Isolation, identification, and Koch's postulate test of bacteria associated with yellow symptoms in african catfish *Clarias gariepinus*

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Abstract. Farming african catfish *Clarias* sp at high stocking densities can foster stress and bacterial infection. In recent times, african catfish have frequently been discovered displaying symptoms of yellow body jaundice, often resulting in mass mortality. The objective of this study was to identify and characterize bacteria linked to jaundice in african catfish. Bacterial isolates were obtained from ten african catfish with yellowish body symptoms, identifying six dominant isolates. Bacteria were examined for Koch's postulates by injecting 0.1 mL of bacteria, with a concentration of 106 CFU mL^{-1}, into african catfish measuring 21.6±1.7 cm in length. The results of the infection test revealed that fish injected with the six bacterial isolates exhibited pathogenic symptoms, including pale body coloration, dropsy, reduced active swimming, fin thinning, ulceration, and mortality. These symptoms indicate that the bacteria are pathogenic to catfish. The study revealed that six bacterial strains were discovered in the internal organs of jaundiced african catfish. These strains were identified as *Staphylococcus pasteuri*, *Salmonella enterica*, *Escherichia coli*, *Enterobacter hormaechei*, *Bacillales bacterium*, and *Aeromonas salmonicida*. The result suggest the presence of bacteria associated with jaundice in african catfish. The outcomes of the Koch's postulate test indicate that the infection of fish with less specific symptons of illness can lead to jaundice.

1 Introduction

The african catfish (*Clarias gariepinus*), locally known in Indonesia as dumbo catfish, is a vital freshwater aquaculture commodity, highly valued by Indonesian communities. There has been a consistent annual increase in demand for this product [1]. According to data from [2] catfish production reached 347,511,480 kg per year. Fish farmers must focus on sustainable production to meet the significant market demands. Catfish is a popular aquaculture commodity due to its delicious taste, ease of culture maintenance, affordable price, and minimal post-harvest waste [3].

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Recently, African catfish have been frequently observed exhibiting yellowish symptoms, often leading to mortality. The proposed hypothesis suggests a connection between the disease and liver function-related metabolic processes. However, isolating bacterial strains associated with the yellowish disease is necessary to confirm the suspicion. In most cases, bacterial infections are the root cause of diseases in dumbo catfish. Bacteria responsible for causing jaundice in dumb catfish include *Pseudomonas fluorescens, Aeromonas hydrophila*, *Escherichia coli*, *Staphylococcus aureus*, and *Micrococcus luteus* [4]. Some bacteria as mentioned above are normal flora bacteria in african catfish, such as *Escherichia coli*, *Salmonella* sp., *Staphylococcus* sp., *Proteus* sp., *Vibrio* sp., *Pseudomonas* sp., *Serratia* sp., *Shigella* sp., and *Enterococcus* sp. [5].

In Nigeria, microorganisms associated with jaundice in *Clarias gariepinus* have been identified, including Salmonella, *Esherichia coli*, Enterococcus, Pseudomonas, Serratia, Klebsiella, Vibrio, Staphylococcus, Streptococcus, Shigella, and Proteus [6]. However, such information has not yet been identified in Indonesia. Therefore, it is necessary to identify the bacteria associated with yellow symptoms by isolating and verifying their involvement in the occurrence of jaundice in African catfish using Koch's postulates.

2 Methods

2.1 Bacterial isolation

Bacterial isolation was performed on ten diseased catfish displaying yellowing symptoms on the body. The liver, kidney, intestine, and gill organs were scraped and cultured on Tryptic Soy Agar (TSA) media. These cultures were then incubated at a temperature between 29-37 °C for 24 hours. Following observation of colony morphology, the growing bacteria were purified using the quadrant method. Subsequently, the bacteria were prepared for testing to determine their pathogenicity through Koch's postulates and were identified.

2.2 Bacterial identification through biochemical tests

Bacterial identification through biochemical testing was conducted on 24-hour post-culture on TSA media, following the procedure outlined by [7]. Identification of bacteria was based on their observed characteristics such as morphology, colour, and biochemical properties. The latter included colony morphology assessment, gram staining, catalase and oxidase tests, O/F test and motility examination.

2.3 Identification of bacteria by biomolecular methods

2.3.1 DNA extraction

Bacteria intended for DNA extraction were cultured in a liquid media of trypticase soy broth (TSB) at a concentration of 10^5-109 CFU mL^{-1}. Subsequently, the cultured bacteria were transferred to 1.5 mL microtubes before being centrifuged at a rate of 14,000 × g for a period of 1 minute, following which the supernatant was carefully removed. The bacterial DNA was then isolated using the extraction method provided in the Bacteria DNA Isolation Kit (Geneaid, Taiwan). Purity and levels of protein contaminants were analyzed using the spectrophotometric method at 260 and 280 nm wavelengths.

2.3.2 PCR amplification

The polymerase chain reaction (PCR) method was used to amplify 16S rRNA with prokaryote-specific oligonucleotide primers 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') [8]. The process began with pre-denaturation at 95 °C for 5 minutes, followed by denaturation at 92 °C for 30 seconds, annealing at 55 °C for 30 s, and DNA elongation at 72 °C for 1 min. This was repeated up to 30 times, with DNA elongation (post PCR) at 72 °C for 5 min and then storage at 4 °C.

2.3.3 Electrophoresis

The bacterial DNA, which was isolated earlier, was subjected to electrophoresis by using 0.4% agarose dissolved in tris-acid acetate (TAE) solution whose quantity was 0.4 g. The first well involved 3 µL of marker DNA, whereas the second well had 3 µL of amplified DNA serving as a positive control. The third well contained negative control, while the fourth well sustained samples numbered 1 to 6. The electrophoresis process went on for half an hour at 100 V. The agarose gel was transferred to an UV-doc device to observe the electrophoresis outcomes. The target DNA band would appear as a clear fluorescent band in line with the positive control band. The absence of a visible band on the agarose suggested that the sample was bacteria free.

2.3.4 DNA sequencing and analysis

The amplified DNA was sequenced at 1st Base Laboratory in Singapore. Trimming techniques were applied during analysis. The amplified DNA was sequenced at 1st Base Laboratory in Singapore. Subsequently, the sequencing results were analyzed using Mega v.10 software (http://www.mwgasoftware.net) and Bioedit v 7.2.5 application (http://mbio.ncsu.edu/Bioedit). In addition, the nucleotide sequences and amino acid residues were compared with other sequences found in gene databases using the BLAST® facility (http://www.ncbi.nlm.nih.gov). The organization of amino acid sequences that encode genes was forecasted utilizing the BLASTX® program and compared with several other species [9] on the NCBI (National Center for Biotechnology Information) database.

2.4 Koch's postulate test

2.4.1 Preparation of fish and rearing containers

The study employed *Clarias* sp., commonly known as catfish, as test subjects. The fish exhibited an average length and weight of 21.57±1.71 cm and 80.75± 9.29 g, respectively. A total of 30 fish were tested, with 5 subjects per bacterial isolate injection. The fish were reared in a glass aquarium measuring 65 × 40 × 40 cm^3 with a water height of 20 cm. Technical abbreviations will be explained when first used. The aquarium was disinfected with chlorine at a concentration of 30 $mg.L^{-1}$, then filled with approximately 15 L of water and an aerator hose was installed for aeration. The test fish were acclimated in the rearing container for three days.

2.4.2 Fish infection

The Koch's Postulate experiment entailed the injection of bacteria into catfish. Specifically, bacteria were obtained from jaundiced fish and individually injected into the catfish. The bacteria were then cultured in TSB media for 24 hours at 30 °C. The injection procedure was carried out intramuscularly. Before bacterial injection, anesthetic solution was administered,

using clove oil solution (*Eugenia aromatica*) at a dose of 0.1 mL^{-1} water. The bacteria were injected at a density of 106CFU mL^{-1} [10] with a dosage of 0.1 mL per fish. The injection of bacteria was administered using a 1 mL syringe through the caudal vein. A Koch's postulate test was undertaken over a duration of 14 days with aeration and siphoning treatment [11]. Throughout this period, changes that occurred in the test fish were observed and recorded at 6-hour intervals.

2.4.3 Bacteria re-isolation

Bacteria injected into the test fish's body were re-isolated from various parts, such as the liver, kidneys, gills, intestines, and wounds found in catfish. The isolated bacteria were subsequently cultured on TSA media. Characteristics, including morphological shape, color, and biochemical properties of bacteria, are observed in order to identify those that grow on the media. The bacterial characterization results were identified using the Cowan and Steel table [7].

2.4.4 Prevalence of bacteria

Prevalence indicates the proportion of fish found to be infected with bacteria out of the total number of fish samples analyzed [12]. The formula to calculate bacterial prevalence is as follows:

$$\text{Prevalence (\%)} = \frac{Number\ of\ fish\ infected\ with\ bacteria}{Number\ of\ fish\ inspected} \quad (1)$$

3 Results and Discussion

3.1 Bacterial isolation and identification results

From the isolation of six fish displaying yellowish symptoms, several bacterial strains were obtained and subsequently analyzed. Six bacterial isolates emerged as dominant from this analysis. The bacteria exhibited round colonies with flat edges and convex elevations in diverse colors, including white, yellow and yellowish white. Bacterial observations were compared with the Cowan and Steel 2003 table and the results were the same at the genus level [7]. Biomolecular testing using the PCR method with universal 16 S rRNA primers showed the presence of *Staphylococcus pasteuri*, *Salmonella enterica*, *Escherichia coli*, *Enterobacter hormaechi*, *Bacillales bacterium*, and *Aeromonas salmonicida* (Table 1).

Table 1. Results of bacterial identification of jaundiced dumbo catfish by biochemical and molecular methods

Test							Results of bacterial identification (biochemical methods)	Species (biomolecular methods)
Isolates	**Gram**	**Form**	**SIM**	**O/F**	**Catalase**	**Oxidase**		
A4G	Negative	Coccus	(+)	F	(+)	(-)	*Enterobacteria*	*Escherichia coli*
C4G	Positive	Coccus	(-)	F	(+)	(-)	*Staphylococcus* sp.	*S. pasteuri*
E4G	Negative	Coccus	(+)	F	(+)	(-)	*Enterobacteria*	*S. enterica*
B2U	Negative	Coccus	(+)	F	(+)	(-)	*Enterobacteria*	*E. hormaechei*
B5L	Positive	Bacillus	(-)	F	(+)	(-)	*Bacillus* sp.	*B. bacterium*
EH2	Negative	Bacillus	(+)	F	(+)	(+)	*Aeromonas* sp.	*A. salmonicida*

Table 2. Alignment results of sequencing results using BLAST

Sample	Name of species	Results of identity (%)*	Access number
A4G	*E. coli*	100%	CP067311.1
C4G	*S. pasteuri*	100%	MT539733.1
E4G	*S. enterica*	91,28 %	KX355298.1
B2U	*E. hormaechi*	95,19 %	LC195004.1
B5L	*B. bacterium*	93,81 %	KJ630306.1
EH2	*A.salmonicida*	100%	X64214.1

*The percentage value of bacterial relatedness is measured by the percent identity metric. Furthermore, an access code is employed for data retrieval from the BLAST database. The percent identity score of the sample revealed a higher degree of bacterial relatedness, attaining a full match with the BLAST results obtained on the National Center for Biotechnology Information (NCBI) database.

Bacteria, the most prevalent prokaryotic organisms in nature, consist of cell walls and cell contents. They exist in three primary forms: rods (bacilli), spheres (cocci), and spirals (spirilla). Some strains display cellular arrangements such as pairs, clusters, and chains or filaments. Generally, bacteria are too small to be seen without a microscope [8]. Bacteria that can be analyzed in laboratory setting*s typically measure between 0.5*-2.0 µm in width and *1*-5 µm in length [13]. The study also investigated various water quality tests, such as dissolved oxygen (DO), pH, temperature, and total ammonia nitrogen (TAN), ranging between 4.0-5.2 $mg.L^{-1}$, 6.9-7.8, 26-28 °C, and 0.029-0.198 $mg.L^{-1}$, respectively. It should be noted that DO represents the amount of oxygen dissolved in the water, while TAN is a measure of the total concentration of ammonia and ammonium ions. According to SNI 6484.4 of 2014, the observed water quality met the standards for optimal fish culture, with values within the recommended ranges: a minimum of 3 $mg.L^{-1}$ for DO, pH between 6.5-8.0, temperature between 25-30 °C, and a maximum of 0.1 $mg.L^{-1}$ for TAN.

3.2 Koch's postulate test results

From the Koch's Postulate test of the six bacteria on dumbo catfish, the observed clinical symptoms are listed in Table 3.

Table 3. Clinical symptoms in fish challenged with the isolated bacteria

Code of isololates	Identification using the biochemical methods	Changes that occur after bacterial infection	
		Morphology	Behaviour
A4G	*Escherichia coli*	Fish affected by this condition had thinning fins, red spots, peeling scales, bleeding, bloating, and excess mucus discharge.	The observed behaviours of the subject fish exhibited decreased appetite, swimming sideways, gasping, less active swimming, and being at the bottom of the water.
C4G	*Staphylococcus pasteuri*.	Symptoms of fish illness included thinned fins, pale body, flaky scales, dropsy, ulcer, and mucus discharge.	Decreased appetite, swimming sideways, gasping, less active swimming, and being at the bottom of the water
E4G	*Salmonella enterica*	Thin fins, pale body, scaly flakes, white spots, inflamed anus,	Decreased appetite, swimming sideways, less active swimming, being at the bottom

Code of isololates	Identification using the biochemical methods	Changes that occur after bacterial infection	
		Morphology	**Behaviour**
		ulceration, dropsy, flaky flesh, and oozing mucus.	of the water.
B2U	*Enterobacter hormaechi*	The fish exhibited symptoms such as thinning fins, red spots, peeling scales, edema, and excessive mucus secretion.	Decreased appetite, swimming sideways, gasping, less active swimming, staying at the bottom of the water and occasionally surfacing.
B5L	*Bacillales bacterium*	The fish exhibited symptoms such as thinning fins, red spots, peeling scales, edema, and excessive mucus secretion.	Decreased appetite, lateral swimming, decreased activity, and staying at the bottom of the water.
EH2	*Aeromonas salmonicida*	Thin fins, pale body, peeling flesh, pistil spots, red ventral fins, secretes a lot of mucus	Decreased appetite, swimming sideways, less active swimming, being at the bottom of the water.

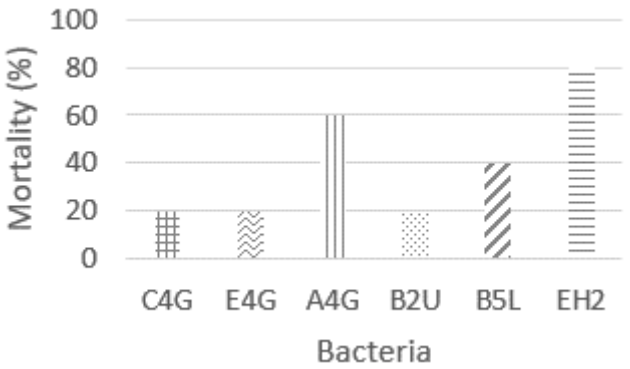

Fig. 1. Prevalence of bacteria in catfish at rearing after Koch's Postulates (C4G= *Staphylococcus pasteuri*, E4G= *Salmonella enterica*, A4G= *Escherichia coli*, B2U= *Enterobacter hormaecheI*, B5L= *Bacillales bacterium*, EH2= *Aeromonas salmonicida*)

The prevalence of bacteria in catfish is presented in Figure 1. Based on the observations, EH2, A4G and B5L show a high prevalence rate of up to 80% with severe clinical symptoms in the fish body. Meanwhile, the isolate codes C4G, E4G and B2U show a low prevalence value of 20% with less severe clinical symptoms. The results of the prevalence value show that the higher the value, the higher the virulence of the bacteria against the catfish. From the re-isolation of bacteria after the challenge test, the results varied. *Staphylococcus pasteuri* bacteria can be isolated from the kidney, *Salmonella enterica* from the intestine, Escherichia coli from the intestine, kidney and liver. Re-isolation of *Enterobacter hormaechi* bacteria was successfully carried out from the intestine, *Bacillales bacterium* from wounds and kidneys, while *Aeromonas salmonicida* bacteria could be obtained from the liver and kidneys. From the re-isolation data, there are two types of bacteria that infect the liver.

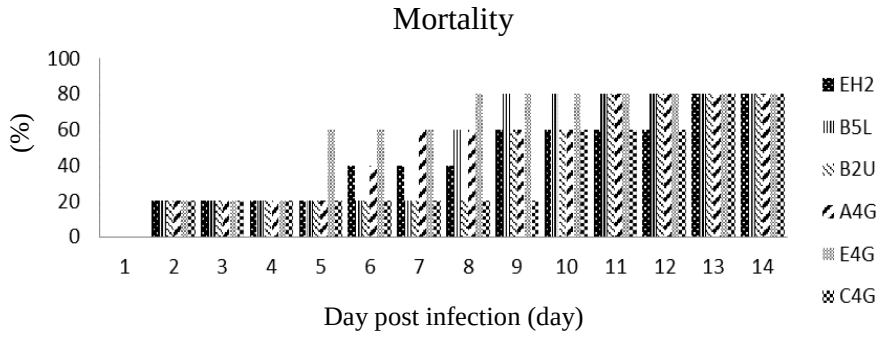

Fig. 2. Mortality rate after Koch's Postulate test (C4G= *Staphylococcus pasteuri*, E4G= *Salmonella enterica*, A4G= *Escherichia coli*, B2U= *Enterobacter hormaecheI*, B5L= *Bacillales bacterium*, EH2= *Aeromonas salmonicida)*

According to [14], *Staphylococcus pasteuri* is a grape-shaped, gram-positive bacterium that is facultatively anaerobic, does not form spores, and is non-motile according to the C4G bacterial isolate. The E4G bacterial isolate concurs with [15] findings, specifically regarding *Salmonella enterica*, which can be found on the skin's surface, gills, and entrails in three different spots on the fish body. Following fish death, infection caused by *Salmonella enterica* can lead to morphological changes and decay including alterations in taste, odour, colour, and mucus formation. *Salmonella enterica* is a non-spore-forming, motile, gram-negative, rod-shaped bacterium that is facultatively anaerobic or aerobic in the Enterobacteriaceae family.

Bacterial isolate A4G reveals the characteristics of *Escherichia coli*, which is a non-spore-forming, rod-shaped bacterium with motile flagella, small-medium size, smooth consistency, and flat edges. It tests positive for indole and produces gas from glucose [16]. The identification results for bacterial isolate B2U match those of *Enterobacter hormaechi*. This gram-negative, oxidase-positive, rod-shaped bacterium was identified as a distinct species in 1989. Additionally, it has been established that *Enterobacter hormaechi* has a wide distribution and can be influenced by environmental conditions [17]. The findings from the analysis of bacterial isolate B5L identified the presence of *Bacillales bacterium*. These bacteria have a gram-positive structure and form endospores that can be round, oval, elliptical, or cylindrical shapes in vegetative cells [18]. Several symptoms of the disease, including red wounds on the mouth and gill covers, red fins, skin rashes, protruding eyes, redness of the anus and swelling of the abdomen, injection scars that develop into ulcers, and skin color fading from grey to pale also identify by [19].

Symptoms of the disease are attributed to *Aeromonas salmonicida* bacterial isolates, specifically bacterial isolate EH2. Aeromonas species belong to the Aeromonadaceae family, and are facultative anaerobic gram-negative bacteria with rod-like shape, 0.3-1.0 μm in diameter and 1.0-3.5 μm in length, approaching the shape of sperms. Cells exist either in unicellular or grouped forms, and are characterized by their gram-negative staining [20]. Molecular identification is achieved through sequencing the 16S rRNA gene, which is commonly utilized to determine bacterial types. The research conducted [21] identified its ability to observe similarities between bacterial species. The sequencing method reveals the lowest similarity between species, of which 91.28% was recorded. The sequencing method reveals the lowest similarity between species, of which 91.28% was recorded. This investigation, however, revealed variable query lengths in the sequencing results of bacterial isolates. Code C4G represents *Staphylococcus pasteuri* with a length of 591 base pairs (bps), while isolate code E4G stands for *Salmonella enterica* with a length of 322 bps. Furthermore, isolate code A4G denotes *Escherichia coli* with 284 bps, isolate code B2U refers to

Enterobacter hormaechi with 396 bps, isolate code B5L represents *Bacillales bacterium* with 179 bps, and isolate code EH2 is indicative of *Aeromonas salmonicida* with a length of 20 bps.

The bacteria found were associated with the yellowish body symptom in catfish. *Staphylococcus pasteuri*, *Salmonella enterica*, *Escherichia coli*, *Enterobacter hormaechi*, *Bacillales bacterium*, and *Aeromonas salmonicida* were tested using Koch's postulate. The test of Koch's postulates with bacteria alone results in the manifestation of symptoms such as pale skin, the development of ulcers and redness in both the ventral and anal regions. However, it does not produce the distinct yellowish body symptoms observed with the bacterial isolates obtained from jaundiced fish.

References

1. N. B. P. Utomo, Susan, M. Setiawati, Jurnal Akuakultur Indonesia **12**, 2 (2013)
2. Ministry of Marine Affairs and Fisheries. Laporan Kinerja Perikanan (LKj) budidaya tahun 2020 (KKP, 2020) (in Indonesian)
3. Damayanti, Agensia penyebab dan profil darah ikan lele dumbo (*Clarias gariepinus*) yang terserang penyakit bakteri, Undergraduate Theses (Diponegoro University, Semarang, 2011) (in Indonesian)
4. G. Ikpi, B. Offem, Rev. Biol. Trop **59**, 2 (2011)
5. T. J. Amande, S. U. Nwaka, IOSR Journal Of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT) **5**, 3 (2013)
6. T. A. Musefiu, E. B. Obuko, A. O. Bolarinwa, Journal of Applied Sciences Research **7**, 7 (2011)
7. G. I. Barrow, R. K. A. Feltham, Cowan and Steel's manual for the identification of medical bacteria (Cambridge University Press, England, 2003)
8. J. R. Marchesi, T. Sato, A. J. Weightman, T. A. Martin, S. J. Hiom, W. E. Wade, Appl. Environ. Microbiol **6**, 4 (1998)
9. N. Saitou, M. Nei, Mol. Biol. Evol **4** (1987)
10. N. Sardjito, O. K. Ningrum, S. B. Radjasa, Prayitno, Journal Of Coastal Development **15**, 3 (2012)
11. S. Rejeki, Triyanto, Murwantoko, Jurnal Perikanan Universitas Gadjah Mada **18**, 2 (2016)
12. Z. Kabata, Parasites and disease of fish cultured in the tropics (Cambridge University Press, England, 1985)
13. A. Irianto, Patologi ikan telestoi (Universitas Gadjah Mada Press, Yogyakarta, 2007) (in Indonesian)
14. T. Kurniasih, Widanarni, Mulyasari, M. Irma, I. A. Zafril, M. A. Lusiastuti, Jurnal Riset Akuakultur **8**, 2 (2013)
15. S. M. Jajere, Vet. World **12**, 4 (2019)
16. A. Fauzan, Isolasi dan karakterisasi dari bakteri ikan laut dalam serta screening potensinya sebagai senyawa antibakteri, Undergraduate Theses (IPB University, Bogor, 2010) (in Indonesian)
17. Z. Wang, L. Duan, F. Lius, Yun Hu, F. Leng, K. Yunchao, Y. Lunguang, S. Hongfei, BMC Veterinary Research **1**, 6 (2020)
18. X. Zhao, O. P. Kuipers, BMC Genomics **1**, 7 (2016)
19. Muslikha, S. Pujiyanto, S. N. Jannah, H. Novita, Jurnal Biologi **5**, 4 (2016)
20. J. G. Holt, N. R. Krieg, P. H. Sneath, J. T. Staley, S. T. Williams, Bergey's Manual of Determinative Bacteriology 9th Ed. (Lippincott, Williams & Wilkins, Baltimore, 1994)
21. G. A. Dewa, M. S. I Dewa, S. I Wayan, Jurnal Sains **33**, 2 (2015)