

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

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Authors' contribution

This work was initiated by AAA and OOO, the laboratory work was carried out by ARO, AKA, IOO and PAA while OOO prepared the manuscript and all the authors read and approved the final manuscript.

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 bacteria 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%), *Enterobacter 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 [1], vitamins and minerals. Fish constitute about 45% of the total amount of protein [2], fish product has a nutrient profile superior to all terrestrial meats like beef, pork and chicken 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 [1]. 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 [1][3]. 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]. The fish *Brycinus longipinnis* a tiny pelagic fish in Nigerian freshwaters has high economic value in the study area. It is used as protein sources by many people of the study area. It is also used as delicious delicacy as condiments in local vegetables' soup, as well as, rice stew. Furthermore, it is occupying a strategic place in the trophic level ecologically in the food pyramid. It is necessary to study this very small but unique aquatic animal for its microbial components to forestall issues relating to public health risk.

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

2 MATERIALS 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° 3' North and 2° 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°C.

2.2 Water Quality Test and Morphometric of Fish Sample

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, Dissolved Oxygen 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°C for 24 hours. Bacteria colonies obtained were purified on Nutrient agar and incubated at 37°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 was grown overnight pelleted at 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 was 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'ACGGCTACCTTGTACGACTT3' The PCR mix comprises of 1µl of 10X buffer, 0.4µl of 50mM MgCl₂, 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. The PCR profile used has initial denaturation temperature of 94°C for 3mins, followed by 30 cycles of 94°C for 60 seconds, 56°C for 60 seconds 72°C for 120 seconds and the final extension temperature of 72°C for 5 minutes and the 10°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°C for 1min followed by 25 cycles of Rapid thermal ramp to 96°C for 10 seconds Rapid thermal ramp to 50°C for 5 seconds and Rapid thermal ramp to 60°C for 4 minutes, then followed by Rapid thermal ramp to 4°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 (1.5×10^8 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°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 Nucleotide Sequence and 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 $245\pm0.2\text{g}$, $8.8\pm0.2\text{cm}$, $9.6\pm0.2\text{cm}$ and $3.4\pm0.1\text{cm}$ respectively. Table 2 shows that water parameters taken has follows; Temperature, Dissolve Oxygen and pH readings of 28.6°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 different 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 *Pseudoalteromonas* spp, two *Enterobacter cloacae*, two *Escherichia coli*, one *Serratia marcescens*, two *Proteus* spp, one *Alcaligenes faecalis*, two *Comamonas* spp. The most occurrence bacteria are *Pseudomonas* spp and *Enterobacter* spp. This is similar to [8] where *Pseudomonas* spp were predominantly found in *Clarias gariepinus*. Table 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 isolated bacteria include the strains of *Pseudomonas aeruginosa*, *Comamonas jiangduensis*, *Escherichia coli*, *Proteus mirabilis*, *Serratia marcescens*, *Alcaligenes faecalis*, *Proteus vulgaris*, *Pseudomonas putida*, *Pseudoalteromona* spp, *Pseudomonas fluorescens*, *Comamonas aquatic* and *Enterobacter cloacae*. Many strains of these bacteria have been isolated from fresh *Clarias gariepinus* and *Oreochromis niloticus* 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.

The work done by [10], conducted on catfish that isolated different microorganisms from the different parts of fish samples such as intestine, gills and skin. *Pseudomonas* and related genera that were aerobic, Gram-negative soil bacteria were obtained. Four species of *Pseudomonas* (*P. fluorescens*, *P. fragi*, *P. lundensis*, and *P. viridiflava*) were the main food spoilage organisms [10].

The size of the amplified band in the study was 1600bp for the bacteria isolates, it was also similar to 1600bp obtained by [8] but contrary to [11] that had 500bp. The blasting search showed that the selected bacteria isolates belong to Gram negative group that is similar with [12]. 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 *Oreochromis niloticus* by [9] who reported 58.3% resistance and 41.7% susceptibility.

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

Morphometric features (cm)	Readings (mean±SD)
Weight	245±0.2g
Standard length	8.8±0.2cm
Total length	9.6±0.2cm
Head length	3.4±0.1cm

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

Parameters	Readings
Temperature (°C)	28.6
Dissolve Oxygen (ppm)	5.5
pH	8.3

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

Isolation site	Genotypic identification	% similarity	Accession number
Gut	<i>Pseudomonas putida</i> strain	95	JQ782512.1
Skin	<i>Enterobacter cloacae</i> strain	97	JX514409.1
Skin	<i>Pseudomonas aeruginosa</i> strain	95	JQ659967.1
Gut	<i>Pseudoalteromonas</i> spp.	79	AB519010.1
Skin	<i>Escherichia coli</i> strain	86	JN578646.1
Gut	<i>Comamonas jiangduensis</i> strain	85	KJ806505.1
Gut	<i>Escherichia coli</i> strain	92	AP010960.1
Skin	<i>Proteus mirabilis</i> strain	94	JX974560.1
Gill	<i>Alcaligenes faecalis</i>	90	GQ856253.1
Gut	<i>Pseudomonas fluorescens</i> strain	78	GU198116.1
Skin	<i>Serratia marcescens</i>	93	AB244453.1
Gill	<i>Proteus vulgaris</i> strain	83	JN384161.1
Gill	<i>Enterobacter</i> spp.	83	FJ608240.1
Gut	<i>Comamonas aquatic</i> strain	84	FJ544370.1

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

Organ	Bacterial isolates
<i>Gut</i>	<i>Pseudomonas putida</i> <i>Pseudoalteromonas spp.</i> <i>Comamonas jiangduensis</i> <i>Escherichia coli</i> <i>Pseudomonas fluorescens</i> <i>Comamonas aquatic</i>
<i>Skin</i>	<i>Enterobacter cloacae</i> <i>Pseudomonas aeruginosa</i> <i>Proteus mirabilis</i> <i>Serratia marcescens</i> <i>Escherichia coli</i>
<i>Gill</i>	<i>Alcaligenes faecalis</i> <i>Proteus vulgaris</i> <i>Enterobacter spp</i>

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

Bacteria	Percentage frequency
<i>Pseudomonas spp</i>	20.0
<i>Pseudoalteromona spp</i>	6.5
<i>Comamona spp</i>	13.5
<i>Escherichia coli</i>	13.5
<i>Proteus spp</i>	13.5
<i>Serratia marcescens</i>	6.5
<i>Alcaligenes faecalis</i>	6.5
<i>Enterobacter spp</i>	20.0

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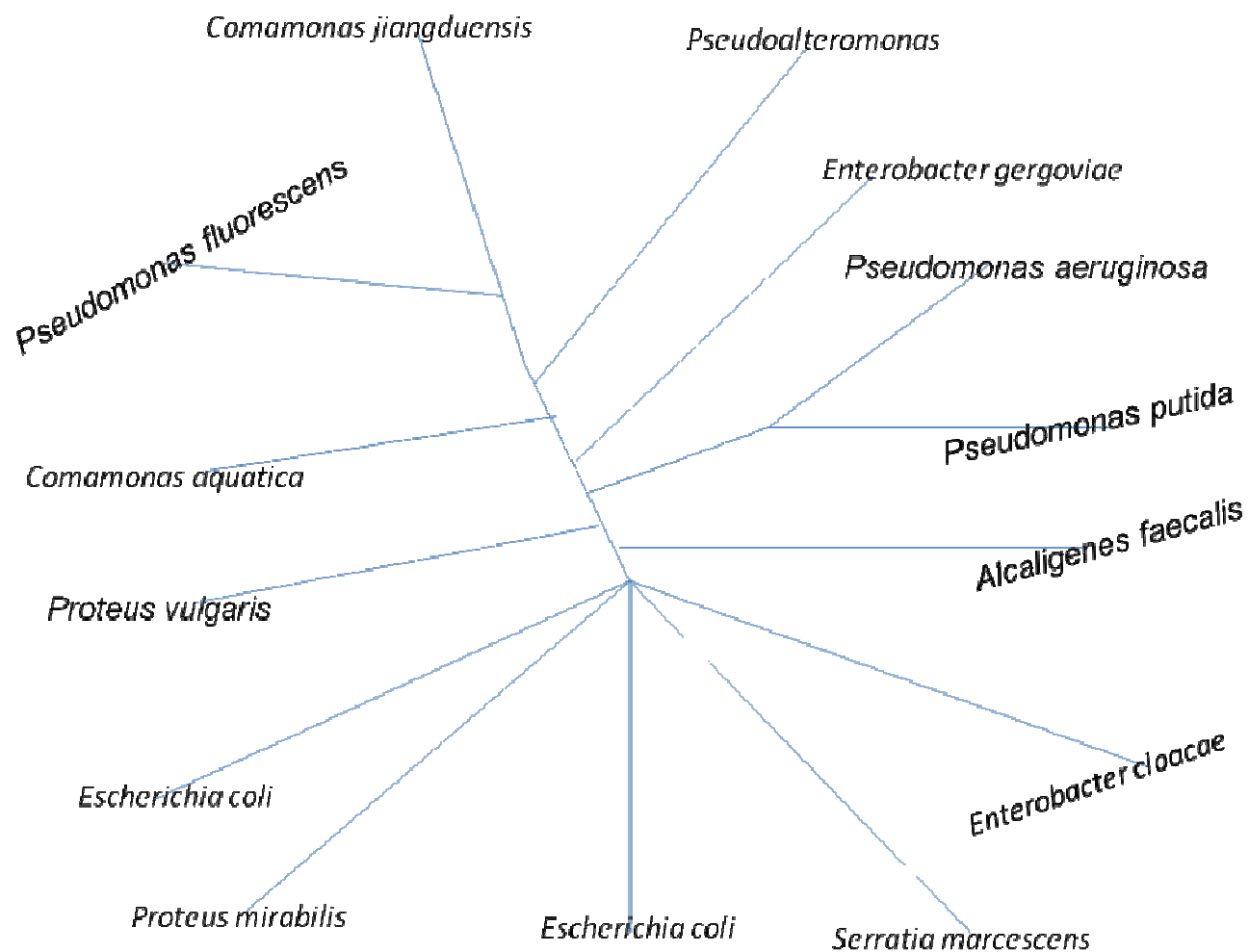


Figure 1 Cluster Analysis for the 14 bacteria isolates

Bacterial strain	Antibiotics							
	OFL	AUG	NIT	CPR	CAZ	CRX	GEN	CXM
<i>Pseudomonas putida</i> strain	S	S	S	S	S	R	S	R
<i>Enterobacter cloacae</i> strain	S	I	S	S	R	R	S	I
<i>Pseudomonas aeruginosa</i> strain	R	R	I	R	R	R	R	R
<i>Pseudoalteromonas</i> spp.	S	R	R	S	S	R	I	R
<i>Escherichia coli</i> strain	S	S	S	S	R	R	I	R
<i>Comamonas jiangduensis</i> strain	R	R	R	R	R	R	S	R
<i>Escherichia coli</i> strain	R	R	I	R	R	R	R	R
<i>Proteus mirabilis</i> strain	S	S	S	S	S	R	S	R
<i>Alcaligenes faecalis</i>	S	R	R	S	R	R	I	R
<i>Pseudomonas fluorescens</i> strain	I	S	I	I	S	R	S	S
<i>Serratia marcescens</i> gene	S	R	R	R	R	R	R	R
<i>Proteus vulgaris</i> strain	S	R	R	S	R	R	S	R
<i>Enterobacter</i> spp.	I	I	I	R	R	R	R	R
<i>Comamonas aquatica</i> strain	S	I	S	I	S	R	S	R

Table 6: Antimicrobial susceptibility pattern of the bacteria isolates stained from *B.longipinnis*

Key:

Resistance (R): 0-10mm
Intermediate (I): 11-16mm
Sensitive (S): 17mm and above

Ofloxacin OFL, Augmentin AUG, Nitrofurantoin NIT, Ciprofloxacin CPR,
Ceftazidime CAZ, Cefuroxime CRX, Gentamicin GEN, Cefixime CXM

Table 7: Percentage Antibiotics susceptibility 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 spp.* is the predominant bacteria found in *B. longipinnis*, with a few strains of *Comamonas spp.*, *Pseudomonas spp.*, *Proteus spp.*, *Escherichia coli*, *Serratia marcescens* and *Enterobacter spp.* 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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