after being injected with *L. grayi* and the control. The infected eel and the health eel showed the different changes in the liver and the intestine. The liver and the intestine experienced the color changes from clear colored to pale colored and greenish to darker color, the worst thing was the liver experienced liver swelling. The disruption of the function of the liver caused by the increase of liver performance to collect, to convert, to accumulate the metabolic substance, to neutralize, and to remove the toxic substance (Szabo *et al*., 2010).

According to the result from this study, the survival rate of eel in control treatment was 100%. Whereas the survival rate in *L. grayi* treatment with density dose of 10^5 CFU/mL was 80%, in *A. hydrophila* treatment with density dose of 10^4 CFU/mL was 20%, and in *S. agalactiae* treatment with density dose of 10^5 CFU/mL was 0%. This all showed that the high-density doses caused high mortality in eel. It was different in *L. grayi* treatment, even though it had high-density doses, it did not cause high mortality in eel (tend to low mortality) and did not significantly different with the control treatment. After *A. hydrophila* and *S. agalactiae* injections, the fish dead in day 2. There was a different cumulative mortality among *A. hydrophila* and *S. agalactiae.*

The highest cumulative mortality in *S. agalactiae* treatment that occurred on day 6 was 80%, meanwhile, in *A. hydrophila* treatment, the mortality symptoms occurred on day 6 was 60%. It showed that the virulence of *S. agalactiae* is higher than *A. hydrophila.* The peak of death occurred on day 6, respectively. This indicated that *A. hydrophila* infection is chronic (Yardimci & Aydin, 2011). Until the 15 days of rearing, the mortality did not occur again in all treatments.

CONCLUSION

According to the result of this study, it has proved that *A. hydrophila* and *S. agalactiae* are virulence to eel *Anguilla bicolor bicolor.*

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