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# GENOTYPIC CHARACTERIZATION OF BACTERIA ASSOCIATED WITH *CLARIAS GARIEPINUS* FROM IDOGO STATION YEWA RIVER AND THEIR HAEMOLYTIC ACTIVITIES

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**ABSTRACT**
Sixteen samples of *Clarais gariepinus* were collected from Idogo Station, Yewa River. Features such as Bacteria were isolate from the gills, skin and gut of the fish samples. A total of 16 bacterial isolates were then selected, thereafter the DNA of the isolates were extracted, followed by PCR amplification of 16S ribosomal RNA gene using 16S rRNA primers. The purification of the PCR product was carried out using ethanol precipitation method, and then sequenced using an automated DNA sequencer. The bacterial Isolates were then streaked on blood agar to determine their haemolytic activities. The result of water analysis showed that Idogo river has temperature of 31.2ºC, Dissolved Oxygen 3.6, pH 7.2. The bacteria isolated from *Clarias gariepinus* were strains of *Citrobacter freundii* 97%,*Proteus penneri* 96%, *Echerichia coli 94%, Morganella morganii 88%, Alcaligenes feacalis 94%, Erwinia tasmaniesis*93%, *Citrobacter braakii* 93%and *Citrobacter amalanoticus* 92%.while 62.5% of bacterial isolates showed partial haemolysis while 6.25% and 31.25% bacteria Isolates showed complete haemolysis and no haemolysis respectively. The result suggests that majority of the bacteria associated with *Clarias gariepinu s*are pathogenic which can result in rapid haemolysis of the red blood cell if consumed by humans.

**KEYWORDS:** *C. gariepinus*, Bacterial isolates, DNA extraction, Sequencing, Heamolytic analysis

## INTRODUCTION

Haemolysis is the situation in which the membranes of the red blood cells (RBCs) are broken down causing the release of haemoglobin and other internal components, into the surrounding fluid (blood plasma). The process occurs naturally as a way of removing dead, damaged or aged RBC from circulation. Catfish is one of the six species included in the U.S. National Animal Genome Project NRSP-8. A number of genome resources have been developed in catfish including a large number of molecular markers (Waldbieser *et al.*, 2001; Karsi *et al.*, 2002), This study further helps to determine the microbial load of fish and the bacteria species present on the skin, gill and gut of the cat fish. High bacterial load was discovered from the fish samples studied and this support the report of Naim and Ahmed (2012). The catfish genome contains one main type of tandem repeats named as Xba elements (Liu and Dunham, 1998) and several types of dispersed repetitive elements with the mariner/Tc1 DNA transposons as the leading type of dispersed repetitive elements (4-5% of the genome), followed by retrotransposons (3-4% of the genome), (Kim *et al.*, 2000; Liu, 2011). Ajayi (2012) reported that *Bacillus spp, Staphylococcus spp, Streptococcus spp, Micrococcus spp*, and members of enterobacteriaceae which include *Escherichia coli* and *Klebsiella spp* were found in the skin of the fresh fish. The author further reported that other complex forms of bacterial species were also encountered in the gills of catfish sample used for this study. At present, a number of genomic tools and resources have been developed in catfish, including bacterial artificial chromosome (BAC) libraries (Quiniou *et al.*, 2003; Wang *et al.,2007).*In pure culture. In situations where bacterial cell concentrations may reach inhibitory concentrations in a test matrix such a catastrophic event becomes significant because of its potential to generate false negative results. In addition, the presence of excess non-target DNA may have a similar inhibitory effect (Hoie *et al*., 1997; Wilson, 1997). This includes *S. aureus, E. coli, Bacillus spp*. The application of polymerase chain reaction (PCR) amplification and specific nucleic acid probes to the detection of fish pathogenic bacteria has generated significant interest (Cascon *et al.*, 1996; Aoki and Hirono, 1995).The alternative pathway activity of complement system can be measured, among other techniques, by the determination of serum hemolytic activity, when this pathway is activated by foreign red blood cells (Yano, 1992). This analysis can be used to evaluate the effects of several factors on the lytic activity of complement system, such as infections, environmental impact and nutrition (Holland & Lambris, 2002).Tsukamoto *et al*. (1990) isolated Proteus species from fresh water. It was reported that *Staphylococcus spp, Escherichia coli* were frequently isolated from the skin of fresh water and concluded that the skin of fresh water fish is the natural habitat of these bacteria. Some investigators reported that the skin of the *Clarias* spp. contained *Klebsiella spp, Pseudomonas spp, Micrococcus spp* as the predominant genera, while others

reported Pseudomonas and Staphylococcus belonging to the family Enterobacteriaceae as the. predominant genera on the skin of fresh water fishes

## MATERIALS & METHODS

### Collection of Samples

The study was carried out at Idogo station, Yewa River Ogun State, Nigeria, located on latitude 06°, 49¹N, and longitude 2°, 54¹E of the Greenwich Meridian. A total of sixteen (16) *Clarias gariepinus* were collected from the study area and morphometrics feature including standard length, total length and head length were measured on the fish in centimeters (cm) after weighing the fish species (gram). Bacteria were swabbed from three different parts of the fish, gills, skins, and guts. Swab sticks were used to streak the bacteria on nutrient agar and incubated for 24hours. The bacterial isolates were then streaked on the Blood Agar and kept inside Refrigerator.

### Water Test and Morphometric feature of fish sample

Morphometric feature of the fish samples which include Standard Length; Total Length; Head Length in centimeter (cm) were measured with the aid of a metre ruler, after the fishes have been weighed on a weighing balance. Temperature, Dissolved Oxygen concetration and pH of water samples were also measured.

### Isolation of Bacteria and Morphological Characterization

Nutrient Agar was weighed according to the manufacture's specifications. The prescription Nutrient agar were weighed and poured into the conical flask in which distilled water were measure accurately and allow the bubbled to dissolved properly before using it. The conical flask was shielded with cotton wool, covered with aluminum foil and agar was autoclaved at $121^0$C for 15 minutes for sterilization. Before pouring into disposable petri - dishes for solidification. The nutrient agar was then allowed to cool for about $40^0$C and incubated.

### Identification of the Bacteria Isolates by 16S rRNA Gene Amplification

Bacterial isolates grown overnight were transferred to eppendorf tube and spunned at 14,000rpm for 2mins. The supernatant was discarded and the DNA was extracted using CTAB method. The DNA was later resuspended in 100µl of sterile distilled water. DNA concentration of the samples were measured and the genomic purity determined. The DNA was further checked on 1.0 % agarose gel and visualised on UV light source. PCR analysis was done using MJ Research Thermal Cycler (PTC-200 model).The primer used for PCR amplification was 16S universal primer for bacteria. The sequence for the forward primer was 5'AGAGTTTGA TCCTGGCTCAG3' and reverse primer was 5'ACGGCTACCTTGTTACGACTT3' The PCR mix comprises of 1µl of 10X buffer, 0.4µl of 50mM $MgCl_2$, 0.5µl of 2.5mM dNTPs, 0.5µl 5mM Forward primer, 0.5µl of 5mM Reverse primer, 0.05µl of 5units/ul Taq with 2µl of template DNA and 5.05µl of distilled water and the PCR profile used has an initial denaturation temperature of $94^0$C for 3mins, followed by 30 cycles of $94^0$C for 60 seconds, $56^0$C for 60 seconds $72^0$C for 120 seconds and the final extension temperature of $72^0$C for 5 minutes and the $10^0$C hold forever. The amplicon was further purified before the sequencing using 2M Sodium Acetate washing techniques. The pellet was resuspend in 5µl sterile distilled water. The PCR mix used includes 0.5µl of BigDye Terminator Mix,1µl of 5X sequencing buffer, 1µl of 16S Forward primer with 6.5µl Distilled water and 1µl of the PCR product making a total of 10µl. The PCR profile for Sequencing is a Rapid profile with the initial Rapid thermal ramp at $96^0$C for 1min followed by 25 cycles of Rapid thermal ramp at $96^0$C for 10 seconds Rapid thermal ramp at $50^0$C for 5 seconds and Rapid thermal ramp at $60^0$C for 4 minutes, then followed by Rapid thermal ramp at $4^0$C and hold forever. The PCR sequence products were purified before the sequencing using 2M Sodium Acetate washing techniques.The pellet was re-suspend in 5 µl sterile distilled water. The combination of 9µl of Hi Di Formamide with 1µl of Purified sequence making a total of 10µl was prepared and loaded on Applied Biosystem (AB1 3130xl model) .

### Antibiotics and Sensitivity

15.2g of Mueller Hinton Agar was dissolved in 400ml[1]of distilled water and autoclaved at 121°C for 15 minutes. It was later brought out and allowed to cool then poured on the petri-dish and allowed to solidify. The plates were later labeled and the slant isolates were introduced into the prepared Mueller hinton agar for culture. Smearing was done to widen the occurrence of the microorganism. The antibiotics known as motor disc antibiotics was placed on the smeared media, in order to view or observe the sensitivity or resistance of the organisms using 20 samples. It was incubated at 37°C for 16 hours. At the end of the incubation there should be enough bacteria to form visible colonies in the areas touched by the inoculating loop, around the antibiotics and read for the zone of inhibitions.

### Nucleotide Sequence and Statistical Analysis

The sequences were aligned to 16S rRNA gene sequences in the Genbank DNA database and the homology of the sequences were analyzed using Basic Local Alignment Search Tool (BLAST) program of the National Centre for Biotechnology Information (NCBI) and determination of the phylogenetic tree was done using CLC software while descriptive statistics was used to analyze data on morphometric and water parameter.

## RESULTS & DISCUSSION

### PCR analysis using16S-rRNA primers and Antibiotics Sensitivity Pattern

The size of the amplified band with 16S universal primer was 1.6Kb for the 16 samples Fig1. The bacteria are five *Citrobacter freundii*, three *Morganella morganii*, two *Alcaligenes faecalis*, and one *Erwinia tasmaniesis*, *Citrobacter braakii, and Citrobacter amalanoticus* Figure 2. showed the population of each bacterial isolate from the skin, *Citrobacter freundii* has a population percentage of 97%, *Morganella morganii* has a population of 88%, *Alcaligenis feacalis* has population percentage of 94%, *Escherichia coli has* percentage of 95%. The population distribution of bacterial isolates in the gut of fish species revealed only *E. tasmaniesis* with the population percentage of 93%. The chart further shows the population distribution of bacterial isolates observed from the gill of fish species. *Proteus penneri* has population percentage of

96% 62.5% of bacterial Isolates showed partial haemolysis while 6.25% and 31.25% bacteria Isolates showed complete haemolysis and no haemolysis respectively.

**TABLE 1:** water quality parameter of African mud Cat Fish In Idogo, Yewa River

| Parameters | Readings |
|---|---|
| Temperature (°C) | 31.2 |
| Dissolve Oxygen (ppm) | 3.6 |
| pH | 7.2 |

**TABLE 2.** Occurrence rate of bacteria isolate of African Catfish

| Bacteria | Occurrence | Percentage |
|---|---|---|
| *Escherichia coli* | 1 | 6.3 |
| *Morganella morganii* | 3 | 25.0 |
| *Alcaligenes feacalis* | 2 | 12.5 |
| *Citrobacter freundii* | 5 | 31.3 |
| *Erwinia tasmaniesis* | 1 | 6.3 |
| *Proteus penneri* | 1 | 6.3 |
| *Citrobacter braakii* | 1 | 6.3 |
| *Citrobacter amalanoticus* | 1 | 6.3 |
| Total | 16 | 100 |

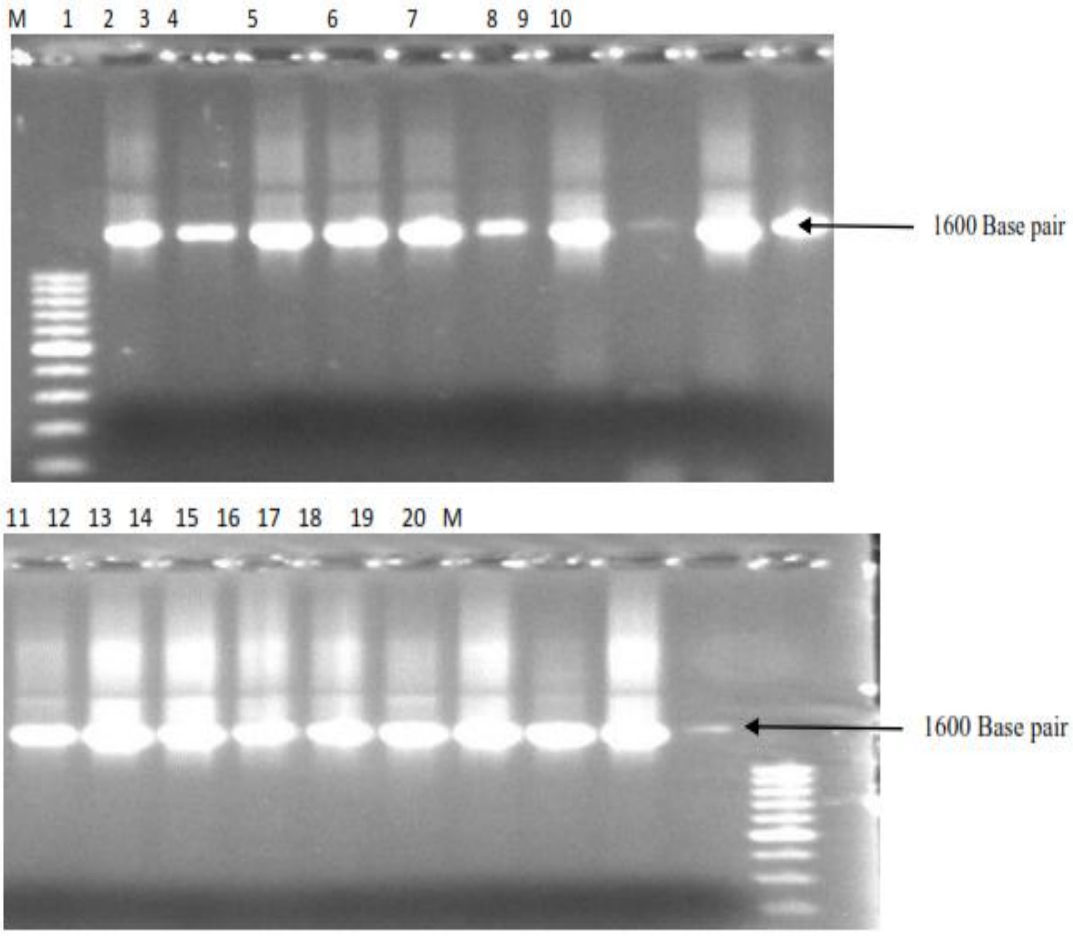


**FIGURE1:** Gel electrophoresis for the 16S primer

**TABLE 3.** Heamolytic pattern of fish bacteria isolate

| Type of heamolytic | Occurrence | Percentage (%) |
|---|---|---|
| Complete heamolysis | 1 | 6.25 |
| Partial heamolysis | 10 | 62.5 |
| No heamolysis | 5 | 31.25 |
| Total | 16 | 100 |

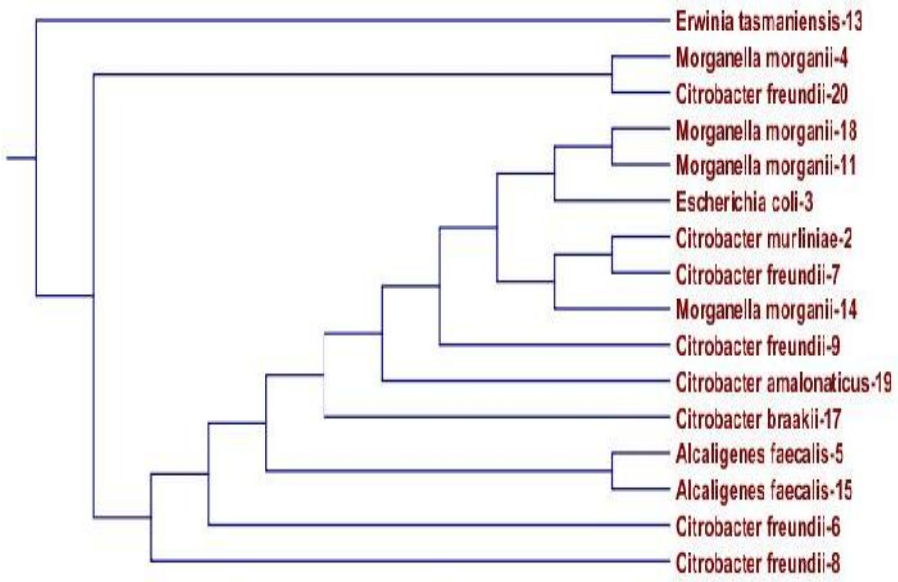


**FIGURE 2**: Dendogram of the Bacteria Isolates

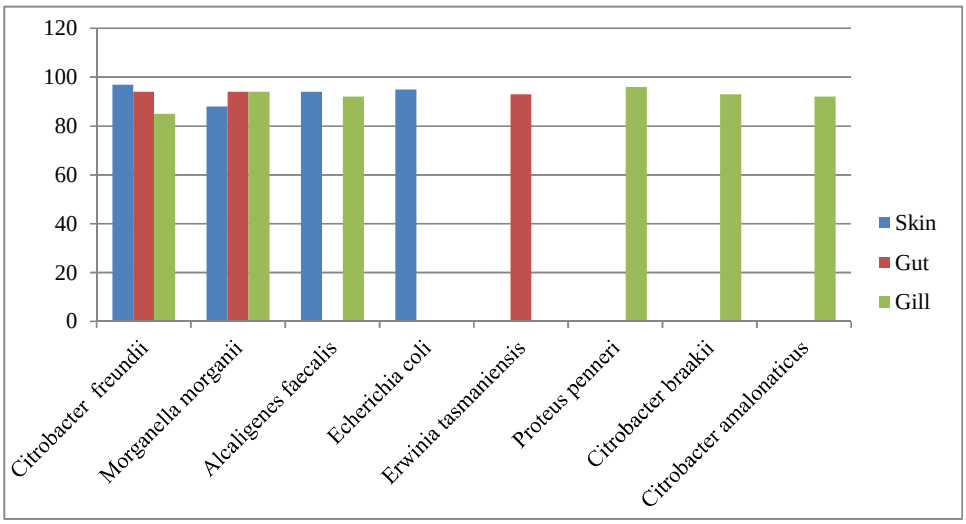


**FIGURE 2:** Distribution of Bacteria isolate across three Substrate of African Cat fish (*Clarias gariepinus*)

**TABLE 4:** Distribution of bacteria isolates in different substrate of *Clarias gariepinus*

| Substrate | Bacteria | Haemolysis | Level of similarity |
|---|---|---|---|
| Skin | *Citrobacter freundii* | ɤ-no haemoloysis | 97% |
| Gill | *Proteus penneri* | ɤ-haemoloysis | 96% |
| Skin | *Escherichia coil* strain | α-In complete | 95% |
| Skin | *Alcaligenes faecalis* | ɤ-no haemoloysis | 94% |
| Skin | *Citrobacter freundii* | β-complete | 94% |
| Gut | *Morganella morganii* | α-In complete | 94% |
| Gut | *Morganella morganii* | α-In complete | 94% |
| Gill | *Morganella morganii* | α-In complete | 94% |
| Gut | *Eriwina tasmaniensis* | α-In complete | 93% |
| Gill | *Citrobacter braakii* | α-In complete | 93% |
| Gill | *Alcaligenes faecalis* | α-In complete | 92% |
| Gill | *Citrobacter amalanoticus* | ɤ-no haemoloysis | 92% |
| Skin | *Citrobacter freundii* | ɤ-no haemoloysis | 91% |
| Skin | *Citrobacter freundii* | α-In complete | 94% |
| Skin | *Morganella morganii* | α-In complete | 88% |
| Gill | *Citrobacter freundii* | α-In complete | 85% |

## DISCUSSION

The mean of morphometric characteristics of the fish species *Clarias gariepinus* studied are significantly different ($p < 0.05$). The total length has the highest value while the length has the least value. According to Meade (1989) physico-chemical parameters such as alkalinity, dissolved oxygen, total hardness pH and temperature are the most common water quality characteristics that will influence fish health and growth. The temperature of the water was slightly higher than the growth range value of 27-29$^{o}$C stated by the Department of water and Forestry (1996). The pH of the water observed in this study falls within the standard range of 6.5- 8.0 (Meade, 1989), which supports microbial growth. The lower the pH of fish flesh the slower in general bacteria decomposition (Tsukamoto *et al*., 1990).DNA sequencing using PCR-based techniques target was 16S ribosomal RNA cells exceeds a certain value, (Barry *et al*., 1990; Wang *et al*., 1992). The 16S rRNA gene (1,500bp) is large enough for informatics purposes. The 16S rDNA gene was amplified by PCR using 16S rDNA specific universal forward and reverse primers, [5'AGAGTTTGATCCTGGCTCAG3'] and [5'A CGGC TACCTTGTTACGACTT3'] respectively against forward primer, (8F; 5'AGGCGGAGCCATACCATG CA3' and reverse primes, (1492R; 5'ATTA AGT AGGTCCGACAGAC3') by Akinyemi and Oyelakin (2014). This implies that genomic sequence can be different between and within species of an organism. The genomic DNA of bacterial isolates of the amplified band in this study was 2400bp for the 16 bacterial isolates. This study further helps to determine the microbial load of fish and the bacterial species present on the skin, gill and gut of *Clarias gariepinus*. High bacterial load was discovered from the fish samples studied and this supports the report of Naim and Ahmed (2012) that pond water bacteria had a reflection in the bacterial composition of the gills and intestine (gut) of cat fish. This high bacterial population may be due to the discharge of waste materials into water bodies upon which the fish species feed or it might result from flooding during rainy season. However this study on Gene sequencing showed that *Citrobacter freundii* occurred mostly in different strains in the fish species. *Morganella morganii* also has high occurrence but lower than that of *C. freundii* in the fish species. *Escherichia coli, Erwinia tasmaniesis, Proteus penneri, Citrobacter braakii* and *Citrobacter amalanoticus* had lower occurrence. From the fish species studied, *C. freundii, Morganella morganii, Alcaligenes faecalis* and *Escherichia coli* were distributed in the skin of the fish. The presence of *E. coli* confirms the report by Tsukamoto *et al*. (1990), that *E. coli* can be observed from the skin of catfish. The highest count observed in *C. freundii* and lowest observed to be *Escherichia coli*, although their value is considerably similar. *C. freundii, M. morganii, Erwinia tasmaniesis* were distributed in the gut of the fish with *Citrobacter freundii* having the highest bacterial count. *M. morganii, C. freundii, Alcaligenes faecalis, Proteus penneri, C. braakii* and *C. amalanoticus* were distributed in the gill with *P. penneri* having the highest bacterial count. Three heamolytic patterns (complete, incomplete and no heamolysis) were revealed by the bacteria isolates of the fish species (*C. gariepinus*) studied. Majority of the bacterial isolates of the catfish are heamolytic and this agrees with findings by Manivasagan *et al.* (2009). This implies that majority of the fish species would cause rapid heamolysis of the red blood cell if consumed by human. Few showed no heamolysis while single bacteria, *Citrobacter freundii*, had complete heamolysis.

## CONCLUSION

The study provided information on the bacteria flora from the gill, gut and skin different relation to their population, distribution and hemolytic pattern, of African mud catfish *Clarais gariepinus* obtained from Idogo station, on Yewa River. The fish species studied was found in be rich to pathogenic bacteria most of which has heamolytic characteristics. The skin part of fish was vulnerable to bacteria which include *Escherichia coli, Morganella morganii, Alcaligenes faecalis, Citrobacter freundii and Citrobacter braakii* and this is because the skin of the fish is usually in direct contact with water. I recommend that research should be conducted to monitor the bacteria isolate of the studied fish species in Idogo station in order to make it less harmful for consumption however, it should be well cooked and preserved before consumption or livestock feeding. The Idogo station fishermen should properly consistence in monitoring the water parameter to reduce the stress of fish species. In order to enhance the survival and good growth rate of fish species in freshwater body and consequently improved the productivity.

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