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***Original Research Article***

# Molecular Characterization and Antibiotic Resistance Profile of Bacteria Associated with *Brycinus longipinnis* from Eggua Station on Yewa River.


## Abstract

Fish is one of the most highly perishable food products, during handling and storage, causing rapid quality deterioration of fresh fish and limits the shelf life of the product. Bacteria isolates were collected from the gills, skins and guts of Brycinus longipinnis from Eggua, station on Yewa River. The isolates were assessed using 16S rRNA gene sequencing method to identify them and to construct the phylogenetic relationship. Sixteen bacteria isolates were selected, their morphological characteristics were determined, the DNA of the isolates were extracted using CTAB method, PCR amplification of 16S ribosomal RNA gene of isolates was carried out using universal primer for bacteria, purification of the PCR product was done using ethanol precipitation, later it was sequenced using an automated DNA sequencer. The sequence data were compared with other gene sequences in GenBank database (NCBI) using a BLAST search to find closely related sequences. Eight different bacteria strains were identified from skins, gills and guts of Brycinus longipinnis. The strains are Pseudomonas spp (15%), Comamonas spp (10%), Escherichia coli (10%), Proteus spp (10%), Entrobacter spp (10%), Pseudoalteromonas spp (5%), Alcaligenes faecalis (5%), and Serratia marcescens (5%) frequency of bacteria. Most of the strains from Brycinus longipinnis were sensitive to Ofloxacin and Ciprofloxacin antibiotics; and resistant to Cefuroxime and Cefixime. It can therefore be concluded that Brycinus longipinnis harbors different species of bacteria.


## 1. INTRODUCTION

Fish is a low fat food, a great source of protein, vitamins and minerals. Fish constitute about 45% of the total amount of protein [1], fish product has a nutrient profile superior to all terrestrial meats of beef, pork and chicken etc., being an excellent source of high quality animal protein and highly digestible energy. It is a good source of sulphur and essential amino acids such as lysine, leucine, valine and arginine. It is therefore suitable for supplementary diets of high carbohydrate contents [2]. Attention has been focused recently on the relationship between fish consumption and reduced incidence of cardiovascular disease. The benefit has been attributed to the nature of the fats in fish. Unlike other fats in other food, it is the only type of fat that supplies omega-3 poly unsaturated fatty acids (PUFA) [3]. PUFAs are essential in lowering blood cholesterol level and high blood pressure. It is able to migrate to alleviate platelet of (cholesterol) aggregation and various arteriosclerosis conditions in adult population. It helps in prevention of asthma, arthritis, psoriasis, and sonic type of cancer [4]. Increase rate of fish consumption has brought up the risen rate of antibiotics use as growth promoter, additive or preventive agents in fish feed and numerous fish farm in this locality [5].

Therefore, the objective of this research work is to identify bacteria flora from gills, guts and skin of *Brycinus longipinnis* from Eggua station on Yewa River through molecular systematic

UNDER PEER REVIEW

using 16S rRNA gene sequence, their antibiotics resistance patterns and construct a clustering analysis to find an evolutionary ties among the microorganisms.

## 2 MATRIALS AND METHODS

### 2.1 Samples collection

The study was carried out at Eggua station on Yewa River which has six landing site. It is situated at $7^0$ 3' North and $2^0$ 55' East. Bacteria samples were taken from the gill, skin and gut of *Brycinus longipinnis* with the use of swab stick. All swab sticks were streaked on both Nutrient agar and Mac Conkey agar by BioMark Laboratory, the samples were later incubated for 18 hours at $37^0$C.

### 2.2 Morphometric of Fish Sample and Water Quality Parameters

The standard length, total length, the head length in centimeter (cm) were measured using a measuring ruler and recorded after weighing the fish sample in grams (g) using sensitive weighing balance. The water quality was tested on temperature, conductivity and pH from difference point on the landing site.

### 2.3. Isolation of Bacteria and Morphological Characterization

Isolation of bacteria from the gills, guts and skin of the fish samples was carried by standard method. Each sample of the gills, guts and skin was inoculated on Nutrient agar and MacConkey agar (Oxoid, UK) and incubated at $37^0$C for 24 hours. Bacteria colonies obtained were purified on Nutrient agar and incubated at $37^0$C for 24 hours for growth pure discrete colonies. Colonial and cellular morphology was performed by phenotypic examination and Gram staining respectively. Each bacteria isolates were biochemically characterized according to [6].

### 2.4 Identification of the bacteria isolates by 16S rRNA gene amplification.

Bacteria isolate grown overnight pelleted 14,000rpm for 2mins, and the DNA was extracted using CTAB method [7]. The DNA was later resuspended in 100µl of sterile distilled water. DNA concentration of the samples were measured and the genomic purity were determined. 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'AGAGTTTGATCCTGGCTCAG3' 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 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 PCR product 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, the initial Rapid thermal ramp to $96^0$C for 1min followed by 25 cycles of Rapid thermal ramp to

UNDER PEER REVIEW

$96^0$C for 10 seconds Rapid thermal ramp to $50^0$C for 5 seconds and Rapid thermal ramp to $60^0$C for 4 minutes, then followed by Rapid thermal ramp to $4^0$C and hold forever. The PCR sequence product was purified before the sequencing using 2M Sodium Acetate washing techniques. The pellet was re-suspended in 5 µl sterile distilled water [8]. 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).

### 2.5 Antibiotic Resistance determination

Antibiotic susceptibilities of the bacterial isolates were analysed by using the Kirby-Bauer disc diffusion method. An overnight culture was standardized to a turbidity equivalent to 0.5 McFarland standards ($1.5\times10^8$cfu/ml) with sterile 1% peptone water. The standardized overnight broth culture was spread on Mueller-Hinton agar plates using sterile swabs. Antibiotic-impregnated discs (Abtek, U.K) were placed on seeded plates and were incubated at $37^o$C for 24 hours. Zones of inhibition were measured after 24 hours of incubation. The strains were classified as 'resistant (R)', 'intermediate sensitive (I)' or 'sensitive (S)' using standard recommendations of Clinical and Laboratory Standards Institute.

### 2.6 Statistical analysis

Corrected 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) in order to determine the bacterial identities and the statistical analysis to determine the phylogenetic tree was done using CLC software while descriptive statistics was used to analyze data on morphometric and water parameter.

## 3 RESULTS AND DISCUSSIONS

### 3.1 Morphometric Characteristics of *Brycinus longipinnis*

Morphometric Characteristics of *Brycinus longipinnis* from Eggua station on Yewa River has indicated in Table 1 shows that fish samples had mean value in weight, standard length, total length and head length of 245g, 4.6±0.2cm, 5.6±0.2cm and 1.0±0.1cm respectively. Table 2 shows that water parameters taken has follows; Temperature, Dissolve Oxygen and pH readings of $28.6^o$C, 5.5ppm and 8.3 respectively.

### 3.2 Molecular characterization by gene sequencing

The size of the amplified band using 16S universal primer was 1.6Kb for the 14 samples. The blasting of the sequence for the isolates shows that there are three types of bacteria species present. The gene sequence of the similarity (%) by BLASTN in GenBank of the NCBI are shown in Table 3. There were eight different bacteria strains that were identified; there were three *Pseudomonas spp, one Pseudo alteromonas spp, two Enterobacter cloacae, two Escherichia coli, one Serratia marcescens, two Proteus spp, one Alcaligenes faecalis, two Comamonas spp.* The most occurrence bacteria is *Pseudomonas spp* and *Enterobacter spp*. Table

UNDER PEER REVIEW

4 shows that the highest bacterial isolates were found in the gut, followed by skin and then the gill. The gut and skin had *Escherichia coli* in common. Cluster analysis showed *Proteus spp, Escherichia coli, Alcaligenes faecalis, Serratia marcescens* were grouped together in the first group and others were grouped together as well.

The taxonomy of many bacteria based on comparative 16S ribosomal RNA sequencing analysis has revealed that some taxa generated on the basis of phenotypical features do not correspond with their phylogenetic relations. Molecular techniques, especially polymerase chain reaction (PCR) based methods are important for the specific characterization and detection of bacterial strains from fishes. In this present study, the genomic DNA from the bacteria isolated from guts, skins and gills of *B. longipinnis* collected from Eggua station on Yewa River were amplified using 16S rRNA primers, followed by sequencing of 16S rRNA gene and BLAST searching at NCBI GenBank database. The isolated bacteria include the strains of *Pseudomonas aeruginosa, Comamonas jiangduensis, Escherichia coli, Proteus mirabilis, Serratia marcescens, Alcaligenes faecalis, Proteus vulgari, Pseudomonas putida, Pseudo alteromona ssp, Pseudomonas fluorescens, Comamonas aquatic* and *Enterobacter cloacae.* Many strains of these bacteria have been isolated from fresh *Clarias gariepinus* and *Oreochromisniloticus* by [9]. It revealed that *B. longipinnis* had the highest frequency of occurrence of *Pseudomonas* spp and *Enterobacter spp* Table 5. The presence of some pathogenic bacterial isolates such as *E. coli* and *Enterobacter cloacae* in *B. longipinnis* from Eggua station on Yewa River reveals the pollution of this river with faecal matter either from sewage disposal or from human activities such as bathing, washing, defecating, etc. Free roaming animals and pets especially sheep, goats and dogs also contribute to faecal contamination of the river. Run-off from roads, parking lots and yards can also carry animal wastes into this river. Birds such as swans, geese and other water fowl can also be significant sources of bacteria into this river and therefore leads to contamination of this fish species with bacteria. This is similar to the previous work of [10] conducted on catfish who isolated different microorganisms from the different parts of fish samples such as intestine, gills and skin. *Pseudomonas* and related genera are aerobic, Gram-negative soil bacteria, some of which can degrade a wide variety of unusual compounds. Four species of *Pseudomonas* (*P. fluorescens, P. fragi, P. lundensis,* and *P. viridiflava*) are the main food spoilage organisms [10]. Figure 1 showed that Comamonas jiangduensis formed separate group while other 13 isolates made up the second group.

This study also showed that *Escherichia coli* is present in both skin and gut of fish, distribution of bacteria is high in gut than skin and gill, *Pseudomonas* spp has the highest frequency distribution of bacteria of *B. longipinnis* in Eggua station on Yewa River.

### 3.3 Antibiotics susceptibility pattern of the bacteria isolates strained from *B. longipinnis*

The antibiotic susceptibility patterns of the bacterial isolates are shown in Table 6 and 7. The level of resistance and sensitivity of these bacteria to clinically relevant antibiotics differs. Among the isolates, 70% bacteria strains were sensitivity to Ofloxacin while 95% showed high resistance rate to Cefuroxime, 60% to Augmentin and Ceftazidime. The results of this study revealed that bacteria associated with *Brycinus longipinnis* show more resistance to commonly used clinical antibiotics than the bacteria isolated from *Clarias gariepinus* and *Oreochromisniloticus* by [9] who reported 58.3% resistance and 41.7% susceptibility.

UNDER PEER REVIEW

161 **Table 1: Mean value of morphometric features of *Brycinus longipinnis*.**

| Morphometric features (cm) | Readings (mean±SD) |
|---|---|
| Standard length | 4.6±0.2cm |
| Total length | 5.6±0.2cm |
| Head length | 1.0±0.1cm |

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165 **Table 2: Water quality parameters of *Brycinus longipinnis*.**

| Parameters | Readings |
|---|---|
| Temperature ($^0$C) | 28.6 |
| Dissolve oxygen (ppm) | 5.5 |
| pH | 8.3 |

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UNDER PEER REVIEW

171 **Table 3: Genotypic bacterial isolates from *B. longipinnis***

| Isolation site | Genotypic identification | % similarity |
|---|---|---|
| Gut | *Pseudomonas putida* strain | 95 |
| Skin | *Enterobacter cloacae* strain | 97 |
| Skin | *Pseudomonas aeruginosa* strain | 95 |
| Gut | *Pseudoalteromonas sp.* | 89 |
| Skin | *Escherichia coli* strain | 86 |
| Gut | *Comamonas jiangduensis* strain | 85 |
| Gut | *Escherichia coli* strain | 92\ |
| Skin | *Proteus mirabilis* strain | 94 |
| Gill | *Alcaligenes faecalis* | 90 |
| Gut | *Pseudomonas fluorescens* strain | 88 |
| Skin | *Serratia marcescens* | 93 |
| Gill | *Proteus vulgaris* strain | 89 |
| Gill | *Enterobacter sp.* | 89 |
| Gut | *Comamonas aquatic* strain | 89 |

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UNDER PEER REVIEW

174 **Table 4: Distribution of bacterial isolates found in different organs of *B. longipinnis***

| ***Organ*** | ***Bacterial isolates*** |
|---|---|
| *Gut* | *Pseudomonas putida* |
| | *Pseudoalteromonas sp.* |
| | *Comamonas jiangduensis* |
| | *Escherichia coli* |
| | *Pseudomonas fluorescens* |
| | *Comamonas aquatic* |
| *Skin* | *Enterobacter cloacae* |
| | *Pseudomonas aeruginosa* |
| | *Proteus mirabilis* |
| | *Serratia marcescens* |
| | *Escherichia coli* |
| *Gill* | *Alcaligenes faecalis* |
| | *Proteus vulgaris* |
| | *Enterobacter sp* |

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UNDER PEER REVIEW

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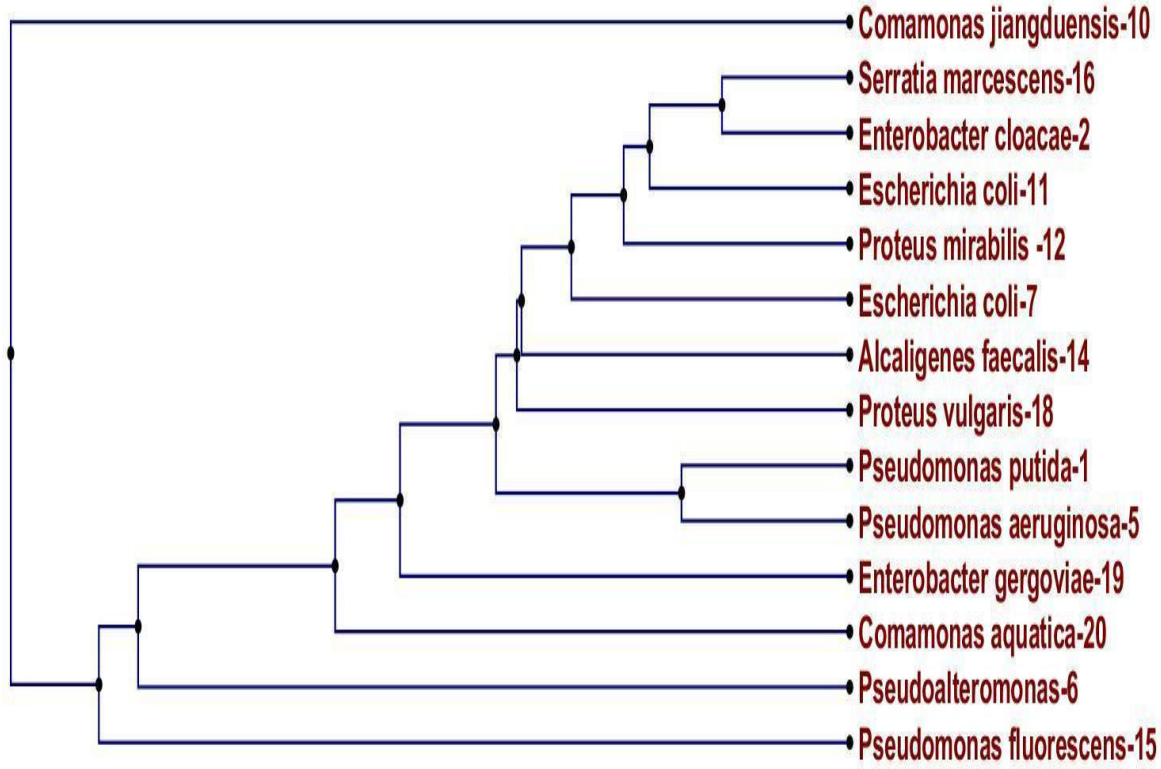
192 **Table: 5 Percentage frequency of bacteria isolates found in *B. longipinnis***

| Bacteria | Percentage frequency |
|---|---|
| *Pseudomonas spp* | 20.0 |
| *Pseudoalteromona spp* | 6.5 |
| *Comamona ssp* | 13.5 |
| *Escherichia coli* | 13.5 |
| *Proteus spp* | 13.5 |
| *Serratia marcescens* | 6.5 |
| *Alcaligenes faecalis* | 6.5 |
| *Enterobacter spp* | 20.0 |

193

UNDER PEER REVIEW

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196 Figure 1 Cluster Analysis for the 14 bacteria isolates

UNDER PEER REVIEW

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| Bacterial strain | Antibiotics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | OFL | AUG | NIT | CPR | CAZ | CRX | GEN | CXM |
| *Pseudomonas putida* strain | S | S | S | S | S | R | S | R |
| *Enterobacter cloacae* strain | S | I | S | S | R | R | S | I |
| *Pseudomonas aeruginosa* strain | R | R | I | R | R | R | R | R |
| *Pseudoalteromonas sp.* | S | R | R | S | S | R | I | R |
| *Escherichia coli* strain | S | S | S | S | R | R | I | R |
| *Comamonas jiangduensis* strain | R | R | R | R | R | R | S | R |
| *Escherichia coli* strain | R | R | I | R | R | R | R | R |
| *Proteus mirabilis* strain | S | S | S | S | S | R | S | R |
| *Alcaligenes faecalis* | S | R | R | S | R | R | I | R |
| *Pseudomonas fluorescens* strain | I | S | I | I | S | R | S | S |
| *Serratia marcescens* gene | S | R | R | R | R | R | R | R |
| *Proteus vulgaris* strain | S | R | R | S | R | R | S | R |
| *Enterobacter sp.* | I | I | I | R | R | R | R | R |
| *Comamonasaquatica* strain | S | I | S | I | S | R | S | R |

198 **Table 6: Antimicrobial susceptibility pattern of the bacteria isolates stained from**
199 ***B.longipinnis***
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202 **Key:**
203 Resistance (R): 0-10mm
204 Intermediate (I): 11-16mm
205 Sensitive (S): 17mm and above
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207 Ofloxacin OFL**,** Augmentin AUG, Nitrofurantoin NIT**,** Ciprofloxacin CPR**,**
208 Ceftazidime CAZ**,** Cefuroxime CRX, Gentamicin GEN, Cefixime CXM
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UNDER PEER REVIEW

**Table 7: Percentage Antibiotics susceptibilty pattern of the bacterial isolates obtained from*B. longipinnis***

| Antibiotics | Sensitivity | | Intermediate | | Resistance | |
|---|---|---|---|---|---|---|
| | Number (n) | (%) | Number (n) | (%) | Number (n) | (%) |
| Ofloxacin | 14 | 70.0 | 1 | 5 | 5 | 25.0 |
| Augmentin | 4 | 20.0 | 4 | 20.0 | 12 | 60.0 |
| Nitrofurantoin | 7 | 35.0 | 5 | 25.0 | 8 | 40.0 |
| Ciprofloxacin | 11 | 55.0 | 3 | 15.0 | 6 | 30.0 |
| Ceftazidime | 8 | 40.0 | 0 | 0.0 | 12 | 60.0 |
| Cefuroxime | 1 | 5.0 | 0 | 0.0 | 19 | 95.0 |
| Gentamicin | 9 | 45.0 | 6 | 30.0 | 5 | 25.0 |
| Cefixime | 1 | 5.0 | 1 | 5.0 | 18 | 90.0 |

## 4. CONCLUSION

The study has clearly shown variation in bacterial species in different parts of *B. longipinnis* collected from Eggua station on Yewa River. The results also revealed that *Pseudomonas sp.* is the predominant bacteria found in *B. longipinnis*, with a few strains of *Comamona ssp, Pseusdo alteromona ssp, Proteus sp, Escherichia coli, Serratia marcescens and Enterobacter sp.* with high level antibiotic resistance. It was also observed that most of the bacterial isolates from *B. longipinnis* shown high rate of resistant pattern.

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UNDER PEER REVIEW

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