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Phenotypic characterization and presumptive identification of bacterial isolates from moribund Nile tilapia (*Oreochromis niloticus* L.)

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Abstract
The general objective of this study was to isolate, characterize and presumptively identify bacterial isolates from moribund Nile tilapia (*Oreochromis niloticus* L.). The rigid sorting of the bacterial colonies based on cultural characteristics has resulted to three different bacteria. Colony A and colony B shared the same cultural characteristics – round with raised margin, small in size, and with entire margin, smooth texture and convex elevation. Colony C was entirely different in shape, margin, texture and elevation. The three bacterial colonies failed to grow in McConkey Agar but luxuriously grow in *Staphylococcus* Selective Agar, therefore, colony A and colony B were presumptively identified under the genus *Staphylococcus*. Colony A and colony B were Gram-positive, spherical in shape, positive to catalase test, and negative to several tests such as citric acid utilization, urea hydrolysis and phenylalanine deamination.

Keywords: Nile tilapia, phenotypic characteristics, colonial characteristics, biochemical test, *Staphylococcus*

1. Introduction
Tilapia (*Oreochromis* spp.), a well-known freshwater fish, has been recognized as a food source for rural communities and industrial aquaculture ventures worldwide. Tilapia culture continues to show a high growth in output and has become the most important aquaculture crop this century [1]. The species is favoured among aquaculturists due to its ability to tolerate a wide range of environmental conditions, fast growth, successful reproductive strategies and ability to feed at different trophic levels [2]. The farming of the Nile tilapia was practiced in more than 100 countries including China, Egypt, Philippines, Indonesia and Thailand which dominated the world production [3].
One of the main problems in fish farms is the occurrence of parasites and bacteria [4]. Parasite and bacterial diseases stand out as important limiting factors to productivity. These diseases may delay growth and cause high rates of mortality in fish [5]. Disease causing pathogens can be found on fish, water, surfaces of aquaculture equipment and facilities, slumps and filter beds. They can be transmitted by water, from fish to fish by vectors and by contaminated feed [6].
Bacteria have been identified and classified on the basis of a variety of characteristics including morphological, growth, tolerance, metabolic, biochemical and genetic. With the fundamental knowledge in isolation technique, growth characteristics of bacteria, staining methods, bacterial nutrition and biochemical activities, it becomes easier for the identification of any unknown bacteria [7]. The general objective of this study was to isolate, characterize and presumptively identify bacterial isolates from moribund Nile tilapia.

2. Materials and Methods
2.1. Isolation of bacterial colonies from moribund Nile tilapia
The kidney of moribund Nile tilapia was serially diluted until 10^{-4} [4] in sterilized distilled water. One millilitre from the 10^{-4} dilution was spread evenly in prepared Nutrient Agar (NA) plate. This procedure was repeated in two more agar plates. The plates were incubated in an inverted position at 37° C for 24 hours.

2.2. Characterization of bacterial colonies
Bacterial colonies grown in NA plates were meticulously sorted based on their appearances on the medium. Cultural characters such as colony size, shape, margin and elevation were considered. Colonies that exhibited different cultural characters were purified, grown in selective medium and subjected to a number of biochemical tests.

2.3. Growth on McConkey Agar
The unknown bacteria were streaked on plate containing MacConkey Agar and the plate was incubated at 33 ˚C for 18 to 24 hours. MacConkey Agar is a selective and differential medium for the isolation of Gram-negative bacteria and the grown isolates are differentiated based on lactose fermentation. Pink colonies will appear if the bacteria are lactose fermenter; no change in color when non-lactose fermenter.

2.4. Growth on *Staphylococcus* Selective Agar
The unknown bacteria were streaked on plate containing *Staphylococcus* Selective Agar and the plate was incubated at 33 ˚C for 18 to 24 hours. The bacterium *Staphylococcus* appears creamy in this selective medium.

2.5. Gram staining
A bacterial smear was prepared by mixing a small amount of fresh growth of each culture with a drop of distilled water in a clean glass slide. The smear was air dried and fixed by heat. The glass slide was labeled properly. The dried smear was stained

with crystal violet for 1 minute and was rinsed thoroughly with tap water. Afterwards, the smear was covered with Gram's iodine for 1 to 2 minutes and was washed with tap water. The smear was decolorized by dripping 95% ethanol and washed immediately. Then, the smear was counterstained with safranin for 45 seconds and washed by tap water. The slides were examined under microscope. Gram positive bacterium should be colored violet to blue while gram negative bacterium should be colored pink to red. Cell size, shape and arrangement were noted also.

2.6. Catalase test

A loopful of the fresh bacterium was transferred in a clean slide. One to two drops of freshly prepared 3% hydrogen peroxide (H_2O_2) were dropped onto the slide. Bubble formation indicates the presence of catalase enzyme.

2.7. Citric acid utilization test

The unknown bacterium was inoculated to Simmon Citrate Agar (SCA) slant using a wire loop by stabbing the butt and streaking on the surface. The slant was incubated at 33 ˚C for 48 hours. Growth and shift of the green color to Prussian blue color means positive utilization of citrate.

2.8. Urea hydrolysis

The unknown bacterium was inoculated heavily to Christensen's Medium (CM) urea broth. The tube was incubated at 35 ˚C for 4 to 6 hours. A red to violet color means positive test for urea hydrolysis.

2.9. Phenylalanine deamination test

The unknown bacterium was inoculated to Phenylalanine Agar (PA) slant. The slant was incubated at 33 ˚C for 18 to 24 hours. Four to five drops of 10% ferric chloride solution were added. The immediate appearance of an intense green color indicates positive phenylalanine deamination.

3. Results and Discussion

3.1. Cultural characterization of the bacterial colonies

Five colonial characters namely shape, size, margin, texture and elevation were considered in order to meticulously sort the bacterial colonies that grow in NA plates. The rigid sorting resulted to three different bacterial colonies. Colony A and colony B shared the same cultural characteristics – round with raised margin, small in size, and with entire margin, smooth texture and convex elevation. Colony C was entirely different in shape, margin, texture and elevation (Table 1).

Table 1: Colonial characteristics of the three bacterial isolates from moribund Nile tilapia.

Colony	Shape	Size	Margin	Texture	Elevation
A	Round w/ raised margin	Small	Entire	Smooth	Convex
B	Round w/ raised margin	Small	Entire	Smooth	Convex
C	Rhizoid	Small	Filamentous	Rough	In growing into medium

3.2. Presumptive identification of the bacterial isolates

The bacterial colonies failed to grow in McConkey Agar, a medium for selective isolation of Gram-negative bacteria. It was already hypothesized that the three colonies were Gram-positive because of growth failure in McConkey Agar. To confirm the Gram-reaction of the bacteria, Gram-staining was done. In the microscope, the bacterial cell appeared blue in colour, an indication of Gram-positive reaction. The three colonies were also grown in *Staphylococcus* Selective Agar; colony A and colony B luxuriously grow in creamy colour but not colony C (Table 2). Therefore, colony A and colony B was presumptively identified under the genus *Staphylococcus*.

Table 2: Growth confirmation of the isolates in selective and differential medium.

Colony	McConkey	*Staphylococcus* Selective Agar
A	-	+
B	-	+
C	-	-

Note: With growth (+); without growth (-)

Few biochemical tests were done in order to elucidate the phenotypic characteristics of the three isolates. Colony A and colony B which were presumptively identified as *Staphylococcus* spp. were positive to catalase test and negative to several tests such as citric acid utilization, urea hydrolysis and phenylalanine deamination (Table 3). The biochemical results of this present study were in harmony with the findings made by Reyes *et al*. [8] when they characterized the *Staphylococcus* spp. isolated from smoked fish. The presumptively identified *Staphylococcus* spp. in this study possessed the enzyme catalase, had no ability to utilize citrate as carbon source and lacked the enzymes urease and amino acid oxidase [8].

Table 3: Results on cell shape, Gram-reaction and other biochemical tests.

Colony	Shape	Gram-reaction	Catalase Test	Citric Acid Utilization Test	Urea Hydrolysis	Phenylalanine Deamination Test
A	Spherical	Gram-positive	+	-	-	-
B	Spherical	Gram-positive	+	-	-	-
C	Rod	Gram-negative	-	-	-	-

Note: Positive result (+); negative result (-)

In the study of Thongkao and Sudjaroen [9], they were able to isolate coagulase *Staphylococcus* in tilapia reared in fish ponds. Methicillin-resistant *S. aureus* was isolated by Atyah and Siti-Zahrah [10] in tilapia grown in cages. According to Caretto *et al.*

[11], *Staphylococcus* spp. is a fish pathogen that can be found in the intestine and feces of fish. European sea brass (*Dicentrarchus labrax*) has been infected by *Staphylococcus* spp., which was marked by the presence of dark body, ulceration, and fin and skin necrosis [12]. In Kenyir Lake, Malaysia, *Staphylococcus* spp. was dominant bacteria in tilapia [13].

4. Conclusion

Colony A and colony B shared the same cultural and biochemical characteristics, and were identified as *Staphylococcus* spp. because of their luxurious and creamy growth in *Staphylococcus* Selective Agar. A number of reports have done that *Staphylococcus* spp. were isolated from Nile tilapia.

4. References

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