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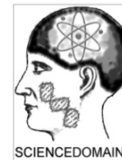

Molecular Characterization and Antibiotic Resistance Profile of Bacteria Associated with *Brycinus longipinnis* from Eggua Station on Yewa River

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Authors' contributions

This work was carried out in collaboration between all authors. This work was initiated by authors AA and OOO. The laboratory work was carried out by authors ARO, AKA, IOO and PAA while author OOO prepared the manuscript. All the authors read and approved the final manuscript.

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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

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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.

Keywords: Bacterial isolate; Brycinus longipinnis; DNA extraction; sequencing; clustering analysis.

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 tropic 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 and spun at 14,000 rpm for 2 mins, the DNA was extracted using CTAB method [7]. The DNA was later resuspended in 100 µl of sterile distilled water. DNA concentration of the samples was measured and the genomic purity was 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 1 µl of 10X buffer, 0.4 µl of 50 mM $MgCl_2$, 0.5 µl of 2.5 mM dNTPs, 0.5 µl 5 mM Forward primer, 0.5 µl of 5 mM Reverse primer, 0.05 µl of 5 units/µl Taq with 2 µl of template DNA and 5.05 µl of distilled water. The PCR profile used has initial denaturation temperature of 94°C for 3 mins, 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 2 M Sodium Acetate washing techniques. The pellet was resuspend in 5 µl sterile distilled water. The PCR mix used includes 0.5 µl of BigDye Terminator Mix, 1 µl of 5X sequencing buffer, 1 µl of 16S Forward primer with 6.5 µl Distilled water and 1µl of the PCR product making a total of 10 µl. The PCR profile for Sequencing is a Rapid profile, the initial Rapid thermal ramp to 96°C for 1 min 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 9 µl of Hi di Formamide with 1µl of Purified sequence making a total of 10 µl 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 DISCUSSION

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±0.2 g 8.8±0.2 cm, 9.6±0.2 cm and 3.4±0.1 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 1.6 Kb 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.

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

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

Table 2. Water quality parameters of *Brycinus longipinnis*

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

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 1600 bp for the bacteria isolates, it was also similar to 1600 bp obtained by [8] but contrary to [11] that had 500 bp. The blasting search showed that the selected bacteria isolates belong to Gram negative group that is similar with [12]. Fig. 1 showed that *Comamonas jiangduensis* formed separate group while other 13 isolates made up the second group.

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

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

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

Organ	Bacterial isolates
Gut	*Pseudomonas putida* *Pseudoalteromonas* spp. *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* spp

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 Tables 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 5. Percentage frequency of bacteria isolates found in *B. longipinnis*

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

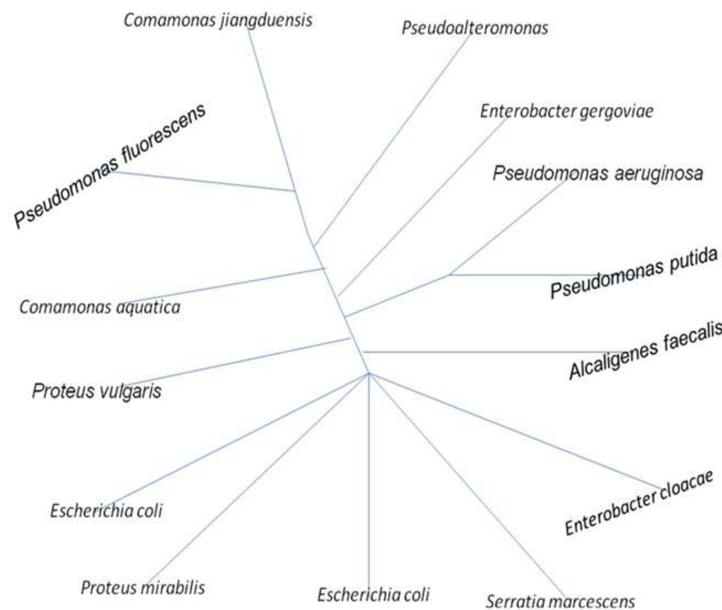

Fig. 1. Cluster analysis for the 14 bacteria isolates

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

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 spp.	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 spp.	I	I	I	R	R	R	R	R
Comamonas aquatica strain	S	I	S	I	S	R	S	R

Key: Resistance (R): 0-10 mm; Intermediate (I): 11-16 mm; 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 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 spp.* is the predominant bacteria found in *B. longipinnis*, with a few strains of *Comamonas* spp, *Pseusdoalteromona* 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.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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