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

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Omni-Akuatika, 14 (2) : 100 – 106 , 2018
ISSN: 1858-3873 print / 2476-9347 online
Research Article
journal homepage: http://ojs.omniaquatika.net

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

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***ABSTRACT***

Bacterial diseases is frequently occur in catfish culture. The aim of this research was to find out the diversity of causative agent associated with bacterial diseases in catfish based on 16S rDNA gene sequences. The combination between exploratory in the field and experiment, method were applied. Seventeen isolates (D01–D17) were gained from kidney and external wound of moribound catfish with NA and GSP medium that were collected from fish pond of Demak Regency, Indonesia. Based on the postulat results showed that four isolates (D07, D10, D11 and D14) that were caused 10–55% of fishes get sick and 0–30% fishes mortal. On the other hand, there were 13 isolates do not cause both sick and mortality of fish. On the basis of sequence 16S rDNA analysis, the result showed that D07, D10, D11 and D14 were closely related to *Aeromonas caviae* (96%), *Aeromonas veronii* (97%.), *Plesiomonas shigelloides* (97%) and *Pseudomonas putida* (96%) respectively. The sensitivity test result indicated that these causative agents have not sensitively to some fish drugs test.

***Keywords***: *Causative agent, Catfish, 16S rDNA, Bacterial diseases*

## 1. Introduction

Catfish (*Clarias gariepinus*) is an important aquacultural species which has been cultured in Demak Regency, Central Java, Indonesia. This area was known as a central catfish producer in central java, Indonesia. However, the decreasing of the production from 21.967,06 tonnes in 2006 to 14.432 tonnes in 2013 (Marine and Fisheries Ministry of Central Java Province, 2014) due to the diseases infection. Therefore increasing the production and development on the sustainable cat fish aquaculture in order to full fill the catfish demand in domestic markets, the fish farmers were applied the intensive culture. However inappropriate managementmay cause some negative impacts, such as out break of bacterial disease (Nguyen *et al*., 2014).

Bacterial disease is still a major problem diseases in catfish culture. Bacterial diseases in the catfish was characterized by pale or blacken of the skin, *haemorrhagic* surround the mouth, fins, and tails, exopthalmia, fin root, body wounds and pale, darken liver and kidneys, red and root antenna (Anyanwu *et al*., 2015; Thanh *et al*., 2009). *Aeromonas salmonicida* (Monir *et al*., 2015), *Vibrio* sp. (Austin and Austin, 2007), *A. hidrophylla* (Anyanwu *et al*., 2015); *A. caviae* and *A. sobria* (Anyanwu *et al*., 2015; Sarjito *et al*, 2017; Thanh *et al*., 2009), *Pseudomonas* spp. (Mao *et al*., 2012), *Edwardsiella ictaluri* (Crumlish *et al*., 2002) were found as causative agents of bacterial diseases on catfish. These pathogens caused high mortality in larvae, fingerling, adult and broodstock (Durborow *et al*., 1998) and in catfish larvae up to 70% (Anyanwu *et al*., 2015).

Preced researches were done by some researchers in attempt to discover the bacterial pathogen on catfish using Polymerase Chain Reaction (PCR) with 16S rDNA gene sequences. However, identification of bacterial pathogen using molecular approach was derived from intensive pond of Demak Regency, Central Java, Indonesia was still need to create the certain design of prevention system and strategy againts this disease.

## 2. Materilas and Methods

*Sampling of catfish and bacterial isolation*

The catfish presumed infected by bacteria collected from production ponds in

http://dx.doi.org/10.20884/1.oa.2018.14.2.553

catfish central production in Demak Regency. Bacterial isolation was admministered in Integrated Laboratory of Diponegoro University, the fish brought with container to keep it alive. Tryptic Soybean Agar (TSA) and Glutamate Starch Penicillin (GSP) medium were used to isolate the bacteria from wound and kidneys with streak method. Bacteria colonies, then were purified by re-struk a single colony to the plate (Brock and Madigan, 1996).

*Postulat koch and characterization*

Seventeen bacteria isolates tested with Postulate Koch to 360 healthy catfish as experimental fish. Acclimatization done dividing the fish into 36 aquarium so there was 10 fishes in every aquarium. Liquid bacteria culture done using zobell liquid medium, injected to fish as many as 0.1 ml with of $10^8$ CFU/ml. The fish injected on the intramuscular and observed for 96 hours to know clinical signs may appear.

From the seventeen bacteria isolates, four isolates were characterised with molecularly approach based on methods Radjasa *et al* (2001). Bacteria extracted from agar plate then suspended in steril water (Sigma, Germany). The Polymerase Chain Reaction was run using Eppendorf Mastercycler (Eppendorf Inc. Germany)with five freezing cycles (-80ºC) and thaw (95ºC). The primer were used to amplify are GM3F (5'AGAGTTTGATCMTGGC-3') and GM4R (5'-TACCTTGTTACGACTT-3') nearly complete 16S rDNA gene. Big Dye Terminator V3.1 dyes and automatic DNA sequencer ABI3130 Genetic Analyzer XL Applied Biosystemsat Macrogen Korea used for sequencing the bacteria DNA. DNA sequences of the bacteria forward was campared to the BLAST (*Basic Local Alignment Search Tool*) on *National Center for Biotechnology Information, National Institute for Health* database USA to gain the homology (Atschul et al. 1997; Radjasa *et al,* 2001). Wheras the phylogentic was constructed with Mega 6 programme (Sarjito *et al,* 2017*).*

*Bacterial sensitivity test*

Bacteria sensitivity test further was applied to the bacteria with in vitro method, the drugs are A ™, B ™, C ™ and D ™. The activity of each drugs showed with clear zone emerge on the bacterial colonies area, afterward the sensitivity test result compared to stantard by National Committee for Clinical Laboratory Standards (NCCLS, 2001)

## 3. Results and Discussion

*Clinical signs of moribound catfish*

Clinical signs of moribound catfish which infected by bacterial diseases from intensive pond of Demak were body wound and pale, haemorhagic surround the mouth, tail, fins and fins root; red and root antenna and dark color in liver and kidneys This morpholocical symptom (pale and body wound, *haemorrhagic* surrounds the mouths, fins, and tails; red and root antenna), the behaviour abnormalities such as decreased appetite, lethargy, body upsite down, swimming imbalance were also observed in tested catfish. The clinical signs above have also been reported by Areerat (1987); Anyanwu *et al*., (2015) and Sarjito *et al.* (2017). These clinical sigs found in the present study has also been observed in naturally-diseased catfish cultured (Areerat, 1987). These clinical symtoms were observed in the present study may due to attachment and colonization of consorsium opportunistic bacterials to fish skin of cat fish (Anyanwu *et al*., 2015).

Seventeen bacterial isolates were gained from kidney, fins root, and body wound of moribound catfish (Table 1). Postulate Koch test results showed that four isolates, namely that D07, D10, D11 and D14 were able caused disease symptom up to 55% of the experimentlly catfish, whilst the three isolates was caused mortality range of 0–30 % (Table 2). The present study also showed that challenged catfish were injected by others isolates and PBS had 100% survival rate and normal behavior. Therefore, these isolates (D07, D10, D11, and D14) were positively confirmed as a causative agent assosiate with bacterial diseases in catfish from Demak. These result also revealed that these causative agent was higher pathogenicity compare to causative agent that was found in catfish from Kendal (Sarjito et al., 2017).

Table 1. Characteristic of isolates bacteria associated with catfish from Demak, Central Java, Indonesia.

| Isolate code | Media | Source | Colony | | |
|---|---|---|---|---|---|
| | | | Colour | Form | Characteristic |
| **D01** | GSP | Kidney | Green | Rounded | Convex |
| **D02** | GSP | Kidney | White | Rounded | Convex |
| **D03** | GSP | Fins root | Cream | Rounded | Convex |
| **D04** | GSP | Anal fin | Yellow | Rounded | Convex |
| **D05** | GSP | fins root | Green | Rounded | Convex |
| **D06** | GSP | fins root | Yellow | Rounded | Convex |
| **D07** | GSP | Kidney | Yellow | Rounded | Convex |
| **D08** | GSP | Fin Dorsal | Yellow | Rounded | Convex |
| **D09** | GSP | Fins root | Yellow | Rounded | Convex |
| **D10** | GSP | Kidney | Yellow | Rounded | Convex |
| **D11** | TSA | Wound | Yellow | Rounded | Convex |
| **D12** | TSA | Kidney | White | Rounded | Convex |
| **D13** | TSA | Anal fin | Yellow | Rounded | Convex |
| **D14** | TSA | Kidney | Yellow | Rounded | Convex |
| **D15** | TSA | Fins root | Yellow | Rounded | Convex |
| **D16** | TSA | Dorsal fin | Yellow | Rounded | Convex |
| **D17** | TSA | Kidney | White | Rounded | Convex |

Table 2. Four selected bacteria suspeccted as causative agent assosiated with bacterial diseases from Demak regency

| Isolates code | Sick catfish (%) | Total Mortality of catfish (%) |
|---|---|---|
| D07 | 40 | 20 |
| D10 | 55 | 30 |
| D11 | 35 | 0 |
| D14 | 20 | 10 |

Table 3. Characteristic of four isolates selected of causative agent assosiated with bacterial disease at catfish (*C. gariepinus*)

| Isolates Code | Media | Source | Colony | | |
|---|---|---|---|---|---|
| | | | Color | Form | Characteristic |
| D07 | GSP | kidney | Yellow | Rounded | Convex |
| D10 | GSP | Kidney | Yellow | Rounded | Convex |
| D11 | TSA | Wound | Yellow | Rounded | Convex |
| D14 | TSA | kidney | Yellow | Rounded | Convex |

Based on the postulat Koch results (Table 2), four isolates (D07, D10, D11 and D14) out of 17 isolates (D01–D17) were choosen for further investigation (Table.3).

Based on the sequence 16S rDNA analysis using Blast Sysytem (Table 4.), the results showed that causative agents of bacterial diseases on catfish from pond of Demak , i.e. : D07, D10, D11 and D14 were closely related to *Aeromonas caviae* (96 %), *Aeromonas veronii* (97%), *Plesiomonas shigelloides* (97%) and *Pseudomonas putida* (96%) respectively. *Aeromonas caviae* and *Aeromonas veronii* were commonly reported as a bacterial pathogen assosiated with motile *Aeromonas septicemia* in African catfish

yanwu *et al.*, 2015), catfish *Clarias* (Arunava *et al.*, 2013), and walking catfish culture *Clarias bratachus* (Areet, 1987). *A. caviae* was also reported as causative agent of bacterial diseases in walking catfish in India (Thomas *et al.*, 2013) and *Rhamdia quelen* (Baldissera *et al.*, 2018). While, *A. veronii* was also found in Malaysian red hybrid tilapia (A*mal et al.*, 2018*), Gold Fish, Carasius auratur,* *Srilangka (Jagoda et al.,* 2017), catfish (Nawaz *et al.*, 2010). Futhermo, *A. veronii* biovar *sobria* was found as a causative agent of Epizootic Ulcerative Syndrome in fish in Bangladesh (Rahman *et al.*, 2002).

Table 4. Analisys of four selected isolates compared with BLAST system

| Isolates | Close Relative | Homology (%) | Acc. Number |
|---|---|---|---|
| D07 | *Aeromonas caviae* | 96 | JQ231158.1 |
| D10 | *Aeromonas veronii* | 97 | MF401516.1 |
| D11 | *Plesiomonas shigelloides* | 97 | CP027852.1 |
| D14 | *Pseudomonas putida* | 97 | HQ162489.1 |

*P. shigelloides* was found in freshwater from Nothern Europe (Krovacek *et al.*, 2000), Grouper (Herrera *et al.,* 2006). *Plesiomonas* sp. have been isolated from freshwater fish and bival (Niedziela *et al.,* 2002). *Pseudomonas* spp. has been reported to be an important fish pathogen that has endangered aquaculture (Mao *et al.*, 2012).

*P. putida* was found as causastive agent of bacterial diseases in rainbow trout (Altinok *et al.*, 2006), Yellow Croaker (*Pseudoschiaena crocea*) (Mao *et al.*, 2012); *Pleurotus eryngii* (Wang et al., 2016). This species was also reported as dominace bacteria in aquatic environment in Congo, India and switzerland (Devarajan *et al.,* 2017). In these present study also confirmed that *P. Putida* was found as a causative agent associated with bacterial diseases on catfish that was intensively cultured in pond of Demak regency.

The phylogenetic of bacteria associated with bacterial diseases of catfish from Demak was seen in figure 1. The relationship between query strain in present study and other related members of the genus *Aeromonas, Pseudomonas and Plesiomonas.* The sensitivity test results (Table 5) showed that four causative agents assosiate with bacterial disease in catfish from Demak.

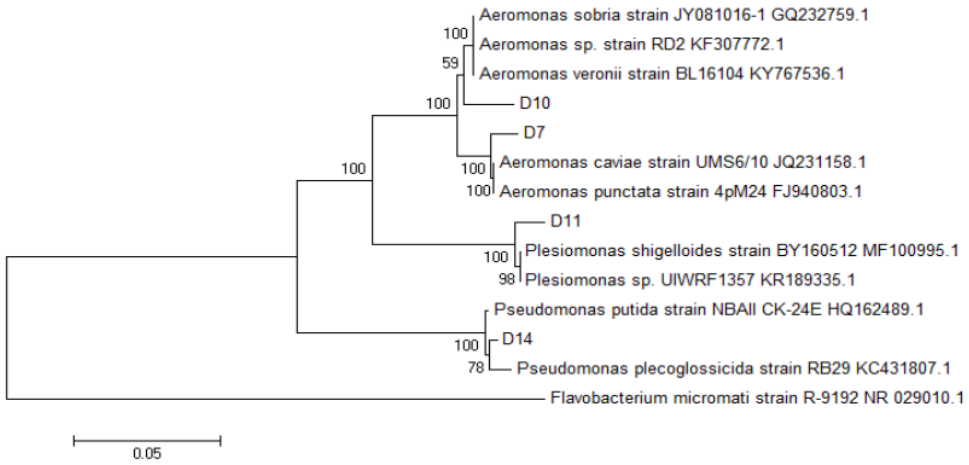


Figure 1. Phylogenetic of the Bacteria Associated with Bacterial Diseases On catfish from Demak Regency.

Tabel 5. The result of senstivity test four causative agent of bacterial diseases on catfish in Demak regency

| Drugs | A ™ | | | | | | B ™, | | | | | | Drug C™ | | | | | | D ™. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Times (Hour) | | | | | | | | | | | | | | | | | | | | | | | |
| | 24 | | | 48 | | | 24 | | | 48 | | | 24 | | | 48 | | | 24 | | | 48 | | |
| Isolate Code | Dosage (µl) | | | | | | | | | | | | | | | | | | | | | | | |
| | 1 | 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 |
| D07 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| D10 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| D11 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |
| D14 | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R | R |

Table 5 shows that *A. caviae* (D07), A. *veronii* (D10), *Plesiomonas shigelloides* (D11, *P. putida* (D14), were not sensitively to drug A 6, B ™, C ™ and D ™. This was indicated by the clear zone around the paper discs on all the bacteria with diameter of 0–1,2 mm. Based on the criteria of National Committee for Clinical Laboratory Standards (2001), the resistant bacteria was characterized by the diameter of clear zone ranged between 0–10 mm. The present researchrevealed that the four causative agent associated with bacterial dieseases in catfish from Demak were resistence to some fish drug tested. The previous study also reported that genus *Aeromonas* (Shinha *et al.*, 2004; Ashiru *et al.*, 2011), *A. caviae* (Motyl *et al.*, 1985) and *Pseudomonas* spp (Devarajan *et al.*, 2017) were reported a resistence to antibiotic. This resistance occurs may caused by irrational dosage administration during culture process (Sukenda *et al.*, 2008) in order to prevent and combat the bacterial diseases. The similar result also reported by Sarjito *et al* . (2017) that the causative agent assosiate with bacterial diseases with catfish from kendal was not sensitively to some fish drug.

## 4. Conclusion

Causative agents assosiate with bacterial diseases in catfish from Demak regency were *A. Caviae* (D07), *A. veronii* (D10), *Plesiomonas shigelloides* (D11), and *P. Putida* (D14). These causative agents was indicated that it was not sensitively to four fish drugs tested. Clinical signs of catfish intected by bacterial diseases were exopthalmia, body wound and pale, *haemorhagic* surround the mouth, tail, fins and fins root; red and root antenna and dark color in liver and kidneys.

## Acknowledgments

This study is part of research grants funded by Directorat for Research and Community Services, Directorate General of Education Strengthening Research Ministry of Research, Technology and High Education. Fiscal year 2016. Number : 022/SP2H/LT/DRPM/II/2016; February 17th 2016. On this portunity, the authors would like to thank to Dean of Fisheries and Marine Sciences Faculty, UNDIP, Head of Integrated Laboratory of Dipone ro University. Aquaculture Laboratory of Fisheries and Marine Sciences Faculty, UNDIP.

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