KORESPONDENSI PAPER

JUDUL : The Diversity of Causative Agent Associated With Bacterial Diseases on Catfish (*Clarias gariepinus*) with Molecular Based from Kendal, Indonesia

JURNAL : 6th International Seminar on New Paradigm and Innovation of Natural Sciences and its Application, Vol. 23, No. 7, 2017

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The Diversity of Causative Agent Associated With Bacterial Diseases on Catfish (*Clarias gariepinus*) with Molecular Based and Their Sensitivity to Fish Drugs from Kendal, Indonesia

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Abstract

Causative agents diversity of bacterial diseases of catfish was isolated from Kendal Regency, Indonesia and based on postulat Koch results. Twenty bacteria (K01 – K20) were isolated from external wound and kidney of moribound catfish with TCBS, NA and GSP medium. The postulat results showed that three isolates (K6, K14 and K19) were weaken up to 60% of fishes and caused 10 – 20 % mortality. On the other hand, there were 14 isolates that did not demosntrated their virulence. Based on 16S rDNA sequence analysis, strain K6, K14 and K19 were closely related to *Aeromonas sobria* (97%.), *Pseudomonas plecoglossicida* (96%.), *Aeromonas caviae* (96%.) respectively. Sensitivity test to all isolates showed that these causative agents resistant to some fish drugs.

Keywords: Causative agent, bacterial diseases, catfish, 16S rDNA.

1. Introduction

Catfish (*Clarias gariepinus*) is the one of important aquaculture commodity in Kendal Regency. Kendal is one of catfish (*c. gariepinus*) producer in central java , producing approximately 21.967,06 tonnes in 2015.[1] Increasing of catfish demand for domestic markets encourages the fish farmers to increase their production. This was done through intensification and extensification. Unappropriate management of intensive culture would cause a negative impacts, such as bacterial disease outbreaks.[2,3]

Bacterial disease is still a big concern in catfish culture. Bacterial infection in the catfish was characterized by pale nor blacken of the body, *haemorrhagic* surrounds the mouths, fins, and tails, exopthalmia, fin root, body wounds and pale; dark color on liver and kidneys; red and root antenna.[4,5] *Aeromonas hidrophylla, A. caviae* and *A. sobria*;[4,5] *A. salmonicida*; [6] *Vibrio sp.*;[7] *Pseudomonas* spp. [8] *Edwardsiella ictaluri*,[9] were reported as

causative agents of bacterial diseases on catfish. These pathogens caused high mortality in larvae, fingerling, adult and broodstock,[10] and in catfish larvae up to 70%.[4]

Several researches had been performed to find out bacterial pathogen on cat fish based on the 16S rDNA gene sequences.[11, 2, 4] However, to our knowledge, there were limited reports being documented so far describing the application of polymerase chain reaction on the diversity of causative agent associated with bacterial diseases on cat fish derived from intensive pond of Kendal Regency, Central Java, Indonesia. This research is important in designing disease prevention strategy and supporting the catfish production.

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2.Material And Methods

The moribound catfish were collected from intensive culture ponds of central production in Kendal Regency. Moribound catfish were kept in the container and immediately brought to Integrated Laboratory of Diponegoro University for bacterial isolation. Bacteria were isolated from Kidney and the wound by streak method then cultured onto Thio-Sulphate Citrate Bile Salt Sucrose (TCBS), Tryptic Soybean Agar (TSA) and Glutamate Starch Penicillin (GSP) medium. Morphological features of the colonies were randomly picked and purified by re-struk a single colony to the plate.[12]

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Postulate Koch test was conducted to twenty isolates by using 420 healthy catfish as an experimental animals. During acclimatitation experimental catfish were divided into 42 aquariums. Twenty isolates were cultured in zobell liquid medium, then intramusculary injected on healthy catfish with 0.1 mL of inoculants bacterial density of 10^8 CFU/mL. Clinical signs and mortality of catfish were observed for 96 hours.

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Molecular characterization 16S rDNA of three isolates were carried out [13,14,15] . They further explained that the Polymerase Chain Reaction (PCR) was conducted with Eppendorf Mastercycler (Eppendorf Inc. Germany). Two primers, GM3F (5'AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') were used to amplify nearly complete 16S rDNA gene. Prior to PCR, genomic DNA of three causative agent assosiated with bacterial diseases on the catfish for PCR analysis were extracted from cell materials taken from agar plate, suspended in steril water (Sigma, Germany) and subjected to five cycles of freeze (-80ºC) and thaw (95ºC). Then, PCR amplification of partial 16S rDNA gene of bacteria and purification of PCR products were perfomed based on the method of. [13] Sequencing were commenced to gain the alkaline composition that made up DNA sequences. The sequencing was done using the Big Dye Terminator V3.1 dyes and automatic DNA sequencer ABI3130 Genetic Analyzer XL Applied Biosystemsat Macrogen

Korea. Whereas, the determined DNA sequences of causative agent were then compared for homology to the BLAST *(Basic Local Alignment Search Too*l) on *National Center for Biotechnology Information, National Institute for Health* database USA[6]

Three causative agent were tested their sensitivity to four fish drugs (A ™, B ™, C ™ and D ™) in vitro based on method[17]. Inhibition of the causative agents by each drugs were demonstrated by production of clear zone in the bacterial colonies area. Sensitivity test was evaluated according_standard developed by National Committee for Clinical Laboratory Standards.[18]

2. Results and Discussion

Clinical signs of moribound catfish which affected by bacterial diseases from intensive pond of Kendal were *haemorrhagic* surrounds the mouth, fins, and tail. Exopthalmia; fin root; body wounds and pale; dark color in liver and kidney. This morpholocical symptom (body wound and pale, *haemorrhagic* surrounds the mouths, fins, and tails; red and root antenna), the behaviour abnormalities such as body upsite down, lethargy, decreased appetite, increased mucus production, swimming imbalance, deficiency in oxygen uptake were also found in experimental catfish. The clinical signs above have been reported [19,20,4]. These clinical signs observed in the present study has also been observed in naturally-diseased catfish cultured.[19] These clinical signs were observed in the present study may due to attachment and colonization of many opportunistic pathogens to fish skin of cat fish.[4]

Twenty bacterial isolates were obtained from kidney, fins root, and body wound of moribound catfish (Table 1.). Postulate Koch test results indicated that three isolates namely K6, K14 and K19 were able caused disease symptom up to 60 % of the challenged catfish, whilst the three isolates was caused mortality range of 10 – 20 % (Table 2). he present study also found that experimentally catfish were injected by others isolates and PBS had 100% survival rate and normal behaviour. Therefore, these isolates (K6, K14 and K19) were positively confirmed as a causative agent assosiate bacterial diseases in catfish from intensive culture pond that is located at Kendal Regency. These mortality rate was lower than that reported,[4] in catfish *A. sobria* infected (30%) and *A. caviae*- infected (20-30%). Moreover, *A. sobria* was less pathogenicity compare to *A. hydrophila* on Stinging Catfish Shing (*Heteropneustes fossilis*).[6] The occurance pathogenicity of these bacteria on the fish was mainly contributed by the haemolysin and aerolysin.[21,22]

Based on the postulat Koch results (Table 2), three isolates (K6, K14 and K19) out of 22 isolates (K1 – K20) were choosen for further investigation (Table.3).

On the basis of sequence 16S rDNA analysis using Blast Sysytem (Table 4.), the results showed that causative agents of bacterial diseases on catfish from pond of kendal, i.e., K6, K14 and K19 were closely related to *Aeromonas sobria* (97%.), *Pseudomonas plecoglossicida* (96%.), *Aeromonas caviae* (96%.) respectively. Both of *Pseudomonas* spp. and *Aeromonas* spp. was naturally found in the aquatic environment, represent the Gram-negative commensal bacteria and include in pathogenic bacteria of fish.[12,7] The presents study revealed that the causative agents associated with bacterial diseases of catfish that was intensively cultured at Kendal ponds, were closely related to *A. sobria* (K6), *P. plecoglossicida* (K14),and *A. caviae* (K19). *A. sobria* and *A. caviae* were commonly reported as a bacterial pathogen assosiated with motile aeromonas septicemia in African cat fish,[4] catfish, *Clarias*; [11] haemorrhage of Tra catfish, *Pangasianodon hypophthalmus* [5], and walking cat fish culture, *Clarias bratachus*.[19] *A. sobria* was also reported in walking catfishes, *C. batrachus*; [21] Nordmann and Poirel, [22] catfish in India,[11] and in China as a aquatic pathogenic bacteria. While, *A. veronii* biovar *sobria* was also found as a causative agent of Epizootic Ulcerative Syndrome in fish in Bangladesh. [23]

Pseudomonas spp. has been reported to be an important fish pathogen that has endangered aquaculture.[8] *P. plecoglossicida* was found as causastive agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*.[24,25] Out break of this disease often occurs after seeds was introduced in culture ponds, and it also could found at any development stage during culture.[24] *P. plecoglossicida* was also found in natural environment.[26] On the other hand, *P. plecoglossicida* could become as a non pathogenic bacteria that was potencially produce an anti-tumor drugs;[27] and as a candidate of probiotic.[24] However, inthe present study also confirmed that *P. plecoglossicida* was found as a causative agent associated with bacterial diseases on cat fish that was intensively cultured in pond of Kendal regency.

The sensitivity test results (Table 4) revealed that three causative agents assosiate with bacterial disease in catfish from Kendal, namely : *A. sobria* (K6), *P. plecoglossicida* (K14), *A. caviae* (K19), were not sensitive to drug A ™, B ™, C ™ and D ™. These were detected by the formation of a clear zone around the paper discs on all three bacteria with diameter of 0 – 1,3 mm. The criteria of resistant bacteria if they have inhibitory zone ranged between 0-10 m.[18] The previous research also found that *Genus Aeromonas;* [28,29] *A. caviae;* [30] and *A sobria*,[31] were resistence to antibiotic. *Pseudomonas* spp. and *Aeromonas* spp. was resistant to multiple drug for 96.6% and 61.9%. [2] This resistance occurs may related to the using of

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fish drugs irrational dosage during aquaculture process,[32] and the content of the active compound (antibiotics) in fish drugs.[33] This resistance occurs when bacteria mutate in one way or another, it will impact on decreasing or losing of effectiveness of the drug, chemical compound or other material to prevent or treat infection.[34,28] Therefore, the three resistant causative agent associated with bacterial diseases on catfish was also caused by the irrational drugs administration during the production process, ie : the prevention and treatment of disease. Moreover, bacterial resistance could also occur due to mutate and selection randomly charge, in this case it act as an agent of antibiotic selection, so it was possible the group multiplication of resistant bacteria and suppressed the growth of bacteria sensitively to the antibiotic properties.[35] Furthermore, resistance to these fish drugs could be transmitted to other bacteria through a group of antibiotic resistant genes, between same genes locus with agents, such as plasmids, transposons, and integrons.[33,26]

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3. Conclusion

Bacterial causative agents in the catfish cultured in central production in Kendal with a molecular approach were *Pseudomonas plecoglossicida*, *Aeromonas veronii and Aeromonas sobria*. The result of sensitivity test obtained that three causative agents have resistant character to four fish drugs. Clinical signs of moribound catfish were *haemorhagic* surrounds the mouth, fins, tail and wound, exopthalmia, fins root, body wound and pale, dark color in liver and kidneys, red and root antenna.

6. Acknowledgments

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7. References and Note

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Table captions

Table 1. Characteristic of Isolates Bacteria Associated on Cat fish from intensive pond of Kendal, Indonesia.

Tabel 2. Percentage of Sick and Mortality of Cat fish (*C. gariepinus*) during Postulates Koch's Performed

Table 3. Three Isolates Selceted of Causative Agent assosiated with Bacterial Disease at Cat fish (*C. gariepinus*)

Table 4. Analisys of Three isolates compared with BLAST system

Tabel 5. The Result of Senstivity Test Three Causative Agent of Bacterial Diseases on Catfish in Kendal Regency

Table 1. Characteristic of Isolates Bacteria Associated on Cat fish from intensive pond of Kendal, Indonesia.

No.	Isolate code	Media	Source	Colony		
				Colour	Form	Characteristic
1	K.1	TCBS	Kidney	Green	Rounded	Convex
2	K.2	TCBS	Kidney	White	Rounded	Convex
3	K.3	TCBS	Fins root	Cream	Rounded	Convex
4	K.4	TCBS	Anal fin	Yellow	Rounded	Convex
5	K.5	TCBS	fins root	Green	Rounded	Convex
6	K.6	TCBS	fins root	Yellow	Rounded	Convex
7	K.7	TCBS	Tail fin	Yellow	Rounded	Convex
8	K.8	TCBS	Fin Dorsal	Yellow	Rounded	Convex
9	K.9	GSP	Fins root	Yellow	Rounded	Convex
10	K.10	GSP	Tail fin	Cream	Rounded	Convex
11	K.11	GSP	Kidney	Pink	Rounded	Convex
12	K.12	GSP	Kidney	White	Rounded	Convex
13	K.13	GSP	Anal fin	Yellow	Rounded	Convex
14	K.14	GSP	Kidney	Yellow	Rounded	Convex
15	K.15	NA	Fins root	Yellow	Rounded	Convex
16	K.16	NA	Dorsal fin	Yellow	Rounded	Convex
17	K.17	NA	Kidney	White	Rounded	Convex
18	K.18	NA	Kidney	Cream	Rounded	Convex
19	K.19	NA	Fins root	White	Rounded	Convex
20	K.20	NA	Tail fin	Yellow	Rounded	Convex

Tabel 2. Percentage of Sick and Mortality of Cat fish (*C. gariepinus*) during Postulates Koch's Performed

No.	Isolates code	Sick cat fish (%)	Total Mortality of cat fish (%)
1.	K6	60	20
2.	K14	35	10
3.	K19	35	10

Table 3. Three Isolates Selceted of Causative Agent assosiated with Bacterial Disease at Cat fish (*C. gariepinus*)

No.	**Isolats Code**	**Media**	**Source**	**Colony**		
				Color	Form	Characteristic
1	K.6	TCBS	fins root	Yellow	Rounded	Convex
2	K14	GSP	Kidney	Yellow	Rounded	Convex
3	K19	TCBS	Wound	Yellow	Irregullar	Convex

Table 4. Analisys of Three isolates compared with BLAST system

No.	Isolates Code	Results	Homologi (%)	No. access
1.	K 6	*Aeromonas* sobria	97	KC210798.1
2.	K14	*Pseudomonas plecoglossicida*	96	KC431807.1
3.	K19	*Aeromonas caviae*	96	JQ231158.1

Tabel 5. The Result of Senstivity Test Three Causative Agent of Bacterial Diseases on Catfish in Kendal Regency

Drugs	A ™						B ™,			Drug C™									D ™.					
	Times (Hour)																							
	24			48			24			48			24			48			24			48		
Isolate	Dosage (µl)																							
Code	6	8	10	6	8	10	2,5	5	7,5	2,5	5	7,5	2,5	5	7,5	2,5	5	7,5	2	4	6	2	4	6
K06	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K14	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K16	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

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REVISED PAPER

The Diversity of Causative Agent Associated With Bacterial Diseases on Catfish (*Clarias gariepinus*) with Molecular Based from Kendal, Indonesia

Deleted: and Their Sensitivity to Fish Drugs

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Abstract

Bacterial disease is still a big concern in intensive catfish culture in Indonesia. The aim of this research was to find out causative agent on cat fish based on the 16S rDNA gene sequences. This research combined between exploratory in the field and experimental method in Laboratory.

Causative agents diversity of bacterial diseases of catfish was isolated from Kendal Regency, Indonesia and based on postulat Koch results. Twenty bacteria (K01 – K20) were isolated from external wound and kidney of moribound catfish with TCBS, NA and GSP medium. The postulat results showed that three isolates (K6, K14 and K19) were weaken up to 60% of fishes and caused 10 – 20 % mortality. On the other hand, there were 14 isolates that did not demosntrated their virulence. Based on 16S rDNA sequence analysis, strain K6, K14 and K19 were closely related to *Aeromonas sobria* (97%.), *Pseudomonas plecoglossicida* (96%.), *Aeromonas caviae* (96%.) respectively. Sensitivity test to all isolates showed that these causative agents resistant to some fish drugs.

Keywords: Causative agent, bacterial diseases, catfish, 16S rDNA.

1. Introduction

Catfish (*Clarias gariepinus*) is the one of important aquaculture commodity in Kendal Regency. Kendal is one of catfish (*c. gariepinus*) producer in central java , producing approximately 21.967,06 tonnes in 2015.[1] Increasing of catfish demand for domestic markets encourages the fish farmers to increase their production. This was done through intensification and extensification. Unappropriate management of intensive culture would cause a negative impacts, such as bacterial disease outbreaks.[2,3]

Bacterial disease is still a big concern in catfish culture. Bacterial infection in the catfish was characterized by pale nor blacken of the body, *haemorrhagic* surrounds the

mouths, fins, and tails, exopthalmia, fin root, body wounds and pale; dark color on liver and kidneys; red and root antenna.[4,5] *Aeromonas hidrophylla*, *A. caviae* and *A. sobria*;[4,5] *A. salmonicida*; [6] *Vibrio sp*.;[7] *Pseudomonas* spp. [8] *Edwardsiella ictaluri*,[9] were reported as causative agents of bacterial diseases on catfish. These pathogens caused high mortality in larvae, fingerling, adult and broodstock,[10] and in catfish larvae up to 70%.[4]

Several researches had been performed to find out bacterial pathogen on cat fish based on the 16S rDNA gene sequences.[11, 2, 4] However, to our knowledge, there were limited reports being documented so far describing the application of polymerase chain reaction on the diversity of causative agent associated with bacterial diseases on cat fish derived from intensive pond of Kendal Regency, Central Java, Indonesia. This research is important in designing disease prevention strategy and supporting the catfish production.

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2.Material And Methods

The moribound catfish were collected from intensive culture ponds of central production in Kendal Regency on May to August 2016. Moribound catfish were kept in the container and immediately brought to Integrated Laboratory of Diponegoro University for bacterial isolation. Bacteria were isolated from Kidney and the wound by streak method then cultured onto Thio-Sulphate Citrate Bile Salt Sucrose (TCBS), Tryptic Soybean Agar (TSA) and Glutamate Starch Penicillin (GSP) medium. Morphological features of the colonies were randomly picked and purified by a single colony to the plate.[12]

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Postulate Koch test was conducted to twenty isolates by using 420 healthy catfish as an experimental animals. During acclimatitation experimental catfish were divided into 42 aquariums. Twenty isolates were cultured in zobell liquid medium, then intramusculary injected on healthy catfish with 0.1 mL of inoculants bacterial density of 10^8 CFU/mL. Clinical signs and mortality of catfish were observed for 96 hours.

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Molecular characterization 16S rDNA of three isolates were carried out [13,14,15] . They further explained that the Polymerase Chain Reaction (PCR) was conducted with Eppendorf Mastercycler (Eppendorf Inc. Germany). Two primers, GM3F (5'AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') were used to amplify nearly complete 16S rDNA gene. Prior to PCR, genomic DNA of three causative agent assosiated with bacterial diseases on the catfish for PCR analysis were extracted from cell materials taken from agar plate, suspended in steril water (Sigma, Germany) and subjected to five cycles of freeze (-80ºC) and thaw (95ºC). Then, PCR amplification of partial 16S rDNA gene of bacteria and purification of PCR products were perfomed based on

the method of. [13] Sequencing were commenced to gain the alkaline composition that made up DNA sequences. The sequencing was done using the Big Dye Terminator V3.1 dyes and automatic DNA sequencer ABI3130 Genetic Analyzer XL Applied Biosystemsat Macrogen Korea. Whereas, the determined DNA sequences of causative agent were then compared for homology to the BLAST *(Basic Local Alignment Search Too*l) on *National Center for Biotechnology Information, National Institute for Health* database USA[6]

Three causative agent were tested their sensitivity to four fish drugs (A ™, B ™, C ™ and D ™) in vitro based on method[17]. Inhibition of the causative agents by each drugs were demonstrated by production of clear zone in the bacterial colonies area. Sensitivity test was evaluated according standard developed by National Committee for Clinical Laboratory Standards.[18]

2. Results and Discussion

Clinical signs of moribound catfish which affected by bacterial diseases from intensive pond of Kendal were *haemorrhagic* surrounds the mouth, fins, and tail. Exopthalmia; fin root; body wounds and pale; dark color in liver and kidney. This morpholocical symptom (body wound and pale, *haemorrhagic* surrounds the mouths, fins, and tails; red and root antenna), the behaviour abnormalities such as body upsite down, lethargy, decreased appetite, increased mucus production, swimming imbalance, deficiency in oxygen uptake were also found in experimental catfish. The clinical signs above have been reported [19,20,4]. These clinical signs observed in the present study has also been observed in naturally-diseased catfish cultured.[19] These clinical signs were observed in the present study may due to attachment and colonization of many opportunistic pathogens to fish skin of cat fish.[4]

Twenty bacterial isolates were obtained from kidney, fins root, and body wound of moribound catfish (Table 1.). postulate Koch test results indicated that three isolates namely K6, K14 and K19 were able caused disease symptom up to 60 % of the challenged catfish, whilst the three isolates was caused mortality range of 10 – 20 % (Table 2). he present study also found that experimentally catfish were injected by others isolates and PBS had 100% survival rate and normal behaviour. Therefore, these isolates (K6, K14 and K19) were positively confirmed as a causative agent assosiate bacterial diseases in catfish from intensive culture pond that is located at Kendal Regency. These mortality rate was lower than that reported,[4] in catfish *A. sobria* infected (30%) and *A. caviae*- infected (20-30%). Moreover, *A. sobria* was less pathogenicity compare to *A. hydrophila* on Stinging Catfish

Shing (*Heteropneustes fossilis*).[6] The occurance pathogenicity of these bacteria on the fish was mainly contributed by the haemolysin and aerolysin.[21,22]

Based on the postulat Koch results (Table 2), three isolates (K6, K14 and K19) out of 22 isolates (K1 – K20) were choosen for further investigation (Table.3).

On the basis of sequence 16S rDNA analysis using Blast Sysytem (Table 4.), the results showed that causative agents of bacterial diseases on catfish from pond of kendal, i.e., K6, K14 and K19 were closely related to *Aeromonas sobria* (97%.), *Pseudomonas plecoglossicida* (96%.), *Aeromonas caviae* (96%.) respectively. Both of *Pseudomonas* spp. and *Aeromonas* spp. was naturally found in the aquatic environment, represent the Gram-negative commensal bacteria and include in pathogenic bacteria of fish.[12,7] The presents study revealed that the causative agents associated with bacterial diseases of catfish that was intensively cultured at Kendal ponds, were closely related to *A. sobria* (K6), *P. plecoglossicida* (K14),and *A. caviae* (K19). *A. sobria* and *A. caviae* were commonly reported as a bacterial pathogen assosiated with motile aeromonas septicemia in African cat fish,[4] catfish, *Clarias*; [11] haemorrhage of Tra catfish, *Pangasianodon hypophthalmus* [5], and walking cat fish culture, *Clarias bratachus*.[19] *A. sobria* was also reported in walking catfishes, *C. batrachus*; [21] Nordmann and Poirel, [22] catfish in India,[11] and in China as a aquatic pathogenic bacteria. While, *A. veronii* biovar *sobria* was also found as a causative agent of Epizootic Ulcerative Syndrome in fish in Bangladesh. [23]

Pseudomonas spp. has been reported to be an important fish pathogen that has endangered aquaculture.[8] *P. plecoglossicida* was found as causastive agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*.[24,25] Out break of this disease often occurs after seeds was introduced in culture ponds, and it also could found at any development stage during culture.[24] *P. plecoglossicida* was also found in natural environment.[26] On the other hand, *P. plecoglossicida* could become as a non pathogenic bacteria that was potencially produce an anti-tumor drugs;[27] and as a candidate of probiotic.[24] However, inthe present study also confirmed that *P. plecoglossicida* was found as a causative agent associated with bacterial diseases on cat fish that was intensively cultured in pond of Kendal regency.

The sensitivity test results (Table 4) revealed that three causative agents assosiate with bacterial disease in catfish from Kendal, namely : *A. sobria* (K6), *P. plecoglossicida* (K14), *A. caviae* (K19), were not sensitive to drug A ™, B ™, C ™ and D ™. These were detected by the formation of a clear zone around the paper discs on all three bacteria with diameter of 0 – 1,3 mm. The criteria of resistant bacteria if they have inhibitory zone ranged between 0-10 m.[18] The previous research also found that *Genus Aeromonas;* [28,29] *A. caviae;* [30] and *A*

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sobria,[31] were resistence to antibiotic. *Pseudomonas* spp. and *Aeromonas* spp. was resistant to multiple drug for 96.6% and 61.9%. [2] This resistance occurs may related to the using of fish drugs irrational dosage during aquaculture process,[32] and the content of the active compound (antibiotics) in fish drugs.[33] This resistance occurs when bacteria mutate in one way or another, it will impact on decreasing or losing of effectiveness of the drug, chemical compound or other material to prevent or treat infection.[34,28] Therefore, the three resistant causative agent associated with bacterial diseases on catfish was also caused by the irrational drugs administration during the production process, ie : the prevention and treatment of disease. Moreover, bacterial resistance could also occur due to mutate and selection randomly charge, in this case it act as an agent of antibiotic selection, so it was possible the group multiplication of resistant bacteria and suppressed the growth of bacteria sensitively to the antibiotic properties.[35] Furthermore, resistance to these fish drugs could be transmitted to other bacteria through a group of antibiotic resistant genes, between same genes locus with agents, such as plasmids, transposons, and integrons.[33,26]

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3. Conclusion

Bacterial causative agents in the catfish cultured in central production in Kendal with a molecular approach were *Pseudomonas plecoglossicida*, *Aeromonas veronii and Aeromonas sobria*. The result of sensitivity test obtained that three causative agents have resistant character to four fish drugs. Clinical signs of moribound catfish were *haemorhagic* surrounds the mouth, fins, tail and wound, exopthalmia, fins root, body wound and pale, dark color in liver and kidneys, red and root antenna.

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Table captions

Table 1. Characteristic of Isolates Bacteria Associated on Cat fish from intensive pond of Kendal, Indonesia.

Table 1. Characteristic of Isolates Bacteria Associated on Cat fish from intensive pond of Kendal, Indonesia.

No.	Isolate code	Media	Source	Colony		
				Colour	Form	Characteristic
1	K.1	TCBS	Kidney	Green	Rounded	Convex
2	K.2	TCBS	Kidney	White	Rounded	Convex
3	K.3	TCBS	Fins root	Cream	Rounded	Convex
4	K.4	TCBS	Anal fin	Yellow	Rounded	Convex
5	K.5	TCBS	fins root	Green	Rounded	Convex
6	K.6	TCBS	fins root	Yellow	Rounded	Convex
7	K.7	TCBS	Tail fin	Yellow	Rounded	Convex
8	K.8	TCBS	Fin Dorsal	Yellow	Rounded	Convex
9	K.9	GSP	Fins root	Yellow	Rounded	Convex
10	K.10	GSP	Tail fin	Cream	Rounded	Convex
11	K.11	GSP	Kidney	Pink	Rounded	Convex
12	K.12	GSP	Kidney	White	Rounded	Convex
13	K.13	GSP	Anal fin	Yellow	Rounded	Convex
14	K.14	GSP	Kidney	Yellow	Rounded	Convex
15	K.15	NA	Fins root	Yellow	Rounded	Convex
16	K.16	NA	Dorsal fin	Yellow	Rounded	Convex
17	K.17	NA	Kidney	White	Rounded	Convex
18	K.18	NA	Kidney	Cream	Rounded	Convex
19	K.19	NA	Fins root	White	Rounded	Convex
20	K.20	NA	Tail fin	Yellow	Rounded	Convex

Tabel 2. Percentage of Sick and Mortality of Cat fish (*C. gariepinus*) during Postulates Koch's Performed

No.	Isolates code	Sick cat fish (%)	Total Mortality of cat fish (%)
1.	K6	60	20
2.	K14	35	10
3.	K19	35	10

Table 3. Three Isolates Selceted of Causative Agent assosiated with Bacterial Disease at Cat fish (*C. gariepinus*)

No.	Isolats Code	Media	Source	Colony		
				Color	Form	Characteristic
1	K.6	TCBS	fins root	Yellow	Rounded	Convex
2	K14	GSP	Kidney	Yellow	Rounded	Convex
3	K19	TCBS	Wound	Yellow	Irregullar	Convex

Table 4. Analisys of Three isolates compared with BLAST system

No.	Isolates Code	Results	Homologi (%)	No. access
1.	K 6	*Aeromonas* sobria	97	KC210798.1
2.	K14	*Pseudomonas plecoglossicida*	96	KC431807.1
3.	K19	*Aeromonas caviae*	96	JQ231158.1

Tabel 5. The Result of Senstivity Test Three Causative Agent of Bacterial Diseases on Catfish in Kendal Regency

Drugs	A ™	B ™,	Drug C™	D ™.

Isolate Code	Times (Hour)																							
	24			48			24			48			24			48			24			48		
	Dosage (µl)																							
	6	8	10	6	8	10	2,5	5	7,5	2,5	5	7,5	2,5	5	7,5	2,5	5	7,5	2	4	6	2	4	6
K06	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K14	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K16	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Important Notification of the 6th ISNPINSA : Additional time for paper revision

From: committee 6thisnpinsa (6thisnpinsa@gmail.com)

To: sarjito_msdp@yahoo.com

Date: Wednesday, December 21, 2016 at 03:46 PM GMT+7

Dear Author of the 6th ISNPINSA,

Regarding to the negotiations of the deadline submission to the ASL Journal, hereby the 6th ISNPINSA committee provide another opportunity and additional time for author to revise and improve the full paper. The revision or improvement may include grammar and providing additional data (result and discussion) that must be based on the **ASL Journal Template** and **peer review guideline** (please check the ASL Journal template and peer review guideline that had been sent by the 6th ISNPINSA committee). For further editing process, herewith we re-attach your full paper.

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REVIEWED PAPER

The Diversity of Causative Agent Associated With Bacterial Diseases on Catfish (*Clarias gariepinus*) with Molecular Based from Kendal, Indonesia

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Bacterial disease is still a big concern in intensive catfish culture in Indonesia. The aim of this research was to find out causative agent on cat fish based on the 16S rDNA gene sequences. This research combined between exploratory in the field and experimental method in Laboratory. Causative agents diversity of bacterial diseases of catfish was isolated from Kendal Regency, Indonesia and based on postulat Koch results. Twenty bacteria (K01 – K20) were isolated from external wound and kidney of moribound catfish with TCBS, NA and GSP medium. The postulat results showed that three isolates (K6, K14 and K19) were weaken up to 60% of fishes and caused 10 – 20 % mortality. On the other hand, there were 14 isolates that did not demosntrated their virulence. Based on 16S rDNA sequence analysis, strain K6, K14 and K19 were closely related to *Aeromonas sobria* (97%.), *Pseudomonas plecoglossicida* (96%.), *Aeromonas caviae* (96%.) respectively. Sensitivity test to all isolates showed that these causative agents resistant to some fish drugs.

Keywords: Causative agent, bacterial diseases, catfish, 16S rDNA.

1. Introduction

Catfish (*Clarias gariepinus*) is the one of important aquaculture commodity in Kendal Regency. Kendal is one of catfish (*c. gariepinus*) producer in central java , producing approximately 21.967,06 tonnes in 2015.[1] Increasing of catfish demand for domestic markets encourages the fish farmers to increase their production. This was done through intensification and extensification. Unappropriate management of intensive culture would cause a negative impacts, such as bacterial disease outbreaks.[2,3]

Bacterial disease is still a big concern in catfish culture. Bacterial infection in the catfish was characterized by pale nor blacken of the body, *haemorrhagic* surrounds the

mouths, fins, and tails, exopthalmia, fin root, body wounds and pale; dark color on liver and kidneys; red and root antenna.[4,5] *Aeromonas hidrophylla*, *A. caviae* and *A. sobria*;[4,5] *A. salmonicida*; [6] *Vibrio sp*.;[7] *Pseudomonas* spp. [8] *Edwardsiella ictaluri*,[9] were reported as causative agents of bacterial diseases on catfish. These pathogens caused high mortality in larvae, fingerling, adult and broodstock,[10] and in catfish larvae up to 70%.[4]

Several researches had been performed to find out bacterial pathogen on cat fish based on the 16S rDNA gene sequences.[11, 2, 4] However, to our knowledge, there were limited reports being documented so far describing the application of polymerase chain reaction on the diversity of causative agent associated with bacterial diseases on cat fish derived from intensive pond of Kendal Regency, Central Java, Indonesia. This research is important in designing disease prevention strategy and supporting the catfish production.

2.Material And Methods

The moribound catfish were collected from intensive culture ponds of central production in Kendal Regency on May to August 2016. Moribound catfish were kept in the container and immediately brought to Integrated Laboratory of Diponegoro University for bacterial isolation. Bacteria were isolated from Kidney and the wound by streak method then cultured onto Thio-Sulphate Citrate Bile Salt Sucrose (TCBS), Tryptic Soybean Agar (TSA) and Glutamate Starch Penicillin (GSP) medium. Morphological features of the colonies were randomly picked and purified by a single colony to the plate.[12]

Postulate Koch test was conducted to twenty isolates by using 420 healthy catfish as an experimental animals. During acclimatitation experimental catfish were divided into 42 aquariums. Twenty isolates were cultured in zobell liquid medium, then intramusculary injected on healthy catfish with 0.1 mL of inoculants bacterial density of 10^8 CFU/mL. Clinical signs and mortality of catfish were observed for 96 hours.

Molecular characterization 16S rDNA of three isolates were carried out [13,14,15] . They further explained that the Polymerase Chain Reaction (PCR) was conducted with Eppendorf Mastercycler (Eppendorf Inc. Germany). Two primers, GM3F (5'AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') were used to amplify nearly complete 16S rDNA gene. Prior to PCR, genomic DNA of three causative agent assosiated with bacterial diseases on the catfish for PCR analysis were extracted from cell materials taken from agar plate, suspended in steril water (Sigma, Germany) and subjected to five cycles of freeze (-80ºC) and thaw (95ºC). Then, PCR amplification of partial 16S rDNA gene of bacteria and purification of PCR products were perfomed based on

the method of. [13] Sequencing were commenced to gain the alkaline composition that made up DNA sequences. The sequencing was done using the Big Dye Terminator V3.1 dyes and automatic DNA sequencer ABI3130 Genetic Analyzer XL Applied Biosystemsat Macrogen Korea. Whereas, the determined DNA sequences of causative agent were then compared for homology to the BLAST *(Basic Local Alignment Search Too*l) on *National Center for Biotechnology Information, National Institute for Health* database USA[6]

Three causative agent were tested their sensitivity to four fish drugs (A ™, B ™, C ™ and D ™) in vitro based on method[17]. Inhibition of the causative agents by each drugs were demonstrated by production of clear zone in the bacterial colonies area. Sensitivity test was evaluated according standard developed by National Committee for Clinical Laboratory Standards.[18]

2. Results and Discussion

Clinical signs of moribound catfish which affected by bacterial diseases from intensive pond of Kendal were *haemorrhagic* surrounds the mouth, fins, and tail. Exopthalmia; fin root; body wounds and pale; dark color in liver and kidney. This morpholocical symptom (body wound and pale, *haemorrhagic* surrounds the mouths, fins, and tails; red and root antenna), the behaviour abnormalities such as body upsite down, lethargy, decreased appetite, increased mucus production, swimming imbalance, deficiency in oxygen uptake were also found in experimental catfish. The clinical signs above have been reported [19,20,4]. These clinical signs observed in the present study has also been observed in naturally-diseased catfish cultured.[19] These clinical signs were observed in the present study may due to attachment and colonization of many opportunistic pathogens to fish skin of cat fish.[4]

Twenty bacterial isolates were obtained from kidney, fins root, and body wound of moribound catfish (Table 1.). Postulate Koch test results indicated that three isolates namely K6, K14 and K19 were able caused disease symptom up to 60 % of the challenged catfish, whilst the three isolates was caused mortality range of 10 – 20 % (Table 2). he present study also found that experimentally catfish were injected by others isolates and PBS had 100% survival rate and normal behaviour. Therefore, these isolates (K6, K14 and K19) were positively confirmed as a causative agent assosiate bacterial diseases in catfish from intensive culture pond that is located at Kendal Regency. These mortality rate was lower than that reported,[4] in catfish *A. sobria* infected (30%) and *A. caviae*- infected (20-30%). Moreover, *A. sobria* was less pathogenicity compare to *A. hydrophila* on Stinging Catfish

Shing (*Heteropneustes fossilis*).[6] The occurance pathogenicity of these bacteria on the fish was mainly contributed by the haemolysin and aerolysin.[21,22]

Based on the postulat Koch results (Table 2), three isolates (K6, K14 and K19) out of 22 isolates (K1 – K20) were choosen for further investigation (Table.3).

On the basis of sequence 16S rDNA analysis using Blast Sysytem (Table 4.), the results showed that causative agents of bacterial diseases on catfish from pond of kendal, i.e., K6, K14 and K19 were closely related to *Aeromonas sobria* (97%.), *Pseudomonas plecoglossicida* (96%.), *Aeromonas caviae* (96%.) respectively. Both of *Pseudomonas* spp. and *Aeromonas* spp. was naturally found in the aquatic environment, represent the Gram-negative commensal bacteria and include in pathogenic bacteria of fish.[12,7] The presents study revealed that the causative agents associated with bacterial diseases of catfish that was intensively cultured at Kendal ponds, were closely related to *A. sobria* (K6), *P. plecoglossicida* (K14),and *A. caviae* (K19). *A. sobria* and *A. caviae* were commonly reported as a bacterial pathogen assosiated with motile aeromonas septicemia in African cat fish,[4] catfish, *Clarias*; [11] haemorrhage of Tra catfish, *Pangasianodon hypophthalmus* [5], and walking cat fish culture, *Clarias bratachus*.[19] *A. sobria* was also reported in walking catfishes, *C. batrachus*; [21] Nordmann and Poirel, [22] catfish in India,[11] and in China as a aquatic pathogenic bacteria. While, *A. veronii* biovar *sobria* was also found as a causative agent of Epizootic Ulcerative Syndrome in fish in Bangladesh. [23]

Pseudomonas spp. has been reported to be an important fish pathogen that has endangered aquaculture.[8] *P. plecoglossicida* was found as causastive agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis*.[24,25] Out break of this disease often occurs after seeds was introduced in culture ponds, and it also could found at any development stage during culture.[24] *P. plecoglossicida* was also found in natural environment.[26] On the other hand, *P. plecoglossicida* could become as a non pathogenic bacteria that was potencially produce an anti-tumor drugs;[27] and as a candidate of probiotic.[24] However, inthe present study also confirmed that *P. plecoglossicida* was found as a causative agent associated with bacterial diseases on cat fish that was intensively cultured in pond of Kendal regency.

The sensitivity test results (Table 4) revealed that three causative agents assosiate with bacterial disease in catfish from Kendal, namely : *A. sobria* (K6), *P. plecoglossicida* (K14), *A. caviae* (K19), were not sensitive to drug A ™, B ™, C ™ and D ™. These were detected by the formation of a clear zone around the paper discs on all three bacteria with diameter of 0 – 1,3 mm. The criteria of resistant bacteria if they have inhibitory zone ranged between 0-10 m.[18] The previous research also found that *Genus Aeromonas;* [28,29] *A. caviae;* [30] and *A*

sobria,[31] were resistence to antibiotic. *Pseudomonas* spp. and *Aeromonas* spp. was resistant to multiple drug for 96.6% and 61.9%. [2] This resistance occurs may related to the using of fish drugs irrational dosage during aquaculture process,[32] and the content of the active compound (antibiotics) in fish drugs.[33] This resistance occurs when bacteria mutate in one way or another, it will impact on decreasing or losing of effectiveness of the drug, chemical compound or other material to prevent or treat infection.[34,28] Therefore, the three resistant causative agent associated with bacterial diseases on catfish was also caused by the irrational drugs administration during the production process, ie : the prevention and treatment of disease. Moreover, bacterial resistance could also occur due to mutate and selection randomly charge, in this case it act as an agent of antibiotic selection, so it was possible the group multiplication of resistant bacteria and suppressed the growth of bacteria sensitively to the antibiotic properties.[35] Furthermore, resistance to these fish drugs could be transmitted to other bacteria through a group of antibiotic resistant genes, between same genes locus with agents, such as plasmids, transposons, and integrons.[33,26]

3. Conclusion

Bacterial causative agents in the catfish cultured in central production in Kendal with a molecular approach were *Pseudomonas plecoglossicida*, *Aeromonas veronii and Aeromonas sobria*. The result of sensitivity test obtained that three causative agents have resistant character to four fish drugs. Clinical signs of moribound catfish were *haemorhagic* surrounds the mouth, fins, tail and wound, exopthalmia, fins root, body wound and pale, dark color in liver and kidneys, red and root antenna.

6. Acknowledgments

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Table captions

Table 1. Characteristic of Isolates Bacteria Associated on Cat fish from intensive pond of Kendal, Indonesia.

No.	Isolate code	Media	Source	Colony		
				Colour	Form	Characteristic
1	K.1	TCBS	Kidney	Green	Rounded	Convex
2	K.2	TCBS	Kidney	White	Rounded	Convex
3	K.3	TCBS	Fins root	Cream	Rounded	Convex
4	K.4	TCBS	Anal fin	Yellow	Rounded	Convex
5	K.5	TCBS	fins root	Green	Rounded	Convex
6	K.6	TCBS	fins root	Yellow	Rounded	Convex
7	K.7	TCBS	Tail fin	Yellow	Rounded	Convex
8	K.8	TCBS	Fin Dorsal	Yellow	Rounded	Convex
9	K.9	GSP	Fins root	Yellow	Rounded	Convex
10	K.10	GSP	Tail fin	Cream	Rounded	Convex
11	K.11	GSP	Kidney	Pink	Rounded	Convex
12	K.12	GSP	Kidney	White	Rounded	Convex
13	K.13	GSP	Anal fin	Yellow	Rounded	Convex
14	K.14	GSP	Kidney	Yellow	Rounded	Convex
15	K.15	NA	Fins root	Yellow	Rounded	Convex
16	K.16	NA	Dorsal fin	Yellow	Rounded	Convex
17	K.17	NA	Kidney	White	Rounded	Convex
18	K.18	NA	Kidney	Cream	Rounded	Convex
19	K.19	NA	Fins root	White	Rounded	Convex
20	K.20	NA	Tail fin	Yellow	Rounded	Convex

Tabel 2. Percentage of Sick and Mortality of Cat fish (*C. gariepinus*) during Postulates Koch's Performed

No.	Isolates code	Sick cat fish (%)	Total Mortality of cat fish (%)
1.	K6	60	20
2.	K14	35	10
3.	K19	35	10

Table 3. Three Isolates Selceted of Causative Agent assosiated with Bacterial Disease at Cat fish (*C. gariepinus*)

No.	Isolats Code	Media	Source	Colony		
				Color	Form	Characteristic
1	K.6	TCBS	fins root	Yellow	Rounded	Convex
2	K14	GSP	Kidney	Yellow	Rounded	Convex
3	K19	TCBS	Wound	Yellow	Irregullar	Convex

Table 4. Analisys of Three isolates compared with BLAST system

No.	Isolates Code	Results	Homologi (%)	No. access
1.	K 6	*Aeromonas* sobria	97	KC210798.1
2.	K14	*Pseudomonas plecoglossicida*	96	KC431807.1
3.	K19	*Aeromonas caviae*	96	JQ231158.1

Tabel 5. The Result of Senstivity Test Three Causative Agent of Bacterial Diseases on Catfish in Kendal Regency

Drugs	A ™						B ™,						Drug C™						D ™.					
	Times (Hour)																							
	24			48			24			48			24			48			24			48		
Isolate Code	Dosage (µl)																							
	6	8	10	6	8	10	2,5	5	7,5	2,5	5	7,5	2,5	5	7,5	2,5	5	7,5	2	4	6	2	4	6
K06	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K14	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K16	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

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The Diversity of Causative Agent Associated with Bacterial Diseases on Catfish (*Clarias gariepinus*) with Molecular Based from Kendal, Indonesia

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Bacterial disease is still a big concern in intensive catfish culture in Indonesia. The aim of this research was to find out causative agent on cat fish based on the 16S rDNA gene sequences. This research combined between exploratory in the field and experimental method in Laboratory. Causative agents diversity of bacterial diseases of catfish was isolated from Kendal Regency, Indonesia and based on postulat Koch results. Twenty bacteria (K01–K20) were isolated from external wound and kidney of moribound catfish with TCBS, NA and GSP medium. The postulat results showed that three isolates (K6, K14 and K19) were weaken up to 60% of fishes and caused 10–20% mortality. On the other hand, there were 14 isolates that did not demosntrated their virulence. Based on 16S rDNA sequence analysis, strain K6, K14 and K19 were closely related to *Aeromonas sobria* (97%.), *Pseudomonasplecoglossicida* (96%.), *Aeromonas caviae* (96%.) respectively. Sensitivity test to all isolates showed that these causative agents resistant to some fish drugs.

Keywords: Causative Agent, Bacterial Diseases, Catfish, 16S rDNA.

1. INTRODUCTION

Catfish (*Clariasgariepinus*) is the one of important aquaculture commodity in Kendal Regency. Kendal is one of catfish (*c. gariepinus*) producer in central java, producing approximately 21.967,06 tonnes in 2015.[1] Increasing of catfish demand for domestic markets encourages the fish farmers to increase their production. This was done through intensification and extensification. Unappropriate management of intensive culture would cause a negative impacts, such as bacterial disease outbreaks.[2,3]

Bacterial disease is still a big concern in catfish culture. Bacterial infection in the catfish was characterized by pale nor blacken of the body, *haemorrhagic* surrounds the mouths, fins, and tails, exopthalmia, fin root, body wounds and pale; dark color on liver and kidneys; red and root antenna.[4,5] *Aeromonashidrophylla, A. caviae* and *A. sobria*;[4,5] *A. salmonicida*;[6] *Vibrio sp*.;[7] *Pseudomonas* spp.[8] *Edwardsiella ictaluri*,[9] were reported as causative agents of bacterial diseases on catfish. These pathogens caused high mortality in larvae, fingerling, adult and broodstock,[10] and in catfish larvae up to 70%.[4]

Several researches had been performed to find out bacterial pathogen on cat fish based on the 16S rDNA gene sequences.[2,4,11] However, to our knowledge, there were limited reports being documented so far describing the application of polymerase chain reaction on the diversity of causative agent associated with bacterial diseases on cat fish derived from intensive pond of Kendal Regency, Central Java, Indonesia. This research is important in designing disease prevention strategy and supporting the catfish production.

2. MATERIAL AND METHODS

The moribound catfish were collected from intensive culture ponds of central production in Kendal Regency on May to August 2016. Moribound catfish were kept in the container and immediately brought to Integrated Laboratory of Diponegoro University for bacterial isolation. Bacteria were isolated from Kidney and the wound by streak method then cultured onto Thio-Sulphate Citrate Bile Salt Sucrose (TCBS), TrypticSoybean Agar (TSA) and Glutamate Starch Penicillin (GSP) medium. Morphological features of the colonies were randomly picked and purified by a single colony to the plate.[12]

Postulate Koch test was conducted to twenty isolates by using 420 healthy catfish as an experimental animals. During acclimatitation experimental catfish were divided into 42 aquariums. Twenty isolates were cultured in zobell liquid medium, then intramuscularly injected on healthy catfish with 0.1 mL of inoculants bacterial density of 10^8 CFU/mL. Clinical signs and mortality of catfish were observed for 96 hours.

Molecular characterization 16S rDNA of three isolates were carried out.[13–15] They further explained that the

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Polymerase Chain Reaction (PCR) was conducted with Eppendorf Mastercycler (Eppendorf Inc. Germany). Two primers, GM3F (5′AGAGTTTGATCMTGGC-3′) and GM4R (5′-TACCTTGTTACGACTT-3′) were used to amplify nearly complete 16S rDNA gene. Prior to PCR, genomic DNA of three causative agent associated with bacterial diseases on the catfish for PCR analysis were extracted from cell materials taken from agar plate, suspended in steril water (Sigma, Germany) and subjected to five cycles of freeze (−80 °C) and thaw (95 °C). Then, PCR amplification of partial 16S rDNA gene of bacteria and purification of PCR products were performed based on the method of Ref. [13]. Sequencing were commenced to gain the alkaline composition that made up DNA sequences. The sequencing was done using the BigDye Terminator V 3.1 dyes and automatic DNA sequencer ABI3130 Genetic Analyzer XL Applied Biosystems at Macrogen Korea. Whereas, the determined DNA sequences of causative agent were then compared for homology to the BLAST *(Basic Local Alignment Search Tool)* on *National Center for Biotechnology Information, National Institute for Health* database USA.[6]

Three causative agent were tested their sensitivity to four fish drugs (A™, B™, C™ and D™) *in vitro* based on method.[17] Inhibition of the causative agents by each drugs were demonstrated by production of clear zone in the bacterial colonies area. Sensitivity test was evaluated according standard developed by National Committee for Clinical Laboratory Standards.[18]

3. RESULTS AND DISCUSSION

Clinical signs of moribound catfish which affected by bacterial diseases from intensive pond of Kendal were *haemorrhagic* surrounds the mouth, fins, and tail. Exopthalmia; fin root; body wounds and pale; dark color in liver and kidney. This morphological symptom (body wound and pale, *haemorrhagic* surrounds the mouths, fins, and tails; red and root antenna), the behaviour abnormalities such as body upsite down, lethargy, decreased appetite, increased mucus production, swimming imbalance, deficiency in oxygen uptake were also found in experimental catfish. The clinical signs above have been reported.[4, 19, 20] These clinical signs observed in the present study has also been observed in naturally-diseased catfish cultured.[19] These clinical signs were observed in the present study may due to attachment and colonization of many opportunistic pathogens to fish skin of cat fish.[4]

Twenty bacterial isolates were obtained from kidney, fins root, and body wound of moribound catfish (Table I). Postulate Koch test results indicated that three isolates namely K6, K14 and K19 were able caused disease symptom up to 60% of the challenged catfish, whilst the three isolates was caused mortality range of 10–20% (Table II). He present study also found that experimentally catfish were injected by others isolates and PBS had 100% survival rate and normal behaviour. Therefore, these isolates (K6, K14 and K19) were positively confirmed as a causative agent associate bacterial diseases in catfish from intensive culture pond that is located at Kendal Regency. These mortality rate was lower than that reported,[4] in catfish *A. sobria* infected (30%) and *A. caviae*-infected (20–30%). Moreover, *A. sobria* was less pathogenicity compare to *A. hydrophila* on Stinging Catfish Shing (*Heteropneustes fossilis*).[6] The occurance pathogenicity of these bacteria on the fish was mainly contributed by the haemolysin and aerolysin.[21, 22] Based on the postulat Koch results (Table II), three isolates (K6, K14 and K19) out of 22 isolates (K1–K20) were choosen for further investigation (Table III).

On the basis of sequence 16S rDNA analysis using Blast System (Table IV), the results showed that causative agents of bacterial diseases on catfish from pond of kendal, i.e., K6, K14 and K19 were closely related to *Aeromonas sobria* (97%.), *Pseudomonas plecoglossicida* (96%.), *Aeromonas caviae* (96%.) respectively. Both of *Pseudomonas* spp. and *Aeromonas* spp. was naturally found in the aquatic environment, represent the Gram-negative commensal bacteria and include in pathogenic bacteria of fish.[7, 12] The presents study revealed that the causative

Table I. Characteristic of isolates bacteria associated on cat fish from intensive pond of Kendal, Indonesia.

No.	Isolate code	Media	Source	Colony		
				Colour	Form	Characteristic
1	K.1	TCBS	Kidney	Green	Rounded	Convex
2	K.2	TCBS	Kidney	White	Rounded	Convex
3	K.3	TCBS	Fins root	Cream	Rounded	Convex
4	K.4	TCBS	Anal fin	Yellow	Rounded	Convex
5	K.5	TCBS	fins root	Green	Rounded	Convex
6	K.6	TCBS	fins root	Yellow	Rounded	Convex
7	K.7	TCBS	Tail fin	Yellow	Rounded	Convex
8	K.8	TCBS	Fin Dorsal	Yellow	Rounded	Convex
9	K.9	GSP	Fins root	Yellow	Rounded	Convex
10	K.10	GSP	Tail fin	Cream	Rounded	Convex
11	K.11	GSP	Kidney	Pink	Rounded	Convex
12	K.12	GSP	Kidney	White	Rounded	Convex
13	K.13	GSP	Anal fin	Yellow	Rounded	Convex
14	K.14	GSP	Kidney	Yellow	Rounded	Convex
15	K.15	NA	Fins root	Yellow	Rounded	Convex
16	K.16	NA	Dorsal fin	Yellow	Rounded	Convex
17	K.17	NA	Kidney	White	Rounded	Convex
18	K.18	NA	Kidney	Cream	Rounded	Convex
19	K.19	NA	Fins root	White	Rounded	Convex
20	K.20	NA	Tail fin	Yellow	Rounded	Convex

Table II. Percentage of sick and mortality of cat fish (*C. gariepinus*) during postulates koch's performed.

No.	Isolates code	Sick cat fish (%)	Total mortality of cat fish (%)
1.	K6	60	20
2.	K14	35	10
3.	K19	35	10

Table III. Three isolates selected of causative agent associated with bacterial disease at cat fish (*C. gariepinus*).

No.	Isolates code	Media	Source	Colony		
				Color	Form	Characteristic
1	K.6	TCBS	Fins root	Yellow	Rounded	Convex
2	K14	GSP	Kidney	Yellow	Rounded	Convex
3	K19	TCBS	Wound	Yellow	Irregular	Convex

Table IV. Analysis of three isolates compared with BLAST system.

No.	Isolates code	Results	Homologi (%)	No. access
1.	K6	*Aeromonas* sobria	97	KC210798.1
2.	K14	*Pseudomonas plecoglossicida*	96	KC431807.1
3.	K19	*Aeromonas caviae*	96	JQ231158.1

Table V. The result of sensitivity test three causative agent of bacterial diseases on catfish in kendal regency.

	A™						B™						Drug C™						D™					
	Times (Hour)																							
	24			48			24			48			24			48			24			48		
	Dosage (μl)																							
Drugs isolate code	6	8	10	6	8	10	2,5	5	7,5	2,5	5	7,5	2,5	5	7,5	2,5	5	7,5	2	4	6	2	4	6
K06	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K14	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K16	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

agents associated with bacterial diseases of catfish that was intensively cultured at Kendal ponds, were closely related to *A. sobria* (K6), *P. plecoglossicida* (K14), and *A. caviae* (K19). *A. sobria* and *A. caviae* were commonly reported as a bacterial pathogen associated with motile aeromonas septicemia in African cat fish,[4] catfish, *Clarias*;[11] haemorrhage of Tra catfish, *Pangasianodon hypophthalmus*,[5] and walking cat fish culture, *Clarias bratachus*.[19] *A. sobria* was also reported in walking catfishes, *C. batrachus*;[21] Nordmann and Poirel,[22] catfish in India,[11] and in China as a aquatic pathogenic bacteria. While, *A. veronii* biovar *sobria* was also found as a causative agent of Epizootic Ulcerative Syndromein fish in Bangladesh.[23]

Pseudomonas spp. has been reported to be an important fish pathogen that has endangered aquaculture.[8] *P. plecoglossicida* was found as causative agent of bacterial haemorrhagic ascites of ayu, *Plecoglossus altivelis.*[24, 25] Out break of this disease often occurs after seeds was introduced in culture ponds, and it also could found at any development stage during culture.[24] *P. plecoglossicida* was also found in natural environment.[26] On the other hand, *P. plecoglossicida* could become as a non pathogenic bacteria that was potentially produce an anti-tumor drugs;[27] and as a candidate of probiotic.[24] However, in the present study also confirmed that *P. plecoglossicida* was found as a causative agent associated with bacterial diseases on cat fish that was intensively cultured in pond of Kendal regency.

The sensitivity test results (Table IV) revealed that three causative agents associate with bacterial disease in catfish from Kendal, namely: *A. sobria* (K6), *P. plecoglossicida* (K14), *A. caviae* (K19), were not sensitive to drug A™, B™, C™ and D™. These were detected by the formation of a clear zone around the paper discs on all three bacteria with diameter of 0–1,3 mm. The criteria of resistant bacteria if they have inhibitory zone ranged between 0–10 m.[18] The previous research also found that *Genus Aeromonas;*[28, 29] *A. caviae;*[30] and *A sobria*,[31] were resistence to antibiotic. *Pseudomonas* spp. and *Aeromonas* spp. was resistant to multiple drug for 96.6% and 61.9%.[2] This resistance occurs may related to the using of fish drugs irrational dosage during aquaculture process,[32] and the content of the active compound (antibiotics) in fish drugs.[33] This resistance occurs when bacteria mutate in one way or another, it will impact on decreasing or losing of effectiveness of the drug, chemical compound or other material to prevent or treat infection.[28, 34] Therefore, the three resistant causative agent associated with bacterial diseases on catfish was also caused by the irrational drugs administration during the production process, i.e.,: the prevention and treatment of disease. Moreover, bacterial resistance could also occur due to mutate and selection randomly charge, in this case it act as an agent of antibiotic selection, so it was possible the group multiplication of resistant bacteria and suppressed the growth of bacteria sensitively to the antibiotic properties.[35] Furthermore, resistance to these fish drugs could be transmitted to other bacteria through a group of antibiotic resistant genes, between same genes locus with agents, such as plasmids, transposons, and integrons.[26, 33]

4. CONCLUSION

Bacterial causative agents in the catfish cultured in central production in Kendal with a molecular approach were *Pseudomonas plecoglossicida*, *Aeromonasveronii and Aeromonas sobria*. The result of sensitivity test obtained that three causative agents have resistant character to four fish drugs. Clinical signs of moribound catfish were *haemorhagic* surrounds the mouth, fins, tail and wound, exopthalmia, fins root, body wound and pale, dark color in liver and kidneys, red and root antenna.

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