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Isolation of bacteria from naturally infected Thai koi and verifying Koch's Postulates by experimental pathogenicity

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Abstract
Aquaculture augments fish production, thereby generating more income and livelihood for the people involved. However, fish disease hinders the smooth ongoing development of the aquaculture industry. They harm the health of fish. The maintenance of a large number of fish crowded together in a small area provides an environment conducive to the development and spread of infectious diseases. In this cramped and crowded environment, fish are more prone to diseases and infections. Fish diseases can be broadly categorized into two types, namely infectious disease, caused by a pathogenic organism such as bacteria, virus, parasite, protozoa, etc., and noninfectious disease caused due to physical, chemical, biological, and handling or procedural changes to the fish. The diseases can be sporadic, epizootic, epizootic, and enzootic. To confirm the infections caused by a pathogenic organism in a host, Koch's Postulates are used. Diseased *Anabas testudineus* fish were collected fish farm, near Guwahati, Assam. Then bacteria were isolated aseptically from the lesion on the body surface, followed by bacterial culture and reinfection on healthy fish. The symptoms, the causative agents, pattern of occurrence, etc were observed. The study tries to focus on healthy aquaculture practices and also helps in improvement in fish production.

Keywords: Aquaculture, infection, lesions, ulcerations

1. Introduction
India is the world-leading fish-producing country in the world. India is now holding the 2nd position among the fish-producing countries in the world after China [1]. Freshwater aquaculture is one of the fastest-growing sectors in India and has the potential for large-scale employment [2]. Fishing in India is a major industry employing 14.5 million people. Fisheries contribute 1.07% of the total GDP of India. According to the National Fisheries Development Board, the Fisheries Industry generates an export yearly earnings of Rs 334.41 billion [3].

The freshwater air-breathing teleost, *Anabas testudineus* is a highly demanded food fish in India due to its great nutritious value, higher market price, higher growth, and taste. It is found throughout Indochina and Southeast Asi [4] A. *Anabas testudineus* has the potential to be a suitable candidate species for fish farming. However, farmed Anabas are prone to various diseases. The common disease of *Anabas testudineus* includes fin rot, gill rot, and skin ulcer. It may be appeared due to the presence of pathogens, especially bacteria, and fungi, low water quality, presence of a higher concentration of ammonia in the water, etc [5]. these infectious diseases hinder the smooth development of the aquaculture industry. They have an ill effect on the health of fish. The maintenance of a large number of fish crowded together in a small area provides an environment conducive to the development and spread of infectious diseases. Bacterial diseases are probably the most common cause of diseases currently found in fish. Bacterial infection in fish farms is harmful not only to the fish but also to the consumer. The major genus of bacteria that are responsible for infection in *Anabas testudineus* is *Staphylococcus*, *Pseudomonas*, *Flavobacterium*, *Escherichia*, *Salmonella,* and *Aeromonas* [5]. The occurrence of diseases has become a primary obstacle to sustainable aquaculture production and this could lead to lesser demand for the species in local markets and, consequently, create a huge economic loss for the fish farmer.

To confirm the infections caused by a pathogenic organism in a host, Koch's Postulates are used. Koch's Postulates are the four criteria designed to establish a relationship between a causative microbe and a disease. The postulates were forwarded by Robert Koch and Friedrich Loffler in 1884, based on earlier concepts described by Jakob Henle [6], and refined and published in 1890. Over 100 years ago, Robert Koch introduced his ideas about how to provide the casual relationship between a microorganism and a disease [7]. A set of guidelines to identify pathogens that could be isolated with the techniques of the day [8]. The Postulates are as follows:

- The microorganism must be found in abundance in all organisms suffering from the disease, but could not be found in a healthy organism.
- The microorganism must be isolated from a diseased organism and grown in pure culture.
- The cultured microorganism should cause disease when introduced into a healthy organism.
- The microorganism must be reisolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

These postulates are generally verified by experimental pathogenicity in a healthy organism (here fish) to know whether the same kind of signs and symptoms appear in the experimental fish as that of the naturally infected fish, which would confirm that there has been a microbial attack on the organism (fish) and hence the symptoms of a disease. Evidence of bacterial diseases is common in a dense population of cultured food or aquarium fish. Predisposition to such outbreaks frequently is associated with poor water quality, organic loading of the aquatic environment, handling, and transport of fish marked temperature changes, hypoxia, or other stressful conditions. Changes occurring in freshwater ecosystems seem to be fundamental in the development of all microorganisms including those pathogenic to fish. As food fish are of great economic importance, therefore the present study was conducted on the fish *Anabas testudineus*.

2. Objectives

As there are only a few studies and research work conducted on the Koch Postulate verification in food fish, especially *Anabas testudineus*, this study was undertaken to perform the isolation of the pathogenic bacteria from *Anabas testudineus* and further verify the Koch's Postulates.

1. To isolate the pathogenic bacteria from naturally infected Anabas fish.
2. To verify Koch's postulates by experimental pathogenicity.

3. Materials and Methods

3.1 Collection and maintenance of naturally infected fish: In February, a few diseased *Anabas testudineus* fish were collected at an interval of ten days from a fish farm, near Guwahati, Assam. The fish species were brought to the Fish Molecular Biology Laboratory of the Department of Zoology, Gauhati University, in the live condition in polythene bags, filled with water from the sample site for isolation of the pathogen. The symptoms of the disease that were evident were cloudy eyes, hemorrhages, open sores on the body, and excess slime.

3.2 Media preparation

Nutrient Agar (HIMEDIA, M001) was used for the cultivation of less fastidious microorganisms. In 1000 ml of distilled water, 28 grams of media were suspended. The media was heated and sterilized by autoclaving at 15 lbs pressure (121 °C for 15 min). Mixed well before pouring and allowed to cool. The media was then poured into a laminar flow hood. The nutrient agar plates, thus prepared were allowed to dry in the oven to remove moisture.

3.3 Isolation of the bacterium

Firstly, the diseased fish were desensitized with the help of clove oil. Then bacteria were isolated aseptically from the kidney and lesion on the body surface, inside a laminar flow chamber, followed by streaking the same on two nutrient agar bacterial plates. The bacteria were allowed to grow on the nutrient agar plates and the plates were left for incubation in a BOD incubator for 24 hours at 30 °C. After 24 hours of incubation, the bacterial populations were counted. The morphological characteristics of the isolates were examined. Three distinct colonies (two yellow coloured and an orange coloured) were observed in the bacterial plates (one type of colony on the first plate and two types of colonies on the second plate). From the distinct colonies observed, pure culture of each of the two bacterial isolates was obtained, by growing each of them in LB medium in a test tube. They were then kept in a shaker overnight. Repeated streaking was carried out until a single colony was obtained. After 24 hours, there was precipitation of pellets in the test tube.

3.4 Isolation of the bacterial DNA

DNA isolation was carried out using the Qiagen Kit (Protocol in QIAamp DNA Mini Kit)

a) Firstly, 1 ml of bacterial culture was taken in 1.5 ml of MCT.
b) It was then centrifuged for 5 min at 7500 rpm.
c) The pellet was collected and ATL buffer was added.
d) Then Proteinase K was mixed by vortex and incubated at 56 °C for 1 H with occasional vortex.
e) Then, about 4 ml of RNase was added and vortex for 15 sec and incubated at room temperature for two minutes.
f) It was then centrifuged for 15 sec and 200 microlitres of AL buffer was added, vortex for 15 sec, and incubated at 70 °C for 10 min.
g) It was again centrifuged for 15 sec and 200 microlitres of chilled ethanol is added followed by vortex for 15 sec.
h) The mixture was then applied to the spin column and centrifuged at 8000 rpm for one minute.
i) The filtrate was discarded and the mixture was taken in a 2 ml collection tube.
j) Then, 500 microlitres of AW1 is added and centrifuged at 8000 rpm for one minute.
k) The filtrate is discarded and collected in a 2 ml collection tube.
l) 500 microlitres of AW2 was added and centrifuged at 14000 rpm for 3 minutes.
m) The filtrate was discarded and taken in 1.5 ml MCT.
n) Added 200 microlitres of AE or distilled water and incubated at room temperature for one minute.
o) Then, it was centrifuged at 8000 rpm for one minute and the filtrate was discarded and taken in 1.5 ml MCT.

3.5 Agarose gel electrophoresis

Preparation on 0.8 % agarose gel

1. Weighed 0.8 g of agarose gel base and poured into a 250 ml flat bottomed flask.
2. Added 1X Tris Borate EDTA Buffer up to 100 ml.
3. The suspense on was heated in a microwave for 2 minutes.
4. Added required volume of stock solution (10 mg/ml) EtBr when the gel cools down to about 60^{o} C to obtain a working solution of 0.5 mg/ml in the gel and mixed well by stirring to avoid bubbles.
5. The gel is cast on a horizontal gel electrophoresis casting unit with the comb placed in it.
6. 5 µl of DNA ladder was loaded into the first well.
7. 10 µl of PCR product was taken in a parafilm strip and loaded into a subsequent well.
8. The gel was run at 80 V until the dye line is approximately 75 – 80 % of the way down the gel.
9. The power is then turned off, electrodes disconnected and the gel was removed from the gel box.
10. The DNA fragments were then visualized in a UV transilluminator.

Thus, the DNA sample (PCR Product) was run on agarose gel and the DNA fragments were then visualized. A band was obtained at 1500 bp.

3.6 16 Sr RNA sequencing

The genomic DNA was extracted using a genomic DNA Purification Kit. The genomic DNA was amplified with bacterial primers. The 16 S r RNA Gene was amplified from the bacterial genomic DNA. The PCR reaction conditions were:

5 min at 94 °C, followed by 35 cycles (30 s at 94 °C, 30 s at 50 °C, 1 min 30 s at 72 °C) and final extension at 72 °C for 7 min using a thermocycler (Applied Biosystems, USA).

3.7 Experimental pathogenicity

From the pellet precipitated, a reinfection solution was prepared in sterile PBS. The pellet was taken in a 15 ml tube and centrifuged for 10 min at 10000 rpm. Then the supernatant was removed and 200 microlitres of PBS were added and the mixture was centrifuged at 7000 rpm for 10 minutes. The supernatant formed was discarded and again 200 microlitres of PBS was added. This time the mixture was centrifuged at 5000 rpm for 5 min. The supernatant formed was discarded and 100 microlitres of PBS was resuspended, which is the required solution to be injected. Then two injections were taken, the first one control which had only PBS, and the second one Experimental, which had 2 ml of the solution. After that reinfection studies were carried out in healthy *Anabas testudineus* to see whether the same signs and symptoms appear in the naturally infected fish collected.

4. Results

4.1 Observation of signs and symptoms of naturally infected fish

Naturally, infected fish were brought to the laboratory and were observed for their signs and symptoms. The fish exhibited symptoms like lesions on the body surface, hemorrhages, and ulcerations (Fig 1 and 2).

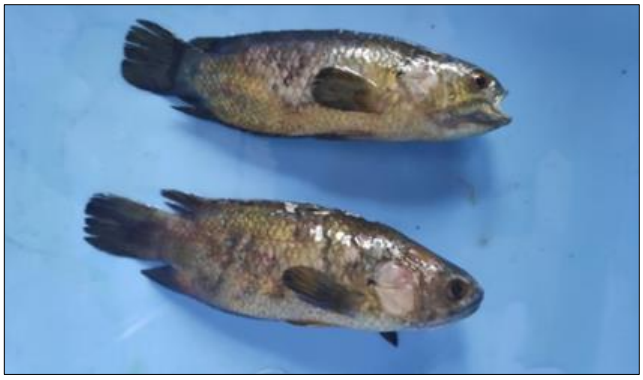

Fig 1: Diseased *Anabas testudineus* showing scale erosion, lesions and hemorrhages

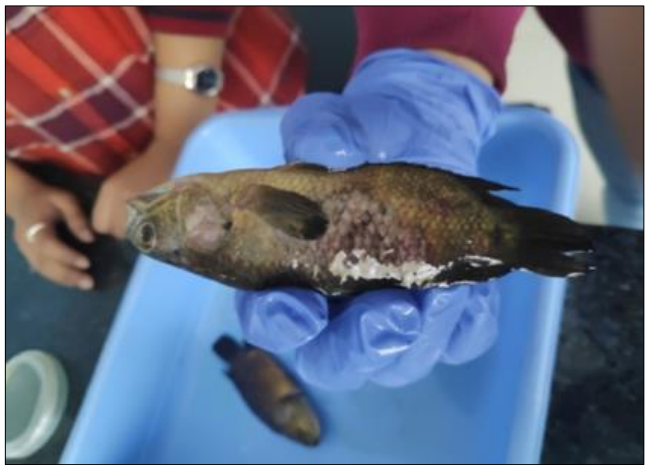

Fig 2: Diseased *Anabas testudineus* showing scale erosion.

4.2 DNA isolation and agarose gel electrophoresis

The reisolated bacteria were streaked repeatedly until the pure culture was obtained (Fig. 3). The pure culture colonies were allowed to grow in the LB Media for 24 H. Then, DNA isolation was carried out using Qiagen Kit, followed by agarose gel electrophoresis. A band at 1500 bp was observed, confirming the presence of the pathogenic bacterial DNA (Fig. 4).

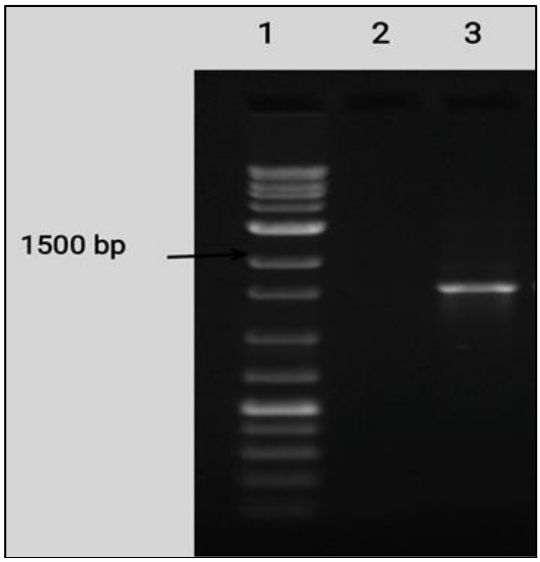

Fig 3: Band observed after agarose gel electrophoresis

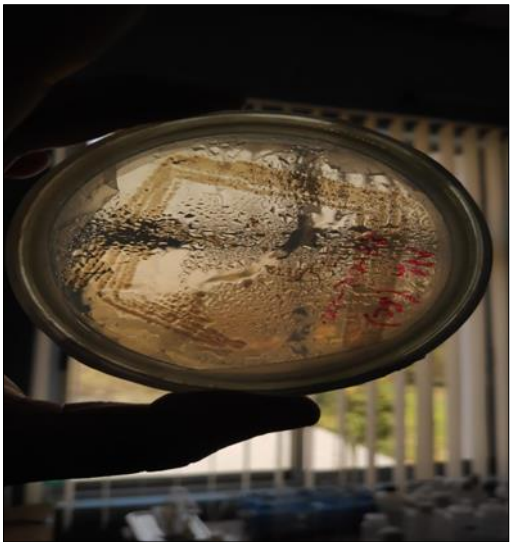

Fig 4: Pure culture of bacteria showing yellowish orange colonies.

4.3. Experimental pathogenicity test

Experimental pathogenicity tests revealed similar symptoms like scale erosion, hemorrhages, and excess slime. Mild symptoms of scale erosion were observed after 24 H and exactly similar symptoms, as were present in the collected naturally diseased fish, were observed after 48 H. After 72 H, the fish was brought to the laboratory in moribund condition for re-isolation studies. A similar isolation procedure was done to re-isolate the bacteria aseptically, as done earlier with the naturally infected fish and bacterial plates were made and kept for incubation in the BOD incubator for 48 H at 28 °C. After 48 Hours, distinct types of colonies were observed (Fig. 3).

For verifying Koch's Postulates sincere efforts were made to isolate the possible pathogenic bacteria from disease-infected fish species of *Anabas testudineus*, which is a commercially important food fish. After the isolation, experimental pathogenicity was carried out to see whether the isolated species of bacteria satisfied Koch's Postulates. Similar signs and symptoms like erosion of scales and lesions over the surface of the body were observed in the healthy fish, when injected with the bacterial isolate, thereby confirming and satisfying Koch's Postulates.

5. Discussion

Aquaculture is an important sector that has contributed immensely to our economy. Despite having such potentiality, fish farming is being confronted with acute problems of fish diseases like fungal diseases, bacterial diseases, and viral diseases. Bacteria, as are the most abundant microorganisms in nature, are responsible for most of the diseases caused in aquaculture. The purpose of this study was to confirm the pathogenicity of bacteria in food fish Anabas. As pathogenic microorganisms retard fish growth, survival, and aquaculture, research work carried out in this area is highly beneficial to mankind. Chhanda et al. (2019) conducted a study on the identification of pathogenic bacteria from infected Anabas. The confirmation of pathogen infection can be understood by verifying Koch's Postulates. Koch's Postulates have been considered a standard for establishing the microbiological etiology of infection and disease. Genomics and molecular biology also help enhance and add to the results obtained, thereby facilitating correct and authentic research work.

6. Conclusion

Fish is a vital source of food for people. It is man's most important source of single protein providing about sixteen percent of the animal protein, consumed by the world's population according to the Food and Agricultural Organization (FAO) of the United Nations (1997). Fish has substantial economic importance. To meet the ever-increasing demand for fish, aquaculture has expanded very rapidly and is now the fastest-growing food-producing industry in the world. Food and Agricultural Organization (FAO, 2000) estimates that by 2030, over half of the fish consumed by the world's people will be produced by aquaculture [11]. The total aquaculture production increased from 10 million tons of fish in 1984 to 38 million tons in 1998 with a growth rate of 11 % per year [11]. Fish diseases pose the most severe threat to aquaculture. Therefore, it is very important to study fish diseases and devise prevention strategies and treatments to curb and control the same. Immense research work has been carried out on diseases of food fish. They studied the signs and symptoms, the type of microorganism causing it, and parameters enhancing their vulnerability to disease. This kind of study greatly helps the aquaculturists to continue smoothly practicing aquaculture and also helps augment aquaculture production.

7. Conflict of interests

All authors agree to the publication of this paper and they do not have any conflict of interests with any party or commercial identity. They have no involvement that might raise questions of bias in this reported work or its conclusions, implications, or opinions.

8. Disclaimer

The products used for this research are commonly and predominantly used products in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation, but the advancement of knowledge. The authors have funded the research in their interest.

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