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MOLECULAR CHARACTERIZATION OF BACTERIA ASSOCIATED WITH AFRICAN CATFISH *Clarias gariepinus* (Burchell, 1822) FROM YEWA-MATA STATION ON YEWA RIVER BY 16S rRNA GENE SEQUENCING METHOD

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ABSTRACT
This study investigates bacteria associated with *Clarias gariepinus*. Five fish samples were collected from Yewa-Mata station on Yewa River and their antibiotic susceptibility pattern determined. The gill, gut and skin surfaces of *Clarias gariepinus* were swabbed and analyzed microbiologically. The bacterial isolates were then characterized by molecular method using 16S rRNA gene sequencing. The antibiotic susceptibility patterns of the isolates were also determined using Kirby-Bauer disc diffusion method. The morphometric characteristics of the fish samples were recorded. The bacteria isolated from the gills, guts and skins were *Comamonas* sp. (25%), *Proteus sp* (15%), *Delftia tsuruhatensis* (5%), *Stenotrophomonas maltophilia* (5%), *Pseudomonas sp.* (5%), *Morganella morganii* (5%), *Candidatus nitrosoarchaeum* (5%), *Aeromonas hydrophila* (5%). *Morganella morganii*, *Proteus sp.* and *Pseudomonas sp.* were most sensitive to Nitrofurantion with a diameter of 17mm, 21mm and 22mm respectively; *Comamonas sp.* and *Aeromonas hydrophila* were most sensitive to Ofloxacin with a diameter of 17mm and 25mm respectively; *Stenotrophomonas maltophilia* was most sensitive to Ciprofloxacin and *Candidatus nitrosoarchaeum* was resistant to all antibiotics used.The mean values of the morphometric characteristics measured for the weight, total length, standard length and head length were 451g ±8.4, 28.54cm ±3.2, 24.93cm ±2.2 and 5.04cm ±0.53 respectively. The study therefore shows that diverse bacterial species exist on the skins, gills and guts of *Clarias gariepinus.*

KEYWORDS: Bacterial strains, *C. gariepinus*, polymerase chain reaction, sequencing, antibiotics.

INTRODUCTION
Fish are susceptible to a wide variety of bacterial pathogens. Many of these bacteria capable of causing disease are considered to be saprophytic in nature. These bacteria only become pathogens when fishes are physiologically unbalanced, nutritionally deficient, or are subjected to other stressors, *i.e*., poor water quality, overstocking, which allows opportunistic bacterial infections to thrive. Some of these bacterial pathogens of fishes are fastidious and require special growth media for laboratory culture. Others grow at different temperatures, dependent upon the aquatic environmental temperature of the fish. *Aeromonas salmonicida* is the most common bacterial pathogen of fishes worldwide (Austin, 2007). There has been a steady increase in the number of bacterial species associated with fish diseases, with new pathogens regularly recognized in scientific literature (Gauger *et al.,* 2002). Molecular techniques are major tools for the analysis of microorganisms from food and other biological substances. The techniques provide ways to screen for a broad range of agents in a single test. Molecular methods vary with respect to discriminatory power, reproducibility, ease of use, and ease of interpretation (Lasker, 2002). The emergence of rapid DNA sequencing methods has vastly improved the advancement of biological and medical research and discovery (Petterson *et al.,* 2009). There are several mechanisms that have evolved in bacteria which confer them with antibiotic resistance. These mechanisms can modify the antibiotic, render it inactive through physical removal from the cell, or modify target site so that it is not recognized by the antibiotic (Berdy, 2012). Therefore, the objective of this work is to identify bacteria strains from *Clarias gariepinus* using molecular techniques and to determine their antibiotic sensitivity patterns using different synthetic antibiotic drugs.

MATERIALS & METHODS
Collection of samples
The study was carried out at Yewa-Mata station on Yewa River which is a one of the major river tributaries of Ogun-Oshun River Basin Authority located in Ogun State, Nigeria. The station lies on Longitude $6^0$57' North to Latitude $2^0$55' East of the equator. Five samples of *C. gariepinus* were used for the study. Samples for microbiological analysis were taken from the gill surfaces, guts and skin surfaces of the fish samples, by swabbing with the aid of sterile swab sticks. The swab sticks were transported to the laboratory on ice pack for microbial analysis. The analysis was carried out within 24 hours of sample collection.
Water Test and Morphometric of Fish Samples
The standard length; total length; dorsal length; head length in centimeter (cm) were measured and recorded after weighing the fish samples in grams (g). The water quality was tested on temperature, conductivity, and pH from all the fish farms visited using Hannah multipurpose

meter and dissolved oxygen meter at the shore and off-shore simultaneously

Microbiological Analysis

The microbial analysis was carried out by streaking each swab stick on the sterile and solidified nutrient agar (Oxoid) and MacConkey agar (Oxoid) plates. The plates were incubated at 37ºC for 48h. Pure cultures of bacterial isolates were obtained by series of sub-culturing on the nutrient agar and MacConkey agar plates. Bacterial isolates were then characterized on the basis of their cultural, morphological and genotypic characteristics.

Extraction of DNA and Amplification of 16S rRNA genes

The genomic DNA of the bacterial isolates were extracted using Bacterial Genomic DNA extraction kit (Norgen Biotek Corporation, Canada), following the manufacturer's specification. Each bacterial isolate was grown in nutrient broth at 37ºC for 24 hours. The concentration and purity of DNA extracted from each isolate were determined using NanoDrop Lite Spectrophotometer. The 16S rRNA genes of the isolated bacteria were amplified by polymerase chain reaction using a pair of 16S rRNA universal primers designated as 27F (5'- AGA GTT TGA TCC TGG CTC AG-3') for forward and 1492R (ACG GCT ACC TTG TTA CGA CTT-3') for reverse (Jiang *et al*., 2006; Haziyamin *et al*., 2012). The primers were synthesized by Integrated DNA Technologies (IDT) Inc, USA. Each of the polymerase chain reactions was performed in a 10.0µl reaction volume containing 2.0µl of template DNA (1µg), 1.0µl of 10X buffer, 0.4µl of 50mM $MgCl_2$, 0.5µl of 2.5mMdNTPs, 0.5µl 5mM Forward primer, 0.5µl of 5mM Reverse primer, 0.05µl of 5units/ul Taq and 5.05µl of nuclease-free water. PCR reactions were carried out in a Thermocycler with denaturation temperature of 94ºC for 3mins, followed by 30 cycles each consisting of denaturation temperature at 94ºC for 60sec, annealing temperature at 56ºC for 60sec and extension temperature at 72ºC for 120sec. Reactions were terminated at final extension of 72ºC for 5mins. The amplified polymerase chain reaction (PCR) products were analysed in a 1.0% (w/v) agarose gel electrophoresis in 1x TAE buffer at 100V for 1 hour. The amplicons were further purified before the sequencing using 2M Sodium Acetate washing techniques. The pellets were re-suspended in 5.0µl sterile distilled water.

Sequencing of the 16S rRNA genes and blasting

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 96^0C for 1min followed by 25 cycles of Rapid thermal ramp to 96^0C for 10 seconds Rapid thermal ramp to 50^0C for 5 seconds and Rapid thermal ramp to 60^0C for 4 minutes, then followed by Rapid thermal ramp to 4^0C and hold forever. The PCR sequence product was 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 AB1 3130xl. Sequence assembly and alignment were carried out, followed by searching the homology in the Genebank DNA database at National Centre for Biotechnology Information (NCBI) using BLASTn search tool to identify the bacterial isolates.

Antibiotic susceptibility test

Antibiotic susceptibility of the bacterial isolates was carried out by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (Bauer *et al.,* 1996). The antibiotics used were nitrofurantion, cipropfloxacin, ceftazidime, cefuroxime, gentamicin, cefixime, ofloxacin and augmentin. Briefly, an overnight culture of each isolate grown in nutrient broth at 37ºC was standardized to a turbidity equivalent to 0.5 McFarland standard (1.5×10^8 cfu/ml) with sterile distilled water. 100µl was spread on Mueller-Hinton agar plates using swabs. Antibiotic-impregnated discs were placed on seeded plates and the plates were incubated at 37ºC for 24 hours. All the assays were carried out in triplicates. Zones of inhibition were measured after 24h of incubation. The strains were classified as 'resistant (R)', 'intermediate sensitive (I)' or 'sensitive (S)' using standard recommendations of Clinical and Laboratory Standards Institute (CLSI, 2006).

RESULTS

Water Test and Morphometric of Fish Samples

The water quality parameters tested for included temperature, conductivity, pH and dissolved oxygen; all of which were taken in-situ at the Yewa-Mata station in river as shown in Table 1 indicates readings to be 29.3^0C, 33.0µs/cm, 6.9 and 3.15ppm for temperature, conductivity, pH and dissolved oxygen respectively.

The mean value of the morphometric characteristics of *Clarias gariepinus* found in Yewa-Mata River as shown in Table 2 indicates that fish samples had a weight, standard length, total length and head length of 451 ±8.4g, 24.93 ±2.2cm, 28.54 ±3.2cm and 5.04 ±0.5cm respectively.

Genotypic characterization of the bacterial isolates

The PCR products obtained following amplification with the 16S rRNA sequences were estimated to be 1,600bp in size (Plate 1). The results of the sequence similarity (%) by BLASTN in GenBank of the National Center for Biotechnology Information (NCBI) library are shown in Table 3.

The quantitative composition of bacteria from various tissues revealed that *Comamonas* species had the highest occurrence in the gill, skin and gut of *Clarias gariepinus* followed by *Proteus* species occurring only in the gill and skin. The percentage of various bacterial species isolated from *Clarias gariepinus* as shown in Figure 2 with *Comamonas* species having the highest percentage of 25% followed by *Proteus* species having 15% while other bacteria strains had a total of 5% each

Antibiotic Sensitivity Pattern of Bacteria Strains

The susceptibility of different strains varied with the type of antibiotic used as shown in Table 4, with most bacteria being resistant to ceftazidime and cafuroxinme while Nitrofurantoin being the most sensitive antibiotic drug. Figure 3 shows the antibiotic resistant pattern of bacteria strained expressed in percentage.

TABLE 1: Water quality parameters of Yewa-Mata River

Water quality parameters	Readings
Temperature (^{0}C)	29.3
Conductivity (µs/cm)	33.0
pH	6.9
Dissolved Oxygen (ppm)	3.15

In-situ

TABLE 2: Mean value of morphometric features of *C. gariepinus* in Yewa-Mata river

Morphometric features	Mean value±SE
Weight (g)	451±8.4
Total length (cm)	28.54±3.2
Standard length (cm)	24.93±2.2
Head length (cm)	5.04±0.53

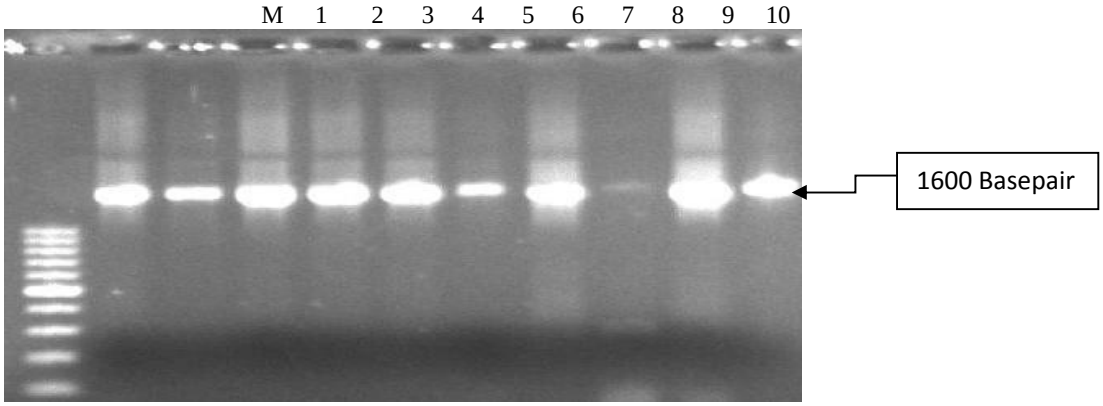

PLATE 1: Gel electrophoresis for the 16S primer
M: Molecular ladder; 1-20-PCR product of bacterial isolates

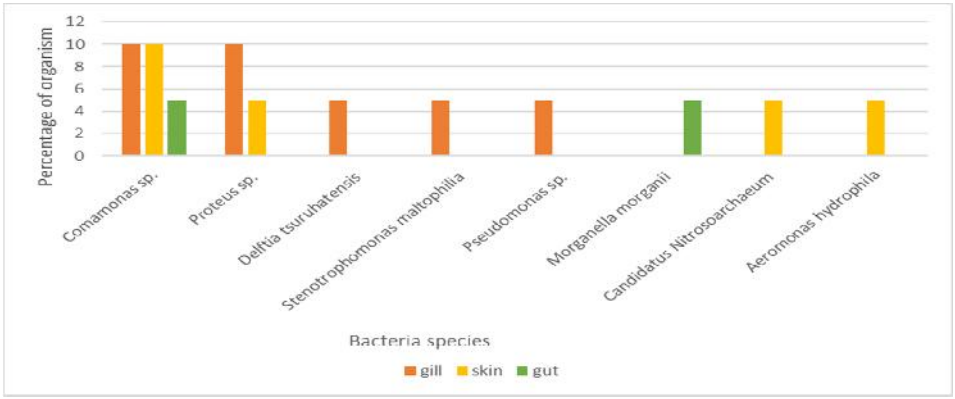

FIGURE 1: Qualitative composition of bacteria from various tissues of the *Clarias gariepinus*

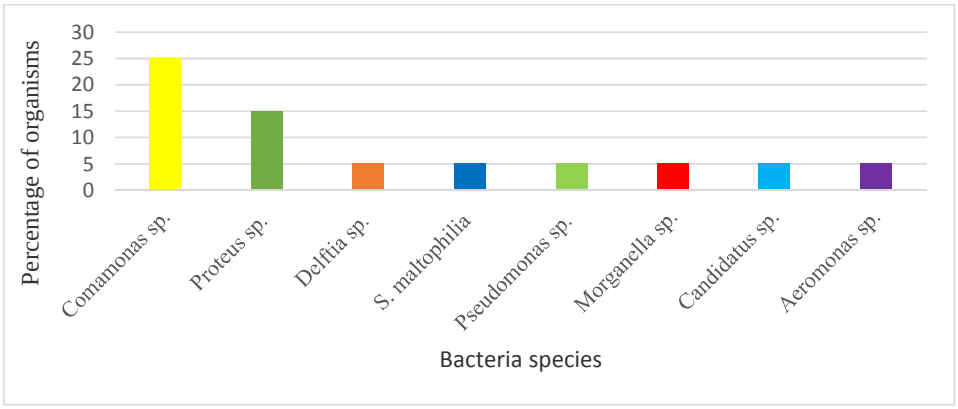

FIGURE 2: Various bacterial species isolated from *Clarias gariepinus*

TABLE 3: Molecular identity of selected bacterial isolated from *Clarias gariepinus*

Isolate code	Substrate	Genotypic identification	(%)Similarity
A1	Gill	*Comamonas testosteroni*	89%
A3		*Proteus hauseri*	91%
A4		*Comamonas testosteroni*	87%
A5		*Delftia tsuruhatensis*	88%
A8		*Stenotrophomonas maltophilia*	82%
A9		*Proteus penneri*	89%
A12		*Pseudomonas sp.*	85%
A17	Gut	*Comamonas testosteroni*	88%
A19		*Morganella morganii*	86%
A20		*Comamonas testosteroni*	87%
A15	Skin	*Proteus penneri*	97%
A22		*Comamonas testosteroni*	81%
A23		*Candidatus Nitrosoarchaeum* limnia	86%
A24		*Aeromonas hydrophila* strain N2	89%

TABLE 4: Antimicrobial susceptibility pattern of bacterial isolates stained from fish samples

	ANTIBIOTIC DRUGS							
Bacteria strains	NIT 300µg	CPR 5µg	CAZ 30µg	CRX 30µg	GEN 10µg	CXM 5µg	OFL 5µg	AUG 30µg
Comamonas testosteroni	S	S	0	0	0	0	S	S
Proteus hauseri	S	16.00	0	0	0	0	0	0
Comamonas testosteronii	0	S	0	0	0	0	S	0
Delftia tsuruhatensis	0	0	0	0	0	0	0	S
Stenotrophomonas maltophilia	S	S	0	0	S	0	S	0
Proteus penneri	15.00	0	0	0	0	0	0	0
Pseudomonas sp.	S	16.00	0	0	0	0	0	0
Comamonas testosteroni	S	16.00	0	0	15.00	0	0	S
Morganella morganii	0	0	0	0	0	0	S	0
Comamonas testosteroni	S	0	0	0	0	0	0	0
Proteus penneri	S	S	0	0	0	0	0	0
Comamonas testosteroni	S	0	0	0	0	0	S	0
Candidatus Nitrosoarchaeum limnia	15.00	0	0	0	16.00	0	0	0
Aeromonas hydrophila	0	15.00	0	0	0	0	S	0

S- sensitive, NIT-nitrofurantion, CPR-ciprpfloxacin, CAZ-ceftazidime, CRX-cefuroxime, GEN-gentamicin, CXM-cefixime, OFL-ofloxacin, AUG-augmentin; Resistant 0-10; Intermediate 11-16; Sensitive 17 & above; Diameter in mm.

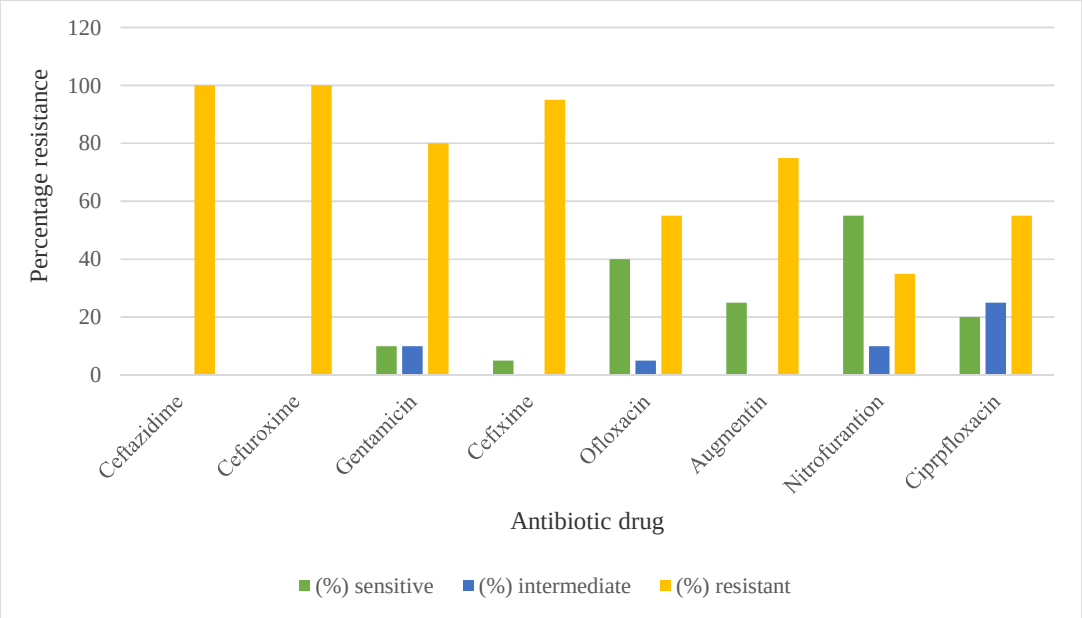

FIGURE 3: Antibiotic resistance pattern of the bacteria strains (in %).

DISCUSSION

The fish microflora contains substantial and complex collection of microorganisms forming a biologically pivotal component of the host body. This microflora is composed of different species of microorganisms which interact with each other. This microflora exerts properties which are potentially damaging or health promoting for the host. The results of this present study revealed that various bacteria are present on the skin, gill and gut of *Clarias gariepinus.* The bacteria isolated from this fish included *Comamonas sp.* (25%), *Proteus sp* (15%), *Delftia tsuruhatensis* (5%), *Stenotrophomonas maltophilia* (5%), *Pseudomonas sp.* (5%), *Morganella morganii* (5%), *Candidatus nitrosoarchaeum* (5%), *Aeromonas hydrophila* (5%). This result is not in line with Akinyemi and Oyelakin (2014) who conducted similar research on the isolation of bacteria isolates from farm-raised catfish *C. gariepinus.*

Comamonas strains was found to have the highest frequency found in the gill, gut and skin of fish with a total of 25% followed by *Proteus* strains which occurred only in the gill and skin with a total of 15% while other strains of bacteria occurred uniformly with a total of 5% for each strain. These bacterial strains found is in line with Cipriano and Dove (2011) who discovered about a dozen bacterial species within the genera *Comomonas, Pseudomonas, Alcaligenes, Moraxella, Acinetobacter*, formed a rich microbial flora on the skin and mucus of healthy fish prior to detection of *A. salmonicida*. The presence of these bacteria could be due to contamination of the water body as previously reported by Adebayo-Tayo *et al*. (2008) and Junaid *et al*. (2010).

According to Cipriano and Dove (2011), human infections caused by pathogen transmitted from fish or the aquatic environment are quite common depending on the season, patients contact with fish and related environment, dietary habits and the immune system status of the exposed individual, there are often bacterial species facultatively pathogenic for both fish and man and may be isolated from fish without apparent symptoms of disease; Aeromonads septicaemia most often caused by *A. hydrophila*, was described as a complication in patients with liver cirrhosis and eye infection in immuno-compromised patients and fatal bacterial pneumonia (Qu *et al*., 2003). The level of resistance and sensitivity of these bacteria to clinically relevant antibiotics-Ceftazidime, Cefuroxime, Gentamicin, Cefixime, Ofloxacin, Augmentin, Nitrofurantion and Ciprpfloxacin differs; all bacteria strains were resistant to Ceftazidime and Cefuroxime. However, Nitrofurantion and Ofloxacin were found to be more effective as most of the bacterial strains were susceptible to these antibiotics. This result is in agreement with findings reported by Akinyemi (2011) who reported that Gentamycin is not absorbed in the gut of the fish, as only application by injection method may be more efficacious, but this option may be laborious in fish husbandry. The antimicrobial resistance in bacterial pathogens is a major impediment to successful therapy, and in several instances, bacterial strains have arisen that are resistant to most available antimicrobial treatments. The public health consequences of antimicrobial resistance to many antibiotics have been debated. However until recently, clear evidence of health risk was not available. The multiple nature of drug resistance of these bacteria creates an extremely serious public health problem and it has always been associated with the outbreak of major epidemic throughout the world (Prescott *et al.*, 2002).

CONCLUSION

The present study shows that diverse bacterial species exist on the skin, gill and gut of *C. gariepinus.* The results also prove that antibiotics are significant chemotherapeutic agents in destroying bacteria present in fish and probably also important in regulating the composition of bacterial communities in fishes. The zone of inhibition revealed the potency of the antibiotics in combating or killing the bacteria. The present investigation disagrees with prophylactic uses of antibiotic because of the development of resistant bacteria; therefore it would be impossible to treat diseases of aquatic organism caused by resistant bacteria and also human diseases with antibiotic-resistance bacteria of animal origin. Hence, the present study implored use of antibiotic to treat diseases, but not as prophylactic use but as a therapeutic tool for curing diseases.

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