



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

Sarjito^{1*}, A. Harjuno Condro Haditomo¹, Desrina¹, Restiana W. Ariyati¹, S Budi Prayitno¹

¹Department of Aquaculture, Fisheries and Marine Science Faculty, Diponegoro University, Tembalang, Semarang, Indonesia

*Corresponding author: sarjito_msdp@yahoo.com

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 management may 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, exophthalmia, 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. hydrophyla* (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

catfish central production in Demak Regency. Bacterial isolation was administered 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-struck 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 (-80°C) and thaw (95°C). 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 Biosystems at Macrogen Korea used for sequencing the bacteria DNA. DNA sequences of the bacteria forward was compared 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). Whereas 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 ATM, BTM, CTM and DTM. The

activity of each drugs showed with clear zone emerge on the bacterial colonies area, afterward the sensitivity test result compared to standart 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, haemorrhagic surround the mouth, tail, fins and fins root; red and root antenna and dark color in liver and kidneys This morphological 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 upside 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 signs found in the present study has also been observed in naturally-diseased catfish cultured (Areerat, 1987). These clinical symptoms were observed in the present study may due to attachment and colonization of consorsium opportunistic bacteria 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 experimentally 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 associate 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 suspected as causative agent associated 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 associated 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 chosen 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 associated with motile *Aeromonas septicemia* in African catfish

(Anyanwu *et al.*, 2015), catfish *Clarias* (Arunava *et al.*, 2013), and walking catfish culture *Clarias bratachus* (Areerat, 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 (Amal *et al.*, 2018), Gold Fish, *Carasius auratur*, in Srilangka (Jagoda *et al.*, 2017), catfish (Nawaz *et al.*, 2010). Futhermore, *A. veronii* biovar *sobria* was found as a causative agent of Epizootic Ulcerative Syndrome in fish in Bangladesh (Rahman *et al.*, 2002).

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

Isolates	Close Relative	Homology (%)	Acc. Number
D07	<i>Aeromonas caviae</i>	96	JQ231158.1
D10	<i>Aeromonas veronii</i>	97	MF401516.1
D11	<i>Plesiomonas shigelloides</i>	97	CP027852.1
D14	<i>Pseudomonas putida</i>	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 bivalve (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 causative 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 dominance 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 associate with bacterial disease in catfish from Demak.

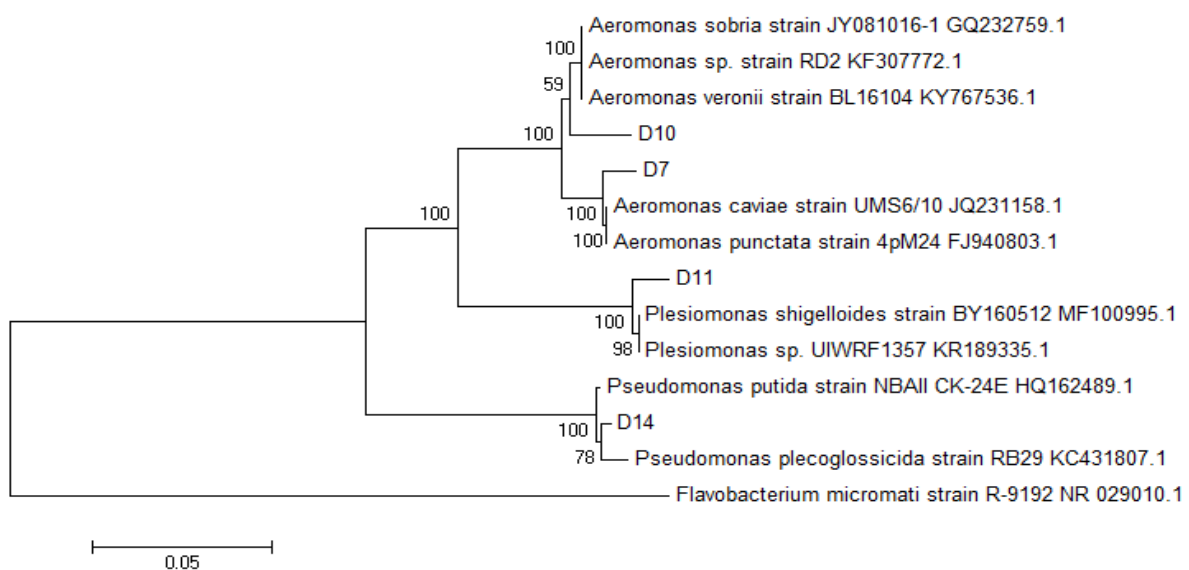


Figure 1. Phylogenetic of the Bacteria Associated with Bacterial Diseases On catfish from Demak Regency.

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

Isolate Code	A TM						B TM						Drug C TM						D TM					
	24						48						Times (Hour)						24					
	24						48						24						48					
	Dosage (µl)						Dosage (µl)						Dosage (µl)						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
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 ATM, BTM, CTM and DTM. 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 research revealed that the four causative agent associated with bacterial diseases in catfish from Demak were resistance 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 resistance 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 associate with bacterial diseases with catfish from kendal was not sensitively to some fish drug.

4. Conclusion

Causative agents associate 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 infected by bacterial diseases were exophthalmia, body wound and pale, *haemorrhagic* 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 Directorate for Research and Community Services, Directorate General of Education Strengthening Research Ministry of Research, Technology and Higher Education. Fiscal year 2016. Number : 022/SP2H/LT/DRPM/II/2016; February 17th 2016. On this opportunity, the authors would like to thank to Dean of Fisheries and Marine Sciences Faculty, UNDIP, Head of Integrated Laboratory of Diponegoro University. Aquaculture Laboratory of Fisheries and Marine Sciences Faculty, UNDIP.

References

- Altinok, I., S. Kayis., E. Capkin. 2006. *Pseudomonas putida* infection in rainbow trout. *Aquaculture*, 261:850-855.
- Amal, M.N.A., C.B. Koh., M. Nurliyana., M. Suhaiba, Z. Nor-Amalina, S. Santha, K.P. Diyana-Nadhiroh, M.T. Yusof, M.Y. Ina-Salwany, dan M. Zamri-Saad. 2018. A case of natural co-infection of Tilapia Lake Virus and *Aeromonas veronii* in a Malaysian red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) farm experiencing high mortality. *Aquaculture*, 485:12-16.
- Anyanwu, M.U., K. F. Chah., V. S. Shoyinka. 2015. Evaluation of pathogenicity of motile *Aeromonas* species in African catfish. *International Journal of Fisheries and Aquatic Studies*, 2(3): 93-98.
- Areerat, S. 1987. Clarias culture in Thailand. *Aquaculture*, 63(4): 355-362.
- Arunava, D. A. Rathore., C. Janani. C, Hemanth., R. A. Balakrishnan. 2013. Diagnosis Of Motile *Aeromonas* Sobria From Cat Fishes With Septicemia By PCR. *IOSR Journal of Agriculture and*

- Veterinary Science (IOSR-JAVS). 2 (6): 87-91.
- Ashiru, A. W., P.O. Uaboi-Egbeni, J.E. Oguntowo., C.N. Idika. 2011. Isolation and Antibiotic Profile of *Aeromonas* Species from Tilapia Fish (*Tilapia nilotica*) and Catfish (*Clarias betrachus*). *Pakistan Journal of Nutrition*. 10 (10): 982-986.
- Atschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, N.D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST : a New Generation of Protein Database Search Programs *Nucleic Acid Res*. 25:3389-3402.
- Austin, B., Austin, D. A. 2007. Bacterial Fish Pathogens. Disease in Farmed and Wild Fish. Fourth edition. Ellis Horwood Limited. Chichester: England. 552 p.
- Baldissera, M.D., C.F. Souza., C.M. Verdi., B.S. Vizzotto., P.H. Doleski., K.L.S. Moreira, M.I.U.M. da Rosca., M.L. da Veiga., B.S. Vizzotto., R.C.V. Santos., B. Baldisserotto. 2018. Cholinergic and adenosinergic systems exert a pro-inflammatory profile in peripheric and splenic lymphocytes of *Rhamdia quelen* experimentally infected by *Aeromonas caviae*. *Aquaculture*, 482:162-166.
- Brock, T. D. N M. T. Madigan. 1991. Biology of Microorganism. 6th ed. New Jersey : Prentice Hall.
- Crumlish, M., T. T. Dung, J. F. Turnbull, N. T. N. Ngoc., H. W. Ferguson. 2002. Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam. *Journal of Fish Diseases*, 25:733–736.
- Devarajan, N., T. Kohler., P. Sivalingam., C. Van Delden., C.K. Mulaji., P.T. Mpiana., B.W. Ibelings., J. Pote. 2017. Antibiotic resistant *Pseudomonas* spp. in the aquatic environment: A prevalence study under tropical and temperate climate conditions. *Water Research*, 115:256-265.
- Durborow, M. R., Thune, L. R., Camus, C. A. 1998. Columnaris Disease, A bacterial Infection Caused by *Flavobacterium columnare*. SRAC Publication. 479 pp.
- Herrera, F.C., J.A. Santos., A. Otero., M.L. Garcia-Lopez. 2006. Occurrence of *Plesiomonas shigelloides* in displayed portions of saltwater fish determined by a PCR assay based on the *hugA* gene. *Int. J. Of Food Microbiology*, 108:233-238.
- Jagoda, S. S. S. De S., K. Honein, A. Arulkanthan, H. Ushio., S. Asakawa. 2017. Genome Sequencing and Annotation of *Aeromonas veronii* Strain Ae52, a Multidrug-resistant Isolate from septicemic gold fish (*Carassius auratus*) in Sri Lanka. *Genomics Data*, 11:46-48.
- Krovacek, K., L.M. Eriksson., C. Gonzales-Rei, J. Rosinsky., I. Ciznar. 2000. Isolation, biochemical and serological characterisation of *Plesiomonas shigelloides* from freshwater in Northern Europe. *Comparative Immunology, Microbiology, & Infectious Diseases*, 23:45-51.
- Marine and Fisheries Ministry of Central Java Province. 2014.
- Mao, Z., Y. Qiu., L. Zheng., J. Chen., J. Yang. 2012. Development of a visual loop-mediated isothermal amplification method for rapid detection of the bacterial pathogen *Pseudomonas putida* of the large yellow croaker (*Pseudosciaena crocea*). *J. Of Microbiological Methods*, 89:179-184.
- Monir, M.D., T. Ahammed., S. C. Borty, N. Bagum, Md. A. Islam and Y. Mahmud. 2015. Pathogenesis Of *Aeromonas* Species In Stinging Catfish Shing (*Heteropneustes fossilis*) Of Bangladesh. *Trend in Fisheries Research*, 4(1) : 2319–4758.
- Motyl, M. R., G. Mckinley., J. M. Jandal, 1985. In vitro susceptibility of *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas caviae* to 22 antimicrobial . *Antimicrobial Agents And Chemotherapy*. 28: 151-153.
- Nawaz, M., S.A. Khan., A.A. Khan., K. Sung, Q. Tran, K. Kerdahi, R. Steele. 2010. Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish. *Food Microbiology*, 27:327-331.
- National Committee for Clinical Laboratory Standards (NCCLS). 2001. Performance Standards for Antimicrobial Disk Approve Standards M100-S11. Wayne, Pa: NCCLS.
- Nguyen, H. N. K., T.T. H. Van., H.T. Nguyen., P.M. Smooker., J. Shimeta, P. J. Coloe . 2014. Molecular characterization of

- antibiotic resistance in *Pseudomonas* and *Aeromonas* isolates from catfish of the Mekong Delta, Vietnam. *Veterinary Microbiology*, 171: 397–405.
- Radjasa, O. K., H. Urakawa., K. Kita-Tsukamoto., K. Ohwada. 2001. Characterization of Psychrotrophic Bacteria in the Surface and Deep-Sea Waters from the Northwestern Pacific Ocean Based on 16S Ribosomal DNA Analysis. *Mar. Biotechnol.* 3: 454 – 462.
- Rahman, M., P.C.Navarro., I. Kühn., G.Huys., J. Swings., Roland Möllby. 2002. Identification and Characterization of Pathogenic *Aeromonas veronii* Biovar Sobria Associated with Epizootic Ulcerative Syndrome in Fish in Bangladesh. *Applied And Environmental Microbiology*, 68(2) : 650–655.
- Sarjito., O.K. Radjasa., S.B. Prayitno., A. Sabdono., S. Hutabarat. 2009. Phylogenetic Diversity of The Causative Agent of Vibriosis Associated with Groupers Fish From Karimunjawa Island Indonesia. *Curr. Res. Bacteriol.* 14-21.
- Sarjito., A. H. C. Haditomo., R. W. Ariyati ., S. B. Prayitno. 2017. The Diversity of Causative Agent Associated with Bacterial Diseases on Catfish (*Clarias gariepinus*) with Molecular Based from Kendal, Indonesia. *Journal of Computational and Theoretical Nanoscience*. 23(7) : 6479-6482.
- Shinha S., T. Shimada., T. Ramamurthy., S.K. Bhattacharya., S. Yamasaki., Y. Takeda., G. B. Nair. 2004. Prevalence, serotype distribution, antibiotic susceptibility and genetic profiles of mesophilic *Aeromonas* species isolated from hospitalized diarrhoeal cases in Kolkata, India. *J. Med. Microbiol.* 53: 527-534.
- Sukenda., L. Jamal., D. Wahjuningrum., A. Hasan. 2008. Use of Chitosan to Prevent *Aeromonas hydrophila* Infection on Catfish *Clarias* sp. *Journal of Indonesia Aquaculture*. 7(2): 159–169.
- Thomas J., N. Madan., K.S.N. Nambi., S.A. Majeed., A.N. Basha., A.S.S. Hameed. 2013. Studies on ulcerative disease caused by *Aeromonas caviae*-like bacterium in Indian catfish, *Clarias batrachus* (Linn). *Aquaculture*, 376-379:146-150.
- Wang G., Y. Gong., Z. Huang., Y. Bian. 2016. Identification of and antimicrobial activity of plant extracts against *Pseudomonas putida* from rot fruiting bodies of *Pleurotus eryngii*. *Scientia Horticulturae*, 212:235-239.