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FIRST CASE STUDY OF COINFECTION *OF Staphylococcus epidermidis* AND *Pseudomonas putida* IN ANGELFISH (*Pterophyllum scalare*) IN SOUTH KALIMANTAN, INDONESIA, WITH THE MAIN CLINICAL SYMPTOM OF LETHARGY

Studi Kasus Pertama Koinfeksi *Staphylococcus epidermidis* dan *Pseudomonas putida* pada Ikan Bidadari (*Pterophyllum scalare*) di Kalimantan Selatan, Indonesia, dengan Gejala Klinis Utama Letargi

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ABSTRACT

Angelfish (Pterophyllum scalare) is a freshwater ornamental fish with high economic value and intensive trade, which increases its susceptibility to infectious diseases. This study reports the first documented case of co-infection by Pseudomonas putida and Staphylococcus epidermidis in angelfish cultured in an aquarium in South Kalimantan, Indonesia. Mortality was recorded in September 2022 and occurred exclusively among angelfish exhibiting lethargy, darkened body coloration, abnormal swimming, and bottom-remaining behavior. Five moribund fish were subjected to clinical examination, post-mortem analysis, bacterial isolation, Gram staining, VITEK 2 Compact identification, and histopathological evaluation of eye and brain tissues. Bacterial isolation revealed P. putida from liver samples and *S.* epidermidis from blood samples. Histopathological findings showed severe cerebellar lesions, including spongiosis, Purkinje cell necrosis, granular layer degeneration, hemorrhage, and bacterial infiltration. Ocular lesions were characterized by cataract formation and bacterial presence within the lens. These findings indicate a systemic bacterial infection with central nervous system involvement. The results suggest that P. putida is the primary pathogen, while S. epidermidis acts as an opportunistic co-infecting bacterium. This report emphasizes the importance of comprehensive diagnostic approaches for mixed bacterial infections in ornamental fish to support effective disease management in aquaculture.

Keywords: Pterophyllum scalare, co-infection, Pseudomonas putida, Staphylococcus epidermidis, histopathology

ABSTRAK

Ikan bidadari (*Pterophyllum scalare*) merupakan ikan hias air tawar bernilai ekonomi tinggi dengan tingkat perdagangan intensif sehingga rentan terhadap penyakit infeksi. Penelitian ini melaporkan kasus pertama koinfeksi *Pseudomonas putida* dan *Staphylococcus epidermidis* pada ikan bidadari yang dipelihara dalam akuarium di Kalimantan Selatan, Indonesia. Kematian ikan terjadi pada September 2022 dan hanya menyerang ikan bidadari dengan gejala klinis berupa letargi, perubahan warna tubuh menjadi gelap, gangguan keseimbangan berenang, serta berdiam di dasar akuarium. Lima ekor ikan moribund diperiksa melalui pemeriksaan klinis, nekropsi, isolasi bakteri, pewarnaan Gram, identifikasi menggunakan VITEK 2 Compact, serta analisis histopatologi jaringan mata dan otak. Hasil isolasi menunjukkan *P. putida* berasal dari organ hati dan *S. epidermidis* dari sampel darah. Pemeriksaan histopatologi memperlihatkan lesi berat pada cerebellum berupa spongiosis, nekrosis sel Purkinje, degenerasi lapisan granular, perdarahan, serta infiltrasi bakteri. Perubahan patologis pada mata berupa katarak dan keberadaan bakteri pada lensa turut teramati. Temuan ini menunjukkan infeksi bakteri sistemik dengan keterlibatan sistem saraf pusat. *Pseudomonas putida* berperan sebagai patogen utama, sedangkan *Staphylococcus epidermidis* bertindak sebagai ko-infeksi oportunistik. Studi ini menegaskan pentingnya diagnosis menyeluruh pada infeksi bakteri campuran pada ikan hias.

Kata Kunci: *Pterophyllum scalare*, koinfeksi, *Pseudomonas putida*, *Staphylococcus epidermidis*, histopatologi

INTRODUCTION

Angelfish (Pterophyllum scalare) is a popular freshwater ornamental fish (Das et al., 2020) with long-standing economic value and is relatively easy to breed (Meneses et al., 2021). This fish, originating from the Amazon River in South America, is the most attractive species in the family Cichlidae (Sushila et al., 2020).

As an ornamental fish that is relatively easy to breed, it is relatively inexpensive and is widely traded among freshwater aquarium enthusiasts. This situation increases the risk of disease spread among angelfish(Gallani et al., 2016). Diseases that are especially common in angelfish include bacterial infections.

Bacterial diseases in freshwater ornamental fish are often reported not just as a single occurrence, but sometimes involve two or more types of bacteria or act as a secondary infection to a viral infection (Sihananto & Wijayanti, 2024), making the recognition of clinical symptoms and diagnosis through proper disease testing crucial in disease control, especially in freshwater ornamental fish.

Among bacterial diseases, Pseudomonas spp. is considered one of the most pathogenic fish pathogens and is responsible for ulcer-type diseases, including ulcerative syndrome (Algammal et al., 2020). Pseudomonas spp. are among the most common bacterial infectious agents of cultured fish and have been reported to cause stress-related diseases in freshwater fish, especially under farming conditions (Lopez et al.. Several studies have reported nearly 100% mortality from Pseudomonas spp. infections in rainbow trout, sea bream, sea bass, and ayu in farm settings (B. Austin & Stobie, 1992).

Pseudomonas spp. Infection is characterized by petechial hemorrhage, darkness of the skin, detached scales, abdominal ascites, and exophthalmia in rainbow trout, African catfish (Clarias gariepinus), and Nile tilapia (Oreochromis niloticus) (Hanna et al., 2014; Ilhan et al., 2006; Omar et al., 2017).

Among Pseudomonas spp., P. putida is a pathogenic bacterium that infects O. niloticus and causes exophthalmia, ascites, and ulceration on the fish's body (Salama et al., 2009). It is also recognized as a major pathogen in rainbow trout fisheries (Ilhan *et al*, 2006; Sakai *et al*, 1989). P. putida is an opportunistic Gram-negative pathogen commonly found in aquatic environments and as a part of the normal gut flora of healthy fish. It belongs to the family Pseudomonadaceae, genus Pseudomonas (Ismail et al., 2023; Oh et al., 2019).

Pseudomonas are ubiquitous inhabitants of oxygenated environments ((Palleroni, 1984) Austin & Stobie, 1992; (B. A. D. A. Austin, 2016) Pseudomonas putida, a member of genetically related fluorescent pseudomonads, is an aerobic, Gram-negative basil shaped bacterium, and has been isolated and identified from diseased ayu (Plecoglossus altivelis altivelis) (Wakabayashi & Nishimori, 1996), yellowtail (Seriola quinqueradiata) (Kusuda & Sugiyama, 1981; Toyoshima, 1976) European eel (*Anguilla anguilla*) (Haenen & Davidse, 2001) oyster toadfish (Opsanus tau) (Roxanna Smolowitz, 1998), large yellow croaker (*Pseudosciaena croce*a), black sea bream (Sparus macrocephalus) (Kubilay & Uluköy, 2004), and Nile tilapia (Oreochromis niloticus) (Eissa et al., 2010; Ismail *et al*, 2023; Zhang et al., 2022)). Moreover, this bacterium is an opportunistic human pathogen responsible for bacteremia (Yoshino & Kitazawa, 2011), and also P.putida is considered the main component of these spoilage microbes with an incidence between 56.7% and 79.0% on spoiled meat (Doulgeraki et al., 2013).

In Turkey, Pseudomonas infection caused by P. putida was first diagnosed in scattered mirror carp (Cyprinus carpio) and goldfish (Carassius auratus) (Aydın et al., 1998). Afterwards, it was reported in cultured rainbow trout (Altinok et al., 2006). P. putida has not been reported as a primary agent in any marine cultured or wild fish species in Turkey. This paper reports the first case of P. putida infection in diseased angel fish (Pterophyllum scalare), with histopathological necrosis in the brain and bacterial cells in the eyes.

A recent study reports that P. putida causes higher mortality in Nile tilapia because it carries virulence-linked genes (ToxA, Nan1, and ExoS) (Alzahrani et al., 2023). P. putida is highly pathogenic for O. niloticus and induces higher mortalities because of detecting toxA, nan1, and exoS virulence-associated genes (Alzahrani et al., 2023). This bacterium in fish shows abnormal swimming, surfacing, resting, and aggression traits (approach, chase, flee, and mouth pushing), as well as abnormal movement (circular and vertical).

(Alzahrani et al., 2023) stated that P. putida caused various behavioral alterations (abnormal swimming, surfacing, resting, and aggression), clinical signs (skin darkness, body hemorrhages, and severe skin ulcerations), and lower survivability (56%). The presence of virulence genes could dominate this [exotoxin A (tox A), nan1, and exoenzyme S (exo S)], which accelerate the infection process (Altinok et al., 2006). isolated P. putida from rainbow trout with exophthalmia, dark pigmentation of the skin, and ulceration, particularly on the dorsal side. P putida paling sensitif dengan menggunakan antibiotik Gentamicin (GN) (10 mcg) dan Ciprofloxacin (CIB) (5 mcg), dan resisten dengan menggunakan antibiotik Amoxicillin (Ax) 25 mcg Ampicillin (AM) 10 mcg, Erythromycin(E) 15 mcg, dan Oxalinic acid (OA) 2 mcg. (Alzahrani et al., 2023).

In addition to P. putida, Staphylococcus epidermidis has been reported as a fish pathogen in some marine and freshwater fish in Japan, Taiwan, and Greece (Kusuda & Sugiyama, 1981; Ismail et al., 2023; Kubilay & Uluköy, 2004). Several severe epizootics have been described in cultured fish, including red sea bream (Chrysophrys major) and yellowtail (Seriola quinqueradiata) in Japan ((Kusuda & Sugiyama, 1981); grass carp (Ctenopharyngedon idella) (Wang et al, 1996) and tilapia (Oreochromis spp.) in Taiwan (Huang et al., 1999) and sea bream (Sparus aurata) and sea bass (Dicentarchus labrax) at different fish farm locations in Greece (Varvarigos, 2001). Sugiyama and Kusuda (1981) thought that the bacteria originated from

water or fish rather than from humans, because of pronounced antigenic differences compared with human strains of S. epidermidis.

This suggestion is supported by ecological studies that have clearly demonstrated the presence of S. epidermidis in aquatic environments (Austin, 2016; Austin, B. and Austin, 1999) Staphylococci may be present in the fish year-round, but a sudden rise in water temperature or other stressors in the aquatic environment triggers the disease. It usually appears in the spring and causes problems throughout the summer (Huang et al., 1999). Staphylococcal infections in fish were also observed by (Fryer, 1993) under specific and severe environmental stress. In fish, typical symptoms of staphylococcal infections include exophthalmia, congestion, and tail ulceration (Kusuda and Sugiyama, 1981). Staphylococcal infections involve systemic disease characterized by septicaemia (Çanak & Timur, 2020; Huang et al., 1999; Ismail et al., 2023; Namvar et al., 2014).

Staphylococcus epidermidis is a bacterium that can cause disease in humans and is usually found in processed fishery products. One sign of opportunistic infections caused by Staphylococcus epidermidis is their incidence among people with weakened immunity. Staphylococcus food poisoning is usually characterized by the sudden Onset of symptoms, including nausea, vomiting, stomach cramps, and diarrhea.

Staphylococcus epidermidis which is known as a coagulase-negative and Gram-positive Staphylococcus, is one of the five significant microorganisms that are located on human skin and mucosal surfaces with the ability of causing nosocomial infections due to the wide usage of medical implants and devices, hence until 1980 S. epidermidis was considered as an opportunistic microorganism, while in accordance to various infections increasement such as cardiovascular, CNS shunts, joints, blood stream infections, etc. (Namvar et al., 2014).

Von Eiff et al., (2002) stated that the most significant virulence factors in S. epidermidis are caused by Biofilms; PIA (polysaccharide intercellular adhesion); Bap (biofilm-associated protein); Poly-γ-glutamic acid (PGA); Toxins, which are staphylococcal enterotoxin-like toxin L (SEIL) and C3 enterotoxin (SEC3); Phenol-soluble modulins (PSMs); Delta-Toxin; Clpxp; and Embp (extracellular matrix binding protein).

In 2022, deaths were reported in angelfish with very distinctive clinical symptoms different from typical disease symptoms, namely the body color becoming darker and swimming sideways, then lying at the bottom of the aquarium, followed by the death of several fish. This condition did not occur in other types of fish in the same aquarium.

This study aimed to determine the cause of death of angelfish through histopathological and bacterial tests. The study reports the first documented co-infection between the bacteria Staphylococcus epidermidis and Pseudomonas putida in angelfish (Pterophyllum scalare) kept in an aquarium in South Kalimantan, Indonesia.

METHODS

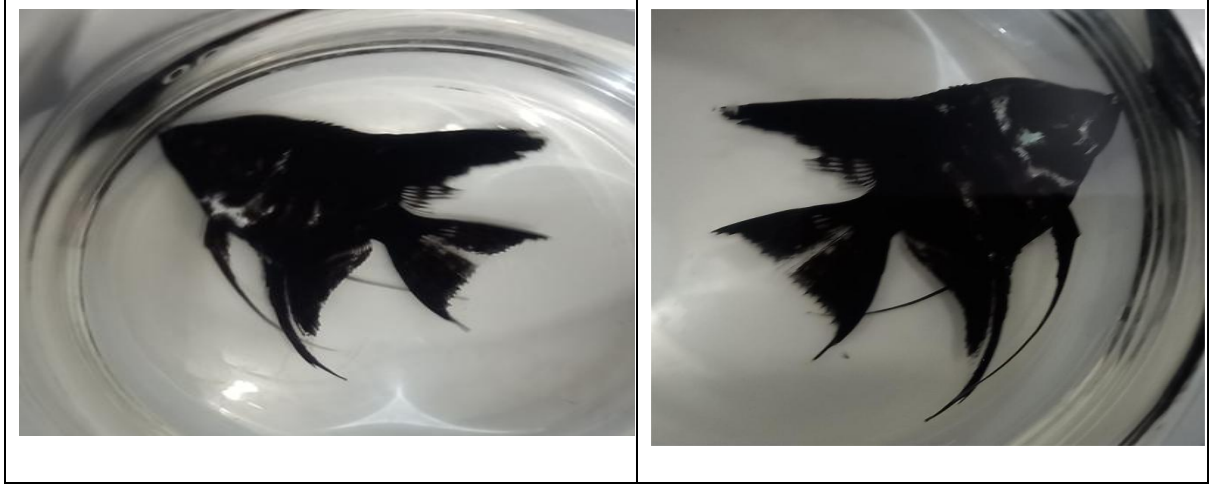


Figure 1. A, B Manfish exhibiting clinical symptoms of blackish discoloration and sinking to the bottom.

Study area

Samples were taken from the Aquarium from the farmer in South Kalimantan Province, Indonesia. The farmer reported mortality only among the angel fish in the aquarium's ornamental fish mix in September 2022.

Samples

Five moribund fish, exhibiting skin discoloration, cataract, abnormal swimming, and lying on the bottom, were collected. Each fish ranged between 10 and 15 g in body weight. Each fish sample was labeled and transported alive to the Mandiangin Freshwater Aquaculture Development Center (MFADC) Testing Laboratory for isolation of the causative agents.

Clinical examination

The clinical examination was performed according to the method described by Noga, n.d.

Post-mortem examination

The post-mortem examination was performed according to the method described by (Heil, 2009).

Isolation and identification of the causative agent

The sampled fish were then necropsied. Samples were taken from suspected bacterial infections using a sterile needle from the liver, streaked onto Tryptic Soy Agar (Oxoid), and incubated at 33 °C for 24 hours. Blood samples were taken using a 1 mL syringe and then streaked on Tryptic soy agar (Oxoid) media, and then incubated at 33º C for 24 hours.

Uji histopatologi

The eyes and brains were removed and fixed in 10% Neutral Buffer Formalin, then processed automatically for 24 hours using an Automatic Tissue Processor RM 2260. They were then blocked using the Leica EG 1150H Hot Plate Embedding Tissue Complete and the Leica EG 1150C Cold Plate Embedding Tissue Complete. Thin sections were cut at 4 µm using a Leica RM 2260 Microtome and stained with routine H&E staining using a Leica st 5020 automatic stainer (IKM No.7.2.10/BPBAT-M). The stained sections were mounted with Entellan (Merck® Germany) and analyzed using an Olympus® Japan CX41 microscope and an Olympus® Japan MD 20 camera.

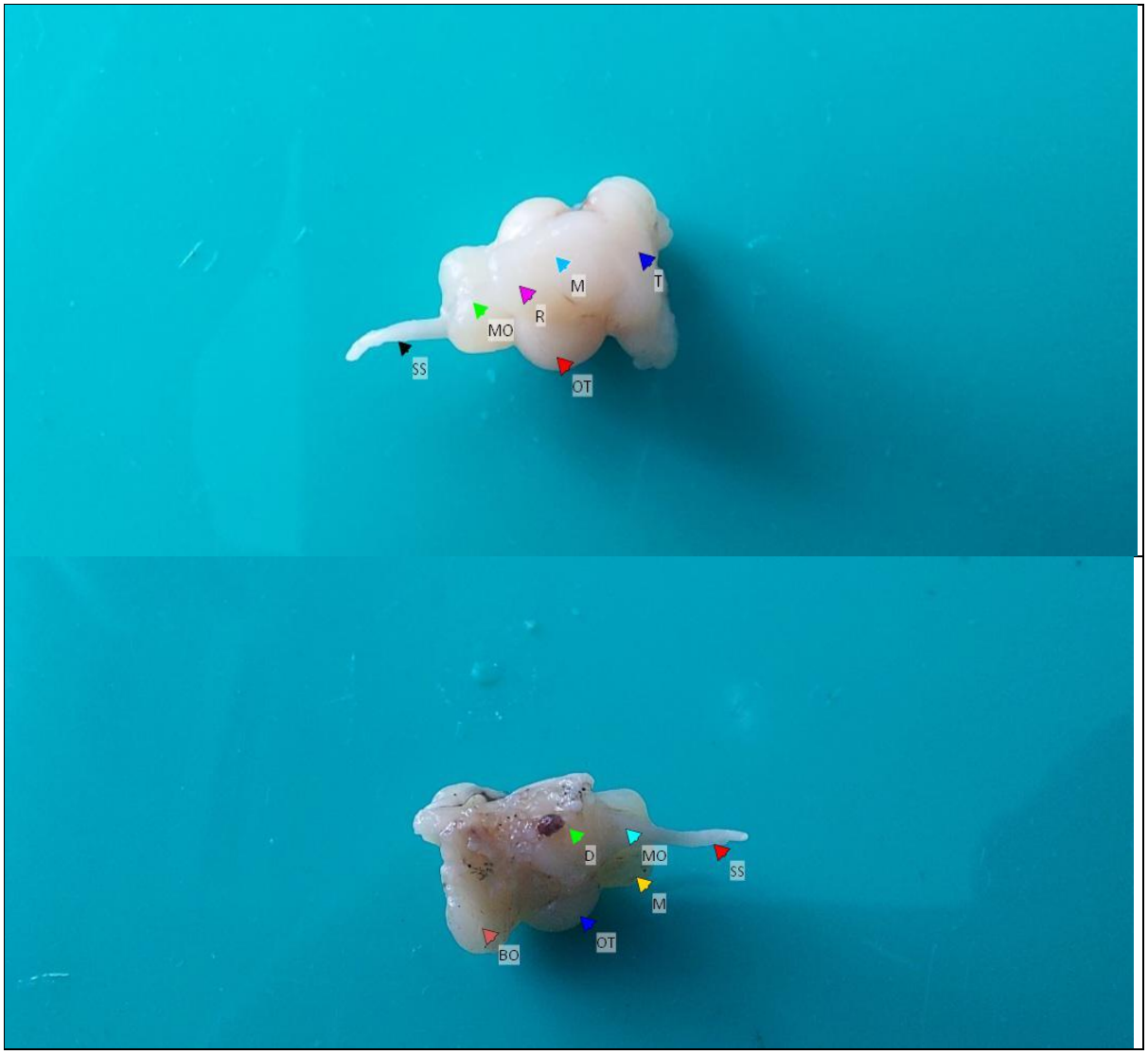


Figure 2. A. The dorsal view of the manfish brain shows: T: Telencephalon, M: Metencephalon, R: Rhombencephalon, OT: Optic Tectum, MO: Medulla Oblongata, SS: Spinal Cord. B: BO: Optic Bulb, OT: Optic Tectum, D: Diencephalon, M: Metencephalon, MO: Medulla Oblongata, SS: Spinal Nerve.

VITEK 2 system identification for recovered isolates:

Gram staining procedures

Gram stain procedures were performed according to the method described by (Lynes, 1976) (C.H. Collins, P.M. Lyne, J.M. Grange, 2004)

VITEK 2 system protocol

For Gram-positive and Gram-negative recovered isolates: A few morphologically similar colonies were picked from a tryptic soy agar plate and then suspended in a sterile test tube containing 3 mL of 0.5% NaCl saline. The optical density of the solution was tested using Densi Chek Pluscalibrator and adjusted to 0.6 McFarland standards. In the VITEK 2 apparatus, a Gram-positive ID card (GP) was set in the cassette with the suspension test tube. Cards were then automatically inoculated with microbial suspensions using an integrated vacuum apparatus. The system's software logged data for entry and initiated the automated pathogen identification process according to the manufacturer's instructions.

Identifikasi dengan VITEK 2 Compact

Identification with Vitek 2 begins by preparing test tubes on the Vitek cassette rack, then filling them with 3 mL of saline solution. Pure bacterial colonies aged 18-24 hours are suspended in 3 mL of saline solution in a test tube using a micropipette, then homogenized. This is followed by preparing a bacterial suspension with a turbidity level of approximately 0.5-0.63 McFarland using a Densicheck device. Next, prepare the Vitek Gram Negative (GN) and Gram Positive cards, then insert the tube connected to the card into the test tube containing the bacterial suspension. After that, the data from the tested samples is entered into the Vitek 2 System software, which is connected to the Vitek 2 Compact device. The cassette rack containing the bacterial sample suspension is inserted into the loading filler chamber of the Vitek 2 Compact device, and the "Start Fill" button is pressed. An alarm will sound, and the filling process indicator will flash when the process is complete. Then move the cassette rack to the incubator chamber and read the results on the Vitek 2 Compact device. The cassette is then removed. The identification process will run automatically for 2-10 hours, and the results will be available automatically.

RESULTS

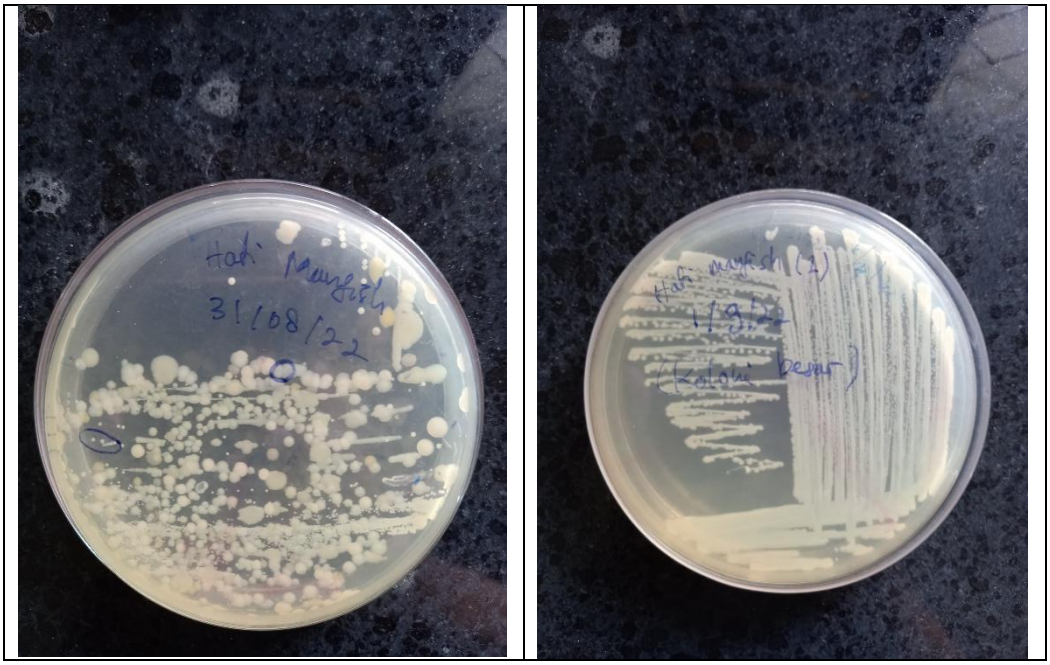


Figure 3. Colony of P. Putida bacteria originating from the liver

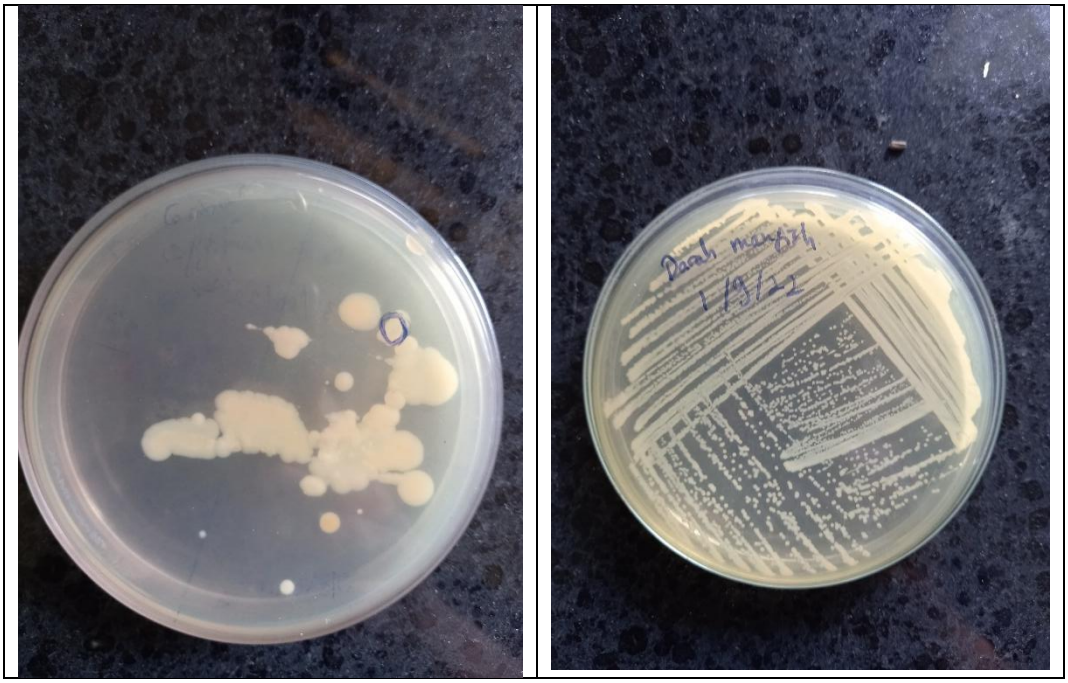


Figure 4. Colony of S. Epidermidis bacteria from blood

Table 1. Phenotypic characteristics of Pseudomonas putida and Staphylococcus epidermidis species isolated from the Angel fish

Characters	P. putida	S Epidermidis
Gram stain	Gram negative	Gram positive
Colony diameter (mm)		1
Morphology		Spherical cocci
Pigmen	Can't produce	Can't produce/white
Motility	motile	Non motile

Oxidase test	+ve	-ve
Fermentation		+ve
Catalase test	+ve	+ve
Coagulase		-ve
H_2S production	-ve	-ve
Urea test	-ve	+ve
Indol	-ve	-ve
Methyl red	-ve	+ve
Vogas-Proskauer (VP) reaction	-ve	+ve
Citrate utilization	+ ve	-ve
Sugars		
Mannitol	-ve	-ve
Glucose	+ve	+ve
Sorbitol	-ve	+ve
Sucrose	-ve	+ve

(+): positive reaction; (-): negative reaction.

DISCUSSION

Pseudomonas agents such as P. fluorescens, P. putida, P. luteola, P. aeroginosa, and P. plecoglossicida have increasingly been reported from diseased cultured and wild fish species all over the world (Altinok et al., 2006; Eissa et al., 2010; Sakai et al., 1989). However, there are no reports of Pseudomonas infections caused by P. putida as the primary agent in cultured freshwater fish in Indonesia.

Fish are aquatic organisms that pose a primary hazard to immune-antioxidant responses, which are disrupted by many stressors, including bacterial infections (Hadwan, 2018). P. putida is one of the pathogenic bacteria that affects juvenile O. niloticus and induces signs of dark pigmentation and petechial hemorrhage on various parts of the body surface (Tartor et al., 2021). and induces ascites, exophthalmia, and ulcers in the body (Salama et al., 2009).

In this study, diseased angel fish showed external clinical symptoms as reported in the large yellow croaker. Although the ulcerative skin lesions on the base of the dorsal fin as reported in rainbow trout (Altınok et al., 2006), externally ulcerative skin lesions around the pelvic and pectoral fins, internally the gross pathology such as white nodules in the kidney and liver observed in the sea bass bear similarities to the large yellow croaker infected with P. putida have not been observed in this study.

Similar gross pathological findings have been reported in large yellow croaker infected with P. plecoglossicida (Zhang et al., 2022). Altinok et al., (2016) reported no internal clinical findings in rainbow trout. Therefore, it may be considered that the clinical signs of Pseudomonas infection caused by P. putida are not specific for different fish species.

In the present study, P. putida strains were resistant to oxytetracycline, erythromycin, streptomycin, kanamycin, and ampicillin, as described by Altinok et al., (2006). However, reported that oxytetracycline is highly effective against Pseudomonas species.

To date, there are no histopathological reports on the infection of freshwater fish species, rainbow trout, with P. putida, nor on ornamental fish, especially angel fish. al. (2006) reported histopathological epithelial necrosis in rainbow trout infected with P. putida; however, in this study, the most prominent pathological changes were observed in the brain and eyes. The necrosis was observed in the brain. Moreover, lens inflammation (cataract), bacterial infiltration of the sclera, brain hemorrhages, and infiltration by cocci and short, rod-shaped bacteria were observed in the diseased angel fish.

With respect to pathogenicity, the pathogenic strains (P. aeruginosa, P. putida, and P. angulliseptica) showed 9-11 protein bands ranging from 23.4 to 100.05 kDa in molecular weight. They possessed two common bands at 29 and 35 kDa. In the same respect, P. putida

produced 60% mortality in O. niloticus (El-Nagar, 2010) and 45% mortality in rainbow trout (Altinok et al., 2006).

The antibiogram sensitivity of P.anguilliseptica, P. putida, and P. aureginosa revealed that almost all of them were highly sensitive to Avatryl and Amikacin and sensitive to Gentamicin, Erythromycin, Novobiocin, and Sulfa-trimethoprim.

(Ismail et al., 2023; Huang et al., 1999; Kubilay & Uluköy, 2004) reported that the Gram stain of Staphylococcus epidermidis revealed gram-positive cocci, found as single cells, pairs, short chains, and irregular clusters of cells (Plat B4), which is similar to that reported by (Austin, 2016).

Staphylococcus epidermidis is among the most common causes of biomaterial-associated infections (BAIs). Also, extracellular polysaccharide production and biofilm formation increase bacterial stability on different surfaces; therefore, antibiotic penetration is prevented (Waldvogel & Bisno, 2000). Vancomycin, linezolid, daptomycin, tigecycline, quinupristin/dalfopristin, and dalbavancin are considered very important for treating S. epidermidis infections. Furthermore, another effective drug for reducing biofilm formation is rifampicin, but it has the disadvantage of promoting rapid antibiotic resistance. It should be noted that low resistance has been observed in streptogramins, linezolid, and tigecycline (Hellmark et al., 2009).

VITEK2 is considered an advanced automated biochemical identification system. Four Staphylococcus epidermidis isolates were identified by the VITEK2 system with98 % probability; analysis time was 7 hours.

The biochemical details are mentioned in. Furthermore, continuous handling may cause skin abrasions and lacerations that serve as portals of entry for pathogenic bacteria. Some of these bacteria can induce systemic disease after replicating and propagating in local lesions (such as skin and musculature), which may explain systemic infection with Staphylococcus epidermidis. Moreover, Staphylococcus epidermidis was isolated from a variety of diseased fresh and marine fish, causing systemic infection, as reported by Kusuda and Sugiyama (1981) and Çanak & Timur (2020).

The external symptoms of the infected fish included congestion and haemorrhages on the pectoral and caudal fins, darkened skin, lethargy, and excessive mucus secretion on the skin and gills. The mouth and lower jaw were haemorrhagic, and the gills were anaemic. Internal symptoms included ascitic fluid in the abdominal cavity, with an enlarged, pale liver showing congested blood vessels with inflamed dark areas. The gallbladder was filled with dark greenish or light yellowish bile. The spleen was also enlarged. Congestion and haemorrhages were seen in some regions of the brain. The stomach was empty in some fish. Although all these symptoms are similar to those observed by (Kubilay & Uluköy, 2004) in sea bass and sea bream, infections with S. epidermidis have been reported to induce different symptoms in tilapia (Huang et al., 1999), such as white and yellowish nodules in the anterior kidney and spleen. On TSA agar at 30 °C, samples from the internal organs, after 18-24 hours, yielded small, convex, regular, white colonies. The isolated bacteria were Gram-positive cocci, seen as single cells or irregular grape-like clusters under a light microscope. All the isolates were identified as S. epidermidis.

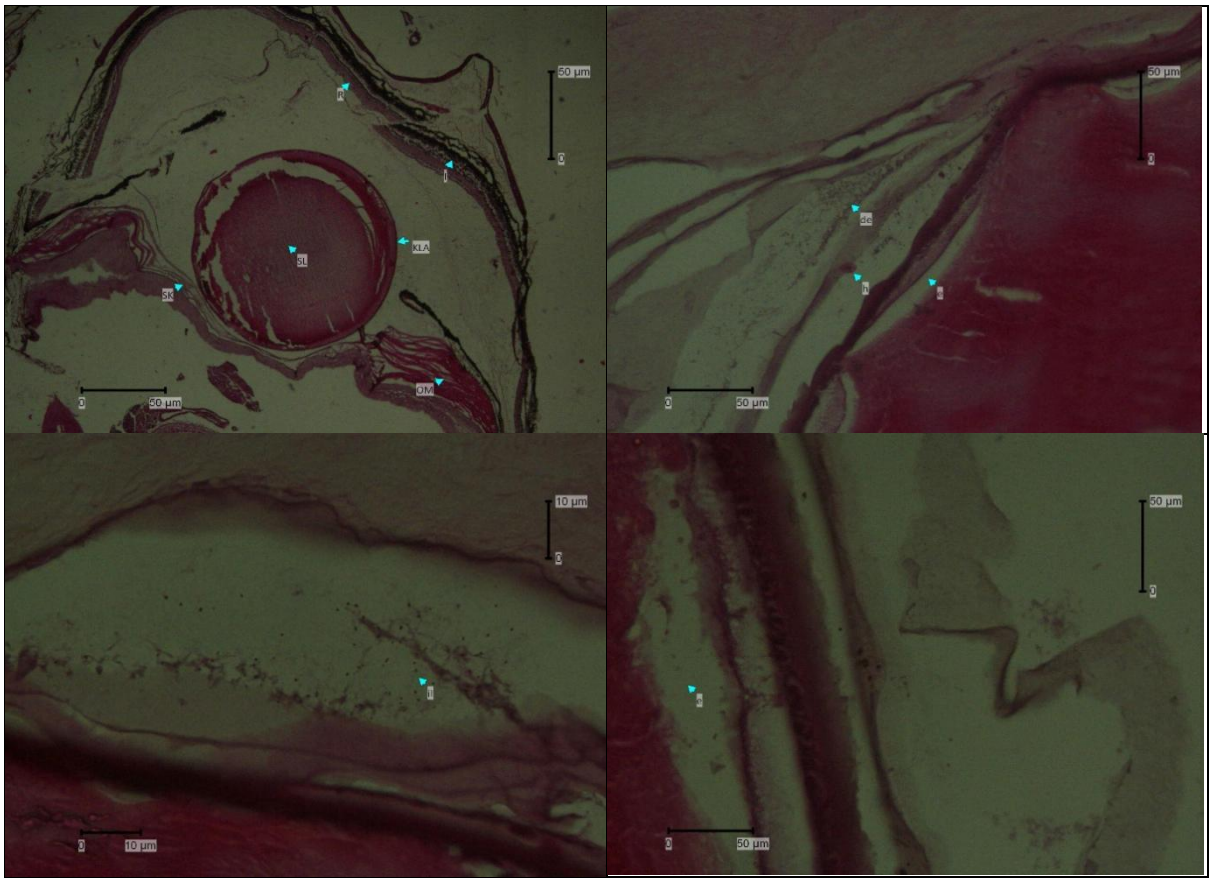
The fish showed symptoms of lethargy, anorexia, swimming sideways, and floating. Macroscopically, the group of fish infected intraperitoneally with a hemolytic strain of S. epidermidis showed redness in the heart and brain, and all fish exhibited congestion in the brain and kidney hemorrhage, as well as acute pericarditis. Sensitivity test results showed that almost all isolates were susceptible to the antibiotics Tetracycline, Penicillin, Streptomycin, Ampicillin, Erythromycin, and Chloramphenicol, and only the hemolytic strain isolates were resistant to Erythromycin. The study concluded that the hemolytic strain of S. epidermidis

causes more severe pathological changes and is resistant to Erythromycin compared with non-hemolytic S. epidermidis strains.

Two species of Staphylococcus have been reported to cause staphylococcosis in fish: *S. aureus* (Shah & Tyagi, 1986) and S. epidermidis (Kusuda & Sugiyama, 1981; Huang et al., 1999) The first reports of pathogenic S. epidermidis strains were from severe epizootics in farmed yellowtail and red sea bream in Japan from July 1976 to September 1977. The description of the disease was not very detailed, but typical signs included exophthalmia, congestion, and ulceration in the fish (Kusuda and Sugiyama,1981).

The results from the identification tests in the present study are in accordance with those reported from the outbreaks in red sea bream and yellowtail in Japan (Kusuda & Sugiyama, 1981; Austin, 2016) and in sea bream and sea bass in Greece (Urku, 2021; Ismail et al., 2023). A characteristic of this disease is that outbreaks are linked to stress, and fish mortality peaks within a few days. However, the condition often persists, and the mortality gradually reappears.

Available information suggests that in aquatic environments, S. epidermidis should be considered an opportunistic bacterium with the potential to become pathogenic to fish under stressful conditions. It is not possible to eliminate bacteria from fish or the environment (Kubilay & Uluköy, 2004; Štrancar et al., 2023; Mohamed et al., 2020) stated that strain was sensitive to chloramphenicol, doxycyclin, lincomycin, pristanamycin, tylosin, enrofloxacin, nitrofurantoin, fusidic acid, and rifampicin.



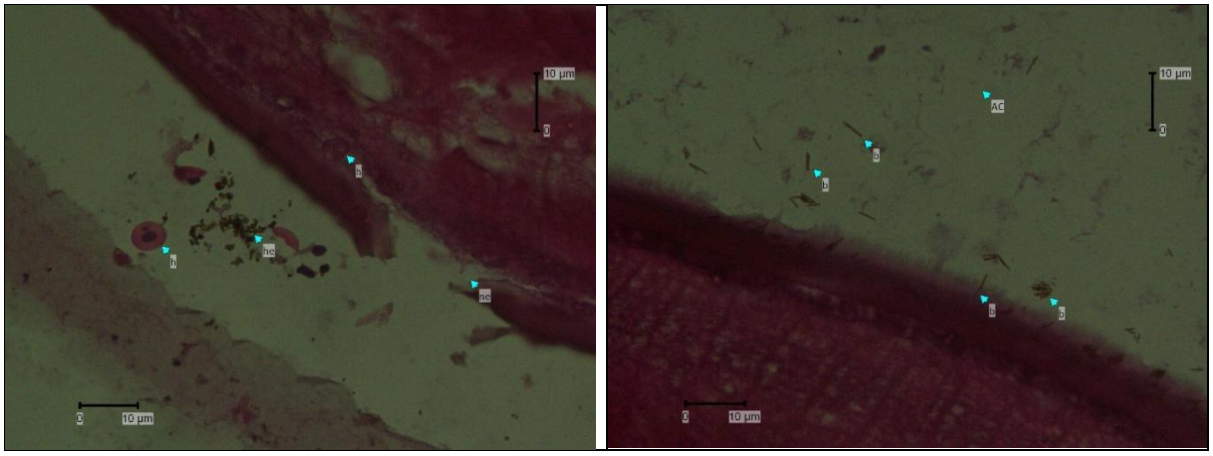


Figure 5. The eyes of manfish affected by disease appear A. Transverse section of the eye of a fish infected with the bacteria S. epidermidis and P. putida (H&E, 40x) B. Leukocyte infiltration in the aqueous chamber (H&E, 400x), C. Cell debris in the aqueous chamber, hemorrhage, and necrosis of cuboidal cells, D. Edema E. Hemorrhage, necrosis in the lens fiber layer, and hemosiderin debris in the aqueous chamber, F. Short rod-shaped bacterial cells in the aqueous chamber.

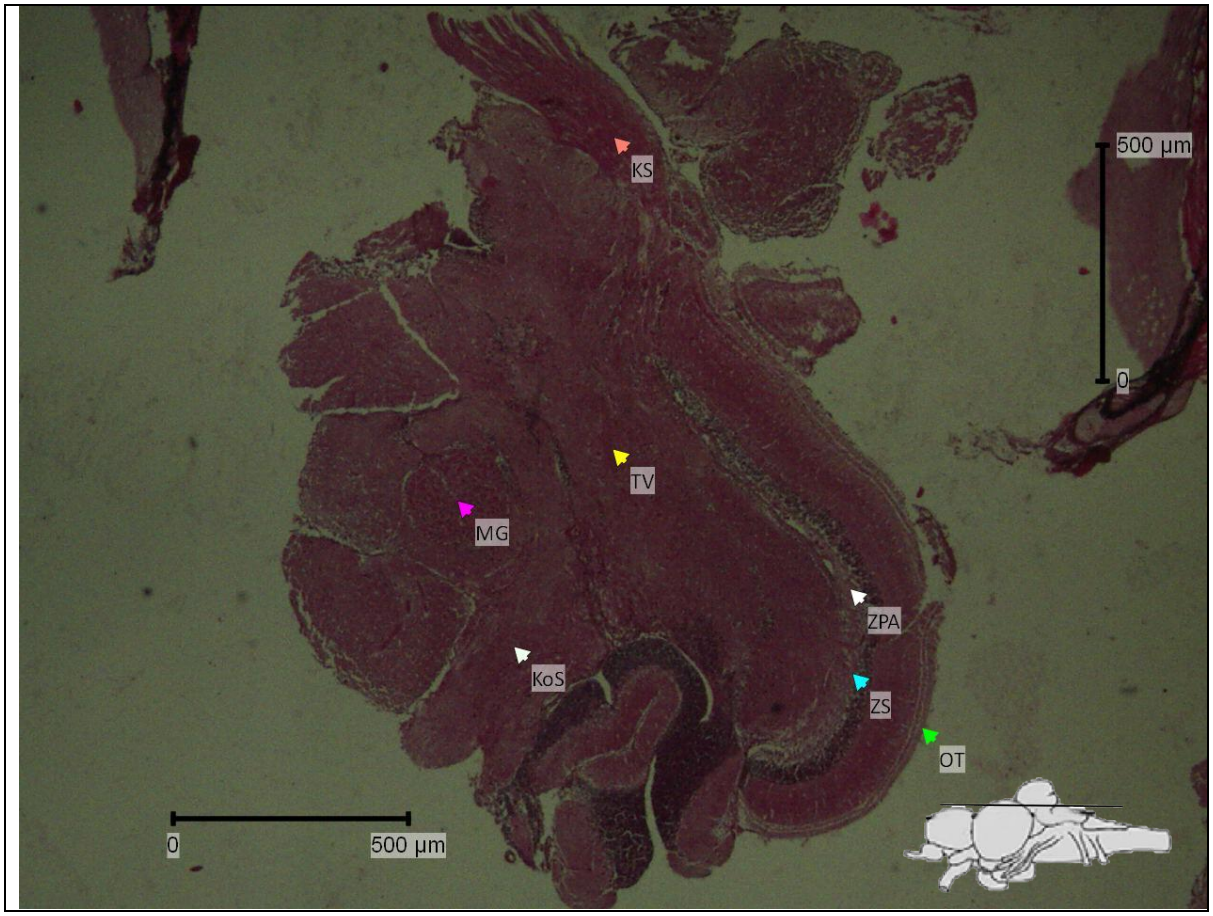


Figure 6. Axial section of the cerebellum of a manfish, OT: Optic Tectum, ZPA: Periventricular Gray Zone, KS: Cerebral Cortex, ZS: Semicircular Zone, KoS: Cerebellar Cortex, TV: Tectal Ventricle, MG: Glomerulosus Migration

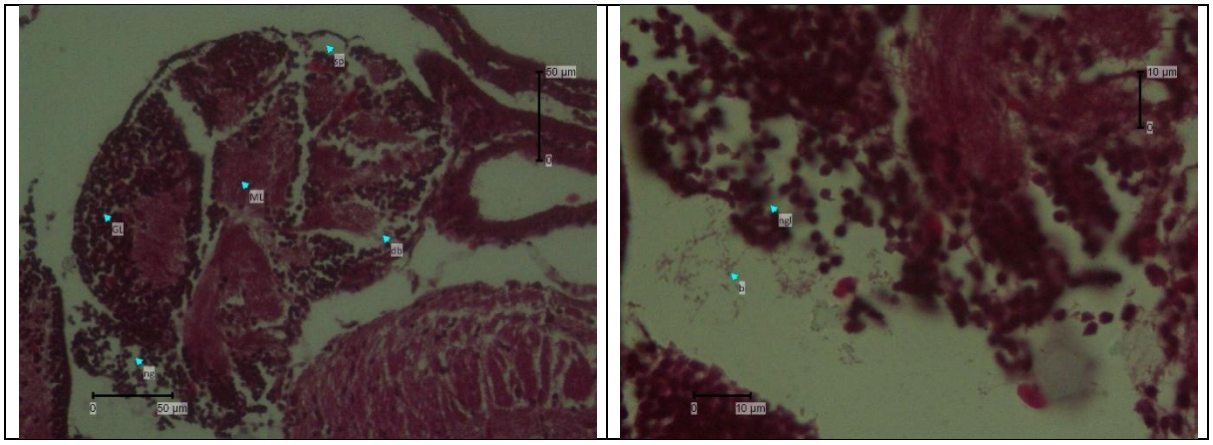


Figure 5. A. GL: Granular Layer, ML: Molecular Layer, sp: spongiosis. Molecular layer (encephalomalacia), Necrosis. Granular layer (Purkinje cell dropout) (cerebellar spongiosis with neuronal necrosis encephalomalacia). db: Debris. (H&E, 400x). B. ngl: Granular layer necrosis, b. short rod-shaped bacteria (H&E, 1000x).

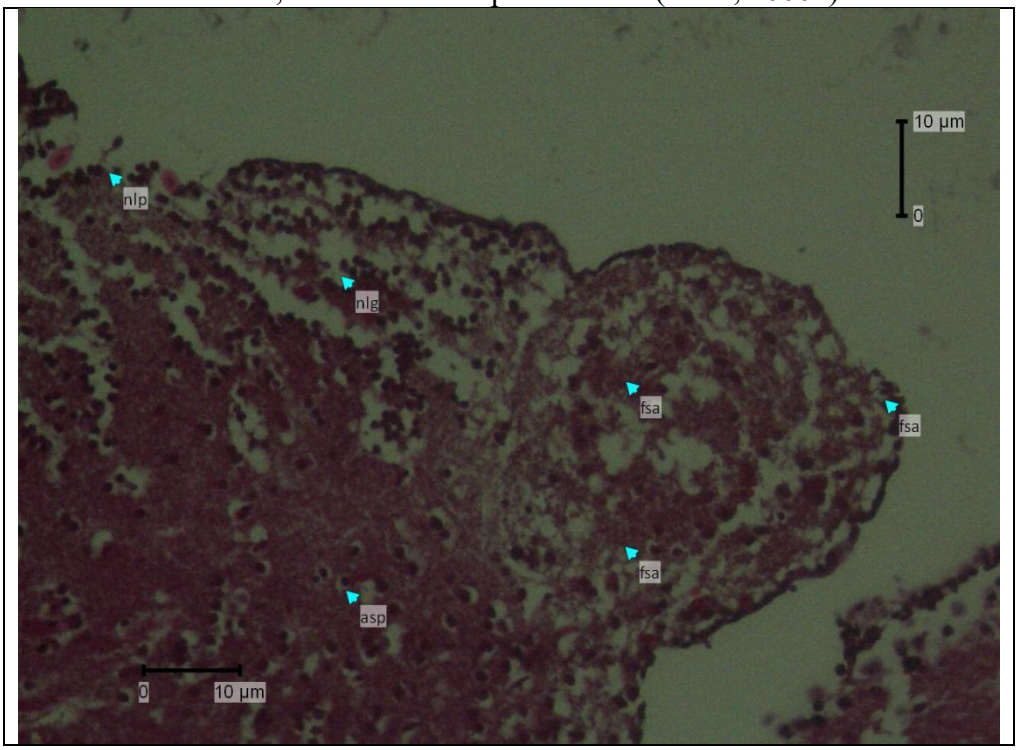


Figure 6. Cerebellum manfish (H&E, 400x), asp: Purkinje cell atrophy, nsp: granular layer necrosis, and fsa: white matter fragmentation.

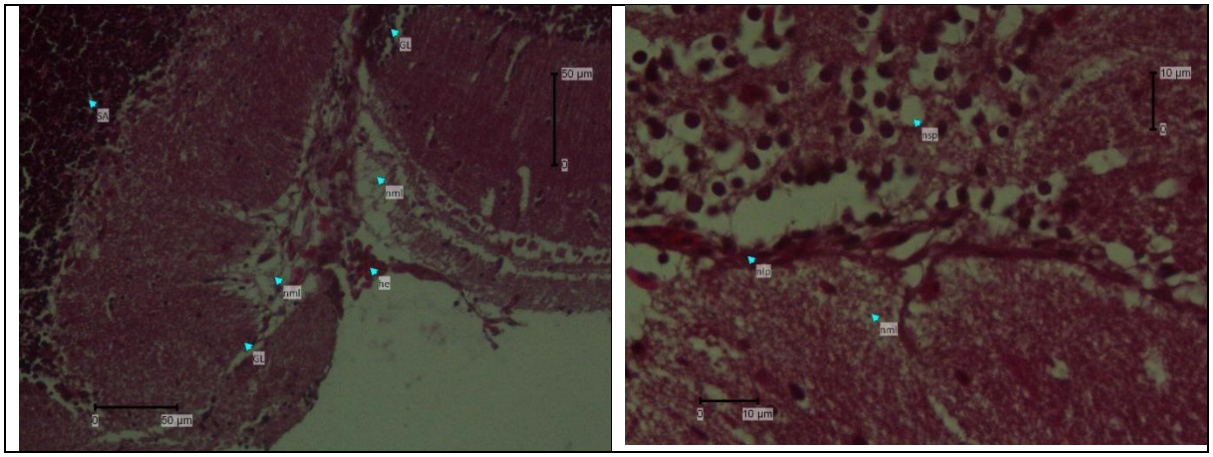
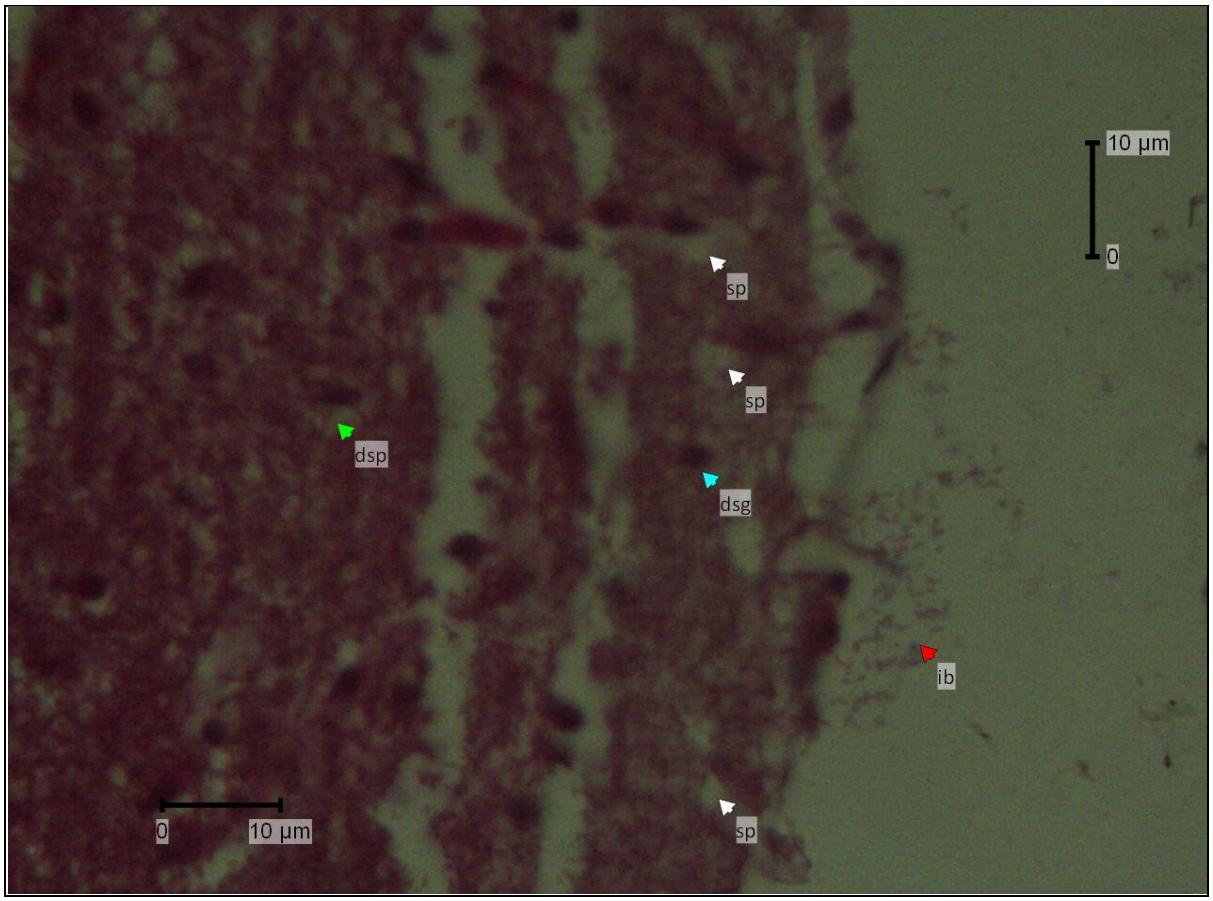


Figure 9. A. GL: Granular Layer, SA: Substansia Alba, nml: Molecular Layer Necrosis, he: Hemorrhage, (H&E, 100x). B. nlp: Purkinje layer necrosis, nml: cerebellum molecular layer necrosis, nsp: Purkinje cell necrosis (H&E, 1000x)



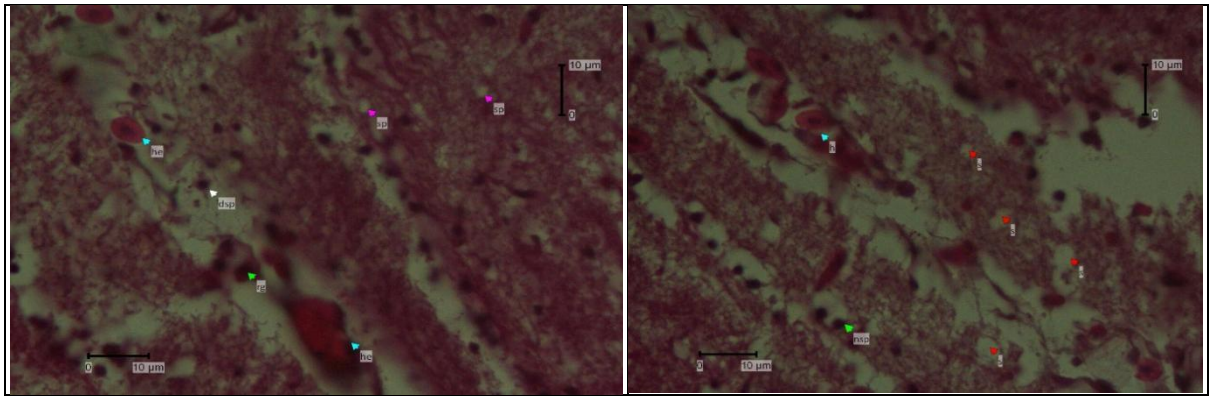


Figure 10. A. dsp: Purkinje cell degeneration, sp: spongiosis, dsg: granular cell degeneration, ib: bacterial cell infiltration, (H&E, 1000x). B. dsp: Purkinje cell degeneration, sp: spongiosis, dsg: granular cell degeneration, he: hemorrhage, (H&E, 1000x). C: h: erythrocytes, dsp: Purkinje cell degeneration (H&E, 1000x).

Figure 7. The brain of a manfish infected with disease, B. The cerebellar folia are collapsed and fragmented, the cortical layer is no longer defined, and numerous empty cavities (vacuoles) throughout this tissue indicate severe structural damage (encephalomalacia), a. Molecular layer: filled with empty holes, the tissue looks like a sponge (spongiosis), nerve fibers are broken, indicating neuropil degeneration due to viral lysis or toxins, b. Purkinje layer: most Purkinje cells are gone; the remaining ones appear shrunken and fragmented, with missing nuclei (karyolysis), a condition called Purkinje cell dropout. c. Granular layer: massive necrosis of cells occurs; they rupture, turn black, and fuse into debris, resulting in granular cell necrosis.

Figure 7, part B, shows A. Spongiosis, characterized by numerous empty cavities that give the tissue a sponge-like appearance. This is likely caused by nerve cell edema or neuron lysis by neurotoxins. B. Tissue necrosis, as seen in the presence of scattered cell nuclei and dead cells. C. Disorganization of the cerebellar folds, which are collapsed and torn.

Figure 8-9. Neuron degeneration in the tissue reduces the function of neurons connecting the cerebellar cortex and medulla and disrupts nerve impulse transmission. The effects on fish include decreased coordination, unbalanced swimming (wobbling), and slower responses to stimuli. Neuron necrosis. Histological characteristics: neuron cell fragmentation, loss of cell boundaries, Cell nuclei rupture or disappear (karyolysis). Effects on tissue include permanent neuron death, disruption of cerebellar nerve signal pathways, and effects on fish, including severe disruption of the central nervous system, uncontrolled movements (spasms or partial paralysis), and increased risk of death if damage spreads—intracellular and intercellular edema. Histological characteristics include widened intercellular spaces, loose and non-compact tissue, and decreased cell density. Effects on tissue include pressure on neurons and glial cells, which accelerates neuronal degeneration and necrosis. In contrast, effects on fish include decreased activity, a tendency to remain at the bottom or surface, and decreased appetite. Visible cerebellar damage shows a combination of degeneration, necrosis, and edema, indicating severe stress due to infection and neurotoxins. Central nervous system disorders directly affect balance, movement coordination, and fish survival. Purkinje cell degeneration, cerebellar granule cell necrosis, and spongiform degeneration and necrosis of nervous tissue are apparent.

Figure 10. A. Damage to the cerebellum tissue of manfish, namely necrosis of Purkinje cells, causes loss of motor control. Degeneration of the granular layer causes uncoordinated swimming movements or irregular swimming. Edema in the cerebellum can increase intracranial pressure and trigger seizures. This is consistent with clinical symptoms such as

whirling, loss of balance, uncontrolled sinking, seizures, inability to find food, and death. Histopathological findings in fish, particularly in the cerebellum or small brain, show severe cerebellar encephalopathy with edema, necrosis, and disorganization of the cerebellar folia.

The VITEK 2 system provided accurate and reliable results for the staphylococcal isolates (Gilad & Schwartz, 2007; Chiquet et al., 2018; Diederen et al., 2006).

Figure 5. Hemosiderin is most commonly found in the anterior part of the lens capsule, including the subcapsular part of the lens capsule. The presence of hemosiderin in the lens is associated with ocular siderosis, a toxic condition caused by foreign bodies, such as iron, in the intraocular space or by chronic intraocular hemorrhage. The presence of iron can damage the lens epithelium, leading to cataracts. In some cases, siderophages, which are hemosiderin-laden macrophages, are observed attached to the lens capsule, some of which are involved in intraocular hemorrhage.

Figure 5 shows short rod-shaped bacteria in the lens tissue. Normally, the lens is avascular and sterile, so the presence of bacteria is a significant pathological finding. The bacteria appear scattered around the lens fibers, accompanied by tissue opacity and disorganization of the lens fibers, indicating the onset of cataract due to a pathological process. This indicates an intraocular infection caused by bacterial penetration from the systemic circulation or surrounding eye tissues, suggesting septicemia or bacteremia in the fish.

Correlation with Microbiological Test Results

Staphylococcus epidermidis is a Gram-positive, coccus (paired/clustered) bacterium. It is generally opportunistic flora and can be pathogenic in fish when the immune system is compromised. It can form biofilms and cause chronic and persistent infections. Pseudomonas putida is a Gram-negative, short rod-shaped bacterium. It is opportunistic and environmental in nature, capable of producing endotoxins (LPS) and proteolytic enzymes, and is often associated with systemic infections and nerve tissue necrosis.

Necrosis in the Cerebellum of Fish

Purkinje cell necrosis, characterized by a cell nucleus undergoing karyolysis/karyorrhexis, cytoplasmic destruction, and Purkinje cells disappearing from the normal layer, indicating severe, irreversible central nervous system damage, is strongly suspected to be caused by Gram-negative bacterial endotoxins. In some cases, it may also be caused by hypoxia and viral diseases.

Substantia Alba Necrosis with characteristic pathological features of myelinated nerve fiber disintegration, empty spaces due to tissue lysis, decreased tissue density capable of causing damage to nerve impulse transmission pathways, and associated with bacterial neurotoxins and systemic inflammatory responses.

Granular layer necrosis with characteristic pathology in the form of shrinking and dying granular cells, dark or missing cell nuclei, and a drastic decrease in cell density. This disrupts signal input to Purkinje cells and exacerbates cerebellar dysfunction. Pathogenesis Mechanism (Eye-Brain Connection).

Systemic bacterial infection (bacteremia/septicemia) occurs when bacteria (especially P. putida) enter the eye (specifically the lens) and the brain (specifically the cerebellum). The bacteria produce endotoxins and proteolytic enzymes, causing damage to the blood-brain barrier. This damage takes the form of interstitial tissue edema, a pale area with a visible cavity between tissues, which can cause fluid to leak from blood vessels due to damage to the blood-brain barrier. Necrosis of cerebellum neurons and damage to eye tissue. This pattern is characteristic of systemic bacterial infections involving the central nervous system.

The functional impact on fish is a disturbance in the visual process. The infected lens causes cloudiness, reducing vision and making it difficult for fish to find food. Meanwhile, a

nervous system disturbance, such as cerebellar necrosis, causes unbalanced swimming, uncoordinated movements, slow responses, and impaired reflexes.

These findings strongly indicate a Systemic bacterial infection (septicemia) caused by Pseudomonas putida, with co-infection by Staphylococcus epidermidis, resulting in bacterial invasion of the eye lens and extensive necrosis in the cerebellum (Purkinje cells, white matter, and granular layer).

Microbiological identification results from blood samples showed the following characteristics: small to medium-sized, round, smooth, convex, white to cream-colored, and relatively slow-growing. Vitek 2 test results showed that the bacteria were Staphylococcus epidermidis.

Staphylococcus epidermidis is an opportunistic bacterium that generally causes mild or secondary bacteremia and is often isolated from blood under conditions of stress or immunosuppression. Other characteristics of S. epidermidis include being a Gram-positive coccus, not strongly invasive to parenchymal tissue, and not producing noticeable pigments. Meanwhile, liver samples showed relatively large colonies. The surface was slightly convex, with a smooth or wet texture, rapid growth, and a grayish color. After testing with Vitek 2, the result was Pseudomonas putida. This bacterium is invasive and systemic, with the liver being the primary target organ in Gram-negative bacterial infections, and it can grow rapidly in parenchymal tissue. Pseudomonas putida is a short Gram-negative rod-shaped bacterium that produces proteolytic enzymes and toxins and can cause necrosis of liver and nerve tissue.

The findings of bacteria in the organs and blood of manfish are consistent with the histopathological changes observed in the eyes and cerebellum, namely, short, rod-shaped bacteria in the lens strongly influenced by Pseudomonas putida, as well as short, rod-shaped bacteria in the periorbital area of the eye.

Purkinje cell necrosis, white matter necrosis, and granular layer necrosis were strongly influenced by Pseudomonas putida, particularly due to its production of endotoxins. Meanwhile, a secondary infection caused by Staphylococcus epidermidis led to mild bacteremia.

Small colonies isolated from fish blood samples were identified as Staphylococcus epidermidis, while large colonies from liver samples were identified as Pseudomonas putida. This was based on colony growth characteristics, tissue origin, and consistency with histopathological findings of necrosis in the central nervous system and the presence of short rod-shaped bacteria. The short-rod shape observed in the lens is highly consistent with Pseudomonas putida, while S. epidermidis was an opportunistic co-infection.

CONCLUSIONS

The abnormal swimming, lying on the bottom, and dark skin symptoms, along with co-infection by the pathogen causing Pseudomonas and Staphylococcal infections, were identified in the diseased angel fish as P. putida and S. epidermidis. It was the first finding. The gross and histopathological findings of the diseased angel fish indicated that P. putida and S. epidermidis are potential pathogens of the cultured fish, especially for ornamental fish.

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