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Hemolysin-Producing Gram-Negative Bacterial Profile in Fish Pond Wastewater

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Abstract- Aquaculture wastewater is increasingly recognized as a hotspot for pathogenic microorganisms. This study investigates the diversity and hemolytic characteristics of Gram-negative bacteria isolated from three fish pond effluents. Standard microbiological methods, including cultural, morphological, and biochemical tests (Gram staining, catalase, oxidase and coagulase), were used for identification, while hemolysin activity was determined using blood agar. The isolates obtained were presumptively identified as Pseudomonas spp., and Klebsiella sp., Pseudomonas sp., isolated from all three ponds showed β-hemolytic activity, whereas Klebsiella sp., showed no hemolytic activity in two water samples tested. The presence of hemolysin-producing Pseudomonas spp., suggests a strong pathogenic potential, as hemolysin is a key virulence factor that enhances bacterial survival and host tissue destruction. The detection of such organisms in fish pond wastewater highlights the risk of bacterial infections in aquaculture and possible transmission to humans. Findings reveal significant levels of hemolysin-producing bacteria, indicating potential threats to environmental safety, fish productivity, and human health. Fish ponds should be regularly screened for possible hemolysin-producing organisms to avoid possible zoonotic diseases ensure better fish production. The use of protective wear should be encouraged while visiting fish ponds.

Keywords: Gram Negative, Bacteria, Hemolysin, Pathogenic, Wastewater

I. INTRODUCTION

Gram-negative bacteria are among the most dominant microbial groups in aquaculture systems due to their adaptability and resilience under varying environmental conditions. Genera such as Aeromonas, Pseudomonas, Klebsiella and Vibrio are frequently isolated from fish pond environments and are recognized for their roles in fish diseases and human infections (Igbinosa & Okoh, 2008). These organisms are capable of producing a range of virulence factors that enhance their survival and pathogenicity in both aquatic hosts and humans. One of the most significant virulence factors produced by these bacteria is hemolysin.

Hemolysins are cytolytic toxins that disrupt host cell membranes, leading to the release of intracellular components and facilitating bacterial invasion (Igbinosa & Okoh, 2008). The presence of hemolysin-producing bacteria in aquaculture wastewater is therefore an important indicator of microbial risk, as it reflects the potential for disease transmission and environmental contamination. Pathogenic organisms are capable of reducing fish production (Buján et al., 2018).

Hemolysins are virulence factors produced by certain Gram-negative bacteria allowing them to cause diseases in humans and animals. Some of the importance of hemolysin production in disease development include; contribution to the pathogenesis of Gram-negative bacteria by damaging host cells and tissues leading to inflammation microsis and other pathological changes.

The production of hemolysin can also influence the severity of diseases caused by Gram-negative bacteria. Examples of hemolysin producing Gram-negative bacteria are certain strains of E. coli, Pseudomonas aeruginosa and Aeromonas hydrophila. The study will inform the development of effective management strategies for reducing the risks of disease associated with hemolysin producing bacteria. It will also provide insights in to the hemolysin production profile of Gram-negative bacteria in fish pond wastewater (Buján et al., 2018).

Aquaculture wastewater systems play a crucial role in the dissemination of these pathogens. In many developing regions, wastewater from fish ponds is often discharged into natural water bodies or reused without adequate treatment, increasing the likelihood of environmental pollution and human exposure (Boyd, 2020). This is particularly concerning in communities where water resources are shared between aquaculture, agriculture, and domestic use.

The global significance of these challenges has been highlighted by the Food and Agriculture Organization (2018), which emphasizes the need for sustainable aquaculture practices and improved biosecurity measures to safeguard both environmental and public health. Despite this, there is still a paucity of data on the distribution and virulence characteristics of bacteria in aquaculture wastewater, particularly in localized settings.

Fish pond wastewater can contaminate surface and ground water sources, harming aquatic ecosystems and human health. The wastewater can contain high-level of nutrients, such as nitrogen and phosphorus, which can lead to eutrophication (Zhang et al., 2018; Purnomo et al., 2022). The contamination of fish pond water has been attributed to feed (animal manure) which contain organic matter and introduce wide range of microorganisms in to the pond (Okpokwasili & Ogbulie, 1993; Njoku et al., 2015).

Fish pond wastewater can also harbor pathogenic microorganisms, Including, bacteria, viruses, and parasites, which can cause waterborne diseases in humans. Effective fish pond waste water management is crucial to migrate the environmental, health and economic risk associated with aquaculture activities.

In Bali metropolis, there is a lot of fish farming going on. The growth of aquaculture industry in Bali has led to an increase in fish pond wastewater generation. Contamination of this culture system has been attributed to poor water quality, high stocking density and the use of animal manure and contaminated feed posing significant, environmental and health risk to local communities (Mukwabi et al., 2019). This research focuses on the isolation and identification of hemolysin-producing Gram-negative bacteria from fish pond wastewater.

II. METHODOLOGY

Sample Collection

The water samples were collected from three different fish ponds in Bali metropolis into sterile bottles at a depth of 10–15 cm from the surface of the water. The samples were labeled accordingly as Sky Pond, Natural Pond, and Eastern Pond. The samples were transported to the Microbiology Laboratory of the Federal Polytechnic Bali and were analyzed within one hour of collection.

Isolation of the Bacteria

The workbench was disinfected using standard aseptic techniques. Nutrient agar, MacConkey agar, and blood agar media were prepared in strict accordance with the manufacturer's instructions to isolate and differentiate the target pathogens. Isolation of the bacterial pathogens from the selected fish pond wastewater was performed using standard serial ten-fold dilutions in sterile normal saline, followed by inoculation onto the agar plates using the pour plate technique (Maduwuba, 2024).

The MacConkey agar plates were prepared, inoculated with the diluted water samples, and incubated at 35°C–37°C for 24–48 hours to selectively isolate the Gram-negative coliforms based on standard diagnostic frameworks (Maduwuba, 2024; Wamala et al., 2018).

Identification of the Isolates

Gram Staining Procedure

Colonies of presumptive Pseudomonas sp., and Klebsiella sp., were picked subsequently and smears were made on clean, grease-free glass slides, heat-fixed by passing them over a flame 3 times, and allowed to air dry.

The standard Gram staining method described by Beveridge (2001) was employed. Crystal violet was applied to the slide for 30 seconds, and the slide was rinsed gently with tap water. Lugol's iodine was then applied to the slide and allowed to act for 30 seconds before being rinsed with water. A decolourizer (95% ethanol) was applied briefly and washed off immediately. Safranin counterstain was applied to the slide for 30 seconds, rinsed, and the slide was allowed to air dry before microscopic examination (Beveridge, 2001).

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Hemolysin Identification

The purified Gram-negative bacterial isolates were screened for hemolytic activity using blood agar supplemented with 5% defibrinated sheep blood. Each isolate was streaked onto the blood agar plates and incubated aerobically at 37°C for 24–48 hours. After incubation, the plates were examined for zones of hemolysis around the bacterial colonies.

Isolates producing a clear zone around the colonies were recorded as β-hemolytic, while isolates without any visible zone of hemolysis were recorded as γ-hemolytic. The procedure was carried out according to standard microbiological methods described by Forbes et al. (2017) and Cheesbrough (2006).

III. RESULT

The table below shows the result of the preliminary identification of bacterial isolates (A, B, C) from three fish ponds (Sky fish pond, Natural fish pond and Eastern fish pond) based on cultural characteristics and biochemical tests. Isolate A and B were found to be positive in all three ponds while Isolate C showed negative growth in Sky Pond but positive growth in Natural Pond and Eastern Pond (Table 1).

Table 1: Cultural characteristics of bacterial isolates from fish pond wastewater

Isolates	Cultural characteristics	Sky Fish Pond	Natural Fish Pond	Eastern Fish Pond
Isolate A	Large, creamy colony	Positive	Positive	Positive
Isolate B	Large, creamy colony	Positive	Positive	Positive
Isolate C	Large, dome-shaped, mucoid colony	Negative	Positive	Positive

Table 2 shows the results of the identification of the isolates using Gram staining and differential biochemical testing, which include catalase, coagulase, and oxidase tests, alongside presumptive identifications.

Table 2: Gram and Biochemical reactions of the isolates found in the fish ponds

Isolates	Gram stain	Catalase test	Coagulase test	Oxidase test	Suspected Organisms
A	Negative	Positive	Negative	Positive	*Pseudomonas* sp.,
B	Negative	Positive	Negative	Positive	*Pseudomonas* sp.,
C	Negative	Positive	Negative	Negative	*Klebsiella* sp.,

Table 3: Identification of isolates by hemolytic reaction

Table 3 below shows the result of the hemolytic reaction of Pseudomonas spp., and Klebsiella sp. Result showed that all the Pseudomonas spp., isolated from the three fish ponds lysed the Red Blood Cells, but the Klebsiella sp., isolated across all two fish ponds did not lyse the Red Blood Cells.

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Table 3. Hemolysin-producing profile of the Gram-negative bacteria from the three fish ponds

Organisms	Hemolytic reaction of isolates from Sky fish pond	Hemolytic reaction of isolates from Natural fish pond	Hemolytic reaction of isolates from Eastern fish pond
Pseudomonas spp.,	β-hemolysis	β-hemolysis	β-hemolysis
Klebsiella sp.,	Not isolated	γ-hemolysis	γ-hemolysis

IV. DISCUSSION

The Pseudomonas spp., found across all ponds exhibit high hemolytic activity (β-hemolysis). This indicates that these strains completely lysed red blood cells and are therefore, pathogenic. This consistent hemolytic reaction suggests that the Pseudomonas strains possess virulence factors associated with pathogenicity, such as the production of hemolysins, capsules and siderophores (Podschun & Ullmann, 2000).

Pseudomonas species are widespread in aquatic environments and can exist as both opportunistic pathogens and commensal organisms (Rahman et al., 2018). Non-hemolytic strains are often associated with normal pond microbiota, contributing to organic matter degradation and maintaining ecological balance.

Klebsiella sp., showed no hemolytic activity in this study. The absence of Klebsiella in the Sky Fish Pond may be due to differences in nutrient availability, water quality, or environmental factors such as pH, temperature, and organic load (Okpokwasili & Ogbulie, 1993).

The absence of hemolysis in this study suggests that the isolate may be environmentally adapted and not of significant health concern. The presence of hemolytic Pseudomonas in all ponds may indicate contamination from organic sources and high nutrient levels in the ponds (Orji et al., 2022).

The detection of hemolytic Pseudomonas spp., is of particular concern for aquatic animal health and public health, as these bacteria can cause diseases in fish and may pose zoonotic risks if the pond water is used for human activities or fish consumption (Ogbulie et al., 2005). Their persistence across different pond types suggests they are well adapted to aquatic environments, possibly forming biofilms or surviving in association with organic matter.

V. CONCLUSION

Fish pond wastewater serves as a reservoir of hemolysin-producing Gram-negative bacteria, posing risks to aquaculture productivity and public health. The fish ponds harbored Gram-negative bacteria (Pseudomonas and Klebsiella). Pseudomonas turned out to be the most prevalent Gram-negative bacteria isolated across all the three fish ponds.

It also consistently demonstrated high hemolysin production across all ponds, indicating strong pathogenic potential. Hemolysin can damage host tissues and facilitate bacterial invasion, posing risks to fish health and potentially to human consumers. Hence, continuous monitoring and improved management practices are essential.

VI. RECOMMENDATIONS

1. Visiting of fish ponds should be highly restricted to authorized personnel to avoid contamination by microbes.
2. Fish ponds should be regularly screened for possible hemolysin-producing microorganisms to avoid possible zoonotic diseases and for better fish production.
3. The use of protective wears (PPE) should be encouraged while visiting fish ponds.

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