

Molecular Characterization and Antibiotic Resistance Profile of Bacteria Associated with *Clarias gariepinus* (Burchell, 1822) From Ebute Iboro Location on Yewa River

ABSTRACT

Bacteria isolates associated with *Clarias gariepinus* were collected from fish sample gotten on Yewa River in Ebute Igbo-Aro. The isolates were assessed using 16S rRNA gene sequencing method to identify the strains and to construct the phylogenetic relationship. The morphometric characteristic such as weight, standard length, total length and head length of fish samples were measured. Bacteria were isolated from the gills, skin and gut of *Clarias gariepinus* using spread plate method. The DNA of the bacteria 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 using ethanol precipitation, thereafter sequenced using an automated DNA sequencer. The homologous of nucleotides was then searched at the gene bank of NCBI and the isolates were identified. The antibiotics sensitivity profile of the bacterial isolates was determined using disc diffusion method. There are three main types of bacteria found. *Pseudomonas spp*, *Serratia spp* and *Aeromonas spp*, were found dominantly on the skin. The sensitivity test showed that the identified bacteria isolates were sensitive to Ofloxacin (5mg), Nitrofurantoin (300mg) and Ciprofloxacin (5mg) which can be used to treat the bacteria isolate to reduce its pathogenic impact on human and to reduce disease outbreak when cultured. The bacteria isolates were 100% resistant to Cefixime (5mg) and Cefuroxime (30mg).

Keywords: Bacterial isolates, *C. gariepinus*, Aquaculture , Antibiotics and Sensitivity.

INTRODUCTION

Fish has been one of the main foods for human for many centuries and still constitute an important part of the diet in many countries [1]. The advantages of fish as food are its easy digestibility and high nutritional value.[1] Fish are an important resource for human worldwide, especially in the rural area[2]. Commercial and subsistence fishers hunt fish in wild fisheries or farm them in ponds or cages in the ocean (aquaculture).

Fish are susceptible to a wide variety of bacterial pathogens. Many of these bacterial capable of causing diseases are considered by some to be saprophytic in nature. These bacteria only become pathogens when fishes are physiologically unbalanced, nutritionally deficient, or there are other stressors, i.e., poor water quality, overstocking, which allow opportunistic bacterial infections to proceed. Disease breaks out in fish tank very quickly and you have to first identify the type of disease before you can take action. The bacteria are transmitted by fish that have made contact with other diseased fish. Bacterial fish disease and infections are very common and are one of the most difficult health problems to deal with [3] Bacteria can enter the fish body through the gills, skin or it can stay on the surface of the body [3] There are four types of bacterial infections. Bacterial gill disease: the gills are the primary target, systemic bacterial disease: bacteria invades

the fish's body and damages internal organs, bacterial body ulcers: lesions on the fish body that can be shallow or deep and fin rot: most likely resulting from environmental stress. [3].

Bacterial disease is one of the most important diseases in captive fishes and a significant cause of high fish morbidity and mortality rate. Different pathogens, especially, gram negative bacteria have been reported to be isolated from naturally-infected fish. Many stress factors could contribute to bacterial infection in captive condition namely, poor water quality, overcrowding, transportation and inadequate nutrition. *Aeromonas hydrophila* is also considered a normal flora as well as a primary and secondary fish pathogen, including ornamental fish. Fish disease has caused substantial economic losses to fish farmers and the fisheries sector. Antibiotics have been used to prevent and treat the bacteria causing disease, but the success of treatment depends on antibiotic susceptibility of etiologic bacteria. Bacteria resistance is an important issue that needs to be considered when choosing an appropriate antibiotic.

Therefore, the objective of this research work is to identify the bacteria isolates using 16S rRNA gene sequencing method and to determine the antibiotics susceptibility profile of the bacteria isolated from gills, guts and skin of *Clarias gariepinus* at Ebute Iboro station on Yewa River.

MATERIALS AND METHODS

Collection of the samples

The study was carried out at Ebute Iboro Yewa River which is a trans-boundary river between Republic of Benin and Nigeria. It lies approximately within latitude 6° 22'N and longitude 2° 50'E of the Greenwich meridian. Twenty samples of *C. gariepinus* were collected at Ebute-Iboro landing site at Yewa River. Samples were collected for microbiological analysis by swabbing the gills, skin and guts of *Clarias gariepinus* with the swab sticks. All swab sticks were streaked on both Nutrient agar and Mac Conkey agar by BioMark Laboratory, the samples were later incubated for 24 hours at 37°C.

Water Test and Morphometric Characteristic of fish samples

The physico-chemical parameters such as temperature (°C), conductivity (µs/cm) and pH of the water body were taken in-situ using Hanna multipurpose meter and recorded. Morphometric feature of fish sample such as weight, standard length and total length was determined.

Isolation of bacteria and Morphological Characterization

The media used in the experiment were weighed out according to the manufacturer's specification; these include Nutrient Agar and MacConkey Agar. The media were properly weighed and dissolved in conical flasks containing distilled water and boiled gently to dissolve the agar completely.. This was sterilized in an autoclave at 121°C for 15 minutes. The media were allowed to cool to 45°C before pouring aseptically into sterile plastic petri dishes for solidification and finally dry in an incubator. Characteristic colonies from original culture on Nutrient agar and Mac Conkey agar plates were picked using sterile Aluminum wire loop and streaked the isolate onto sterile Nutrient and Mac Conkey Agar plates. These plates were incubated at 37°C overnight for pure microbial growth. These isolates were placed to their appropriate agar slant labeled and incubated at 37°C for 24 hours for growth after which they were kept in the refrigerator at 4°C for identification.

Identification of the Bacteria Isolates by 16S rRNA Gene Amplification

Bacteria isolate grown overnight was transferred to eppendorf tube and it was spun down at 14,000 rpm for 2 mins, the supernatant was discarded and the DNA was extracted using CTAB method [8]. The DNA was later resuspended in 100 µl of sterile distilled water. DNA concentration of the samples were measured and the genomic purity were determined. The DNA was further checked on 1.0 % agarose gel and was visualised on UV light source. PCR analysis was done using MJ Research Thermal Cycler (PTC-200 model). The primers used for PCR amplification were 16S universal primers for bacteria. The sequence for the forward primer was 5'AGAGTTTGATCCTGGCTCAG3' and reverse primer was 5'ACGGCTACCTTGTACGACTT3' The PCR mix comprises of 1 µl of 10X buffer, 0.4 µl of 50 mM MgCl₂, 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/ul Taq with 2 µl of template DNA and 5.05 µl of distilled water and the PCR profile used has an 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 amplicon was further purified before the sequencing using 2M Sodium Acetate washing techniques. The pellet was resuspend in 5 µl sterile distilled water. The PCR mix used includes 0.5µl of BigDye Terminator Mix, 1µl of 5X sequencing buffer, 1 µl of 16S Forward primer with 6.5 µl Distilled water and 1 µl 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 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 product was purified before the sequencing using 2M Sodium Acetate washing techniques. The pellet was re-suspended in 5 µl sterile distilled water. The combination of 9 µl of Hi Di Formamide with 1 µl of Purified sequence making a total of 10 µl was prepared and loaded on Applied Biosystem (AB1 3130xl model) .

Antibiotics and Sensitivity

15.2 g of Mueller Hinton Agar was dissolved in 400 ml of distilled water and mixed, then autoclaved at 121 °C for 15 minutes. It was later brought out and allowed to cool then it was poured on the petri-dish and allowed to solidify. The plates were later labelled and the slant isolates were introduced into the prepared Mueller hinton agar for culture in which Smearing was done to widen the occurrence of the microorganism. The antibiotics known as motor disc antibiotics were placed on the smeared media, in order to view or observe how the organisms are been sensitive or resistance to the organism using 20 samples. It was incubated at 37°C for 18-24 hours at the end of the incubation they 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 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.

RESULTS AND DISCUSSION

Morphometric Characteristics of *Clarias gariepinus*

Morphometric Characteristics of *Clarias gariepinus* from Ebute Iboro Location on Yewa River as in Table 2, shows that fish samples had mean value in Weight, Standard Length, Total Length and Head Length of 645g, 42.6±0.2cm, 51.6±0.2cm and 10.6±0.1cm respectively. Table 1 shows that water parameters taken has follows; Temperature, Dissolved Oxygen and pH readings of 28.6°C, 5.5ppm and 8.3 respectively.

Molecular Characterization by Gene Sequencing

The size of the amplified band with 16S universal primer was 1.6Kb for the 20 samples Plate 1, this is similar to Akinyemi and Oyelakin 2014. The blasting of the sequence for the isolates shows that there are ten types of bacteria species present. The bacteria are four *Citrobacter spp*, two *Pseudomonas species*, two *Aeromonas spp*, two *Serratia spp*, and one *Escherichia coli*, *Shigella boydii*, *Pantoea anthophila*, *Bacillus spp*, *Morganella morganii*, *Cronobacter sakakii* each.

Table 4. The four isolates had between 89%-94% homology identity with *Citrobacter freundii*, *Pseudomonas spp* (92%-94% homology), *Serratia spp* 87%-90% homology whereas others had 87% 94% homology. The result of the study for the ten strains showed *Pseudomonas fluorescens*, *lysiniabacillus fusiformis*, *Pseudomonas plecoglossicid* and, *Pseudomonas nitroreducens* to be closely related forming one sub-group. The isolates in the second sub-group are *Pseudomonas fluorescens*, *Pseudomonas nitroreducens* and *Pediococcus acidilactici* while *Pseudomonas putida* and two *Pseudomonas fluorescens species* stand alone.

The characteristics of the 20 isolates of bacteria selected were examined for studies on the basis of their sensitivity or resistivity, to the antibiotic used, the characteristic and taxonomic identification of the 20 bacterial isolates, the PCR sequencing the PCR has 1600bs (size) but after it was observed that 16 bacteria isolate were identified taxonomically the bacteria present in **C1** (*Aeromonas hydrophila*) from the gill, **C2** (*Pantoea anthophila*) from the skin, **C3** (*Cronobacter sakazakii*) from the gill, **C4** *Serratia marcescens* from the gill, **C5** (*Shigella boydii*) from the gut, **C6** (*Pseudomonas sp*) from the skin, **C7** (*Citrobacter freundii*) from the skin, **C8** (*Citrobacter freundii*) skin, **C9** (*Pseudomonas gessardii*) gill, **C10** (*Citrobacter freundii*) skin, **C11** *Serratia liquefaciens* gut, **C12** (*Citrobacter freundii*) skin, **C13** (*Escherichia coli*) skin, **C14** (*Aeromonas hydrophila*) skin, **C15** (*Bacillus sp*) gut, **C16** (*Morganella morganii*) from the gills as showed in table 3.

Antibiotics Sensitivity Pattern of the Bacterial Isolates Strained from *Clarias gariepinus*

The antibiotic sensitivity test was carried out to know how susceptible or resistible the identified bacteria are to the motor disc which had 8 classes of antibiotics around it, using disc diffusion method. The sensitivity test shows that the identified bacterial isolates were 100% resistant to Cefixime (CXM) and Cefuroxime (CRX) followed by Ceftazidime (CAZ) which is 90% resistance. As for the sensitivity test, bacterial isolate were 30% sensitive to the Ofloxacin (OFL), Ciprofloxacin (CPR) and Nitrofurantoin (NIT), followed by Gentamicin (GEN) which is 25%. The zone of inhibition was measured with the use of a transparent ruler, when there is a round clearing of the bacteria around it, it means the organisms is sensitive to it. But when the organisms are growing on it, it means the organisms resistant to it. When they are no round clearing around it is represented as 0 (mm) which is showed in table 5.

Table 1. Water Quality Parameters

Water Parameters	Readings
pH	8.1
Temperature	29.8°
Dissolved Oxygen	5.35ppm

Table 2. Morphometric Characteristics of Ten *C. gariepinus* in Ebute-Iboro

Sample	Weight (g)	Total length (cm)	Standard length (cm)	Head length (cm)
C1	253	40.0	35.5	9.0
C2	364	47.0	42.3	11.0
C3	939	46.0	40.0	10.5
C4	911	48.0	41.0	10.2
C5	965	48.0	41.2	10.5
C6	806	46.5	41.0	10.0
C7	926	49.0	43.0	10.5
C8	871	48.0	42.5	11.0
C9	887	52.0	46.0	11.0
C10	934	45.5	40.0	11.0

Table 3. Occurrence Rate of Bacteria Isolate obtained from the Fish Samples.

Name of organism	No of occurrence	% Occurrence
<i>Citrobacter spp</i>	4	25.00
<i>Eshericha coli</i>	1	6.25
<i>Pseudomonas spp</i>	2	12.50
<i>Shigella boydii</i>	1	6.25
<i>Aeromonas spp</i>	2	12.50
<i>Pantoea anthophila</i>	1	6.25
<i>Cronobacter sakaakii</i>	1	6.25
<i>Serratia spp</i>	2	12.50
<i>Bacillus spp</i>	1	6.25
<i>Morganella morgani</i>	1	6.25

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Table 4. Nearest relatives of various selected bacterial isolates

Isolates	Source		
	Nearest Relatives		% Homologous
C1	Gill	<i>Aeromonas hydrophila</i>	96
C2	Skin	<i>Pantoea anthophila</i>	94
C3	Skin	<i>Cronobacter sakazaki</i>	89
C4	Gill	<i>Serratia marcescens</i>	87
C5	Gut	<i>Shigella boydii</i>	87
C6	Skin	<i>Pseudomonas sp</i>	92
C7	Skin	<i>Citrobacter freundii</i>	90
C8	Skin	<i>Citrobacter freundii</i>	94
C9	Gill	<i>Pseudomonas gessardii</i>	94
C10	Skin	<i>Citrobacter freundii</i>	89
C11	Gut	<i>Serratia ligufatiens</i>	90
C12	Skin	<i>Citrobacter freundii</i>	90
C13	Skin	<i>Esherichia coli</i>	94
C14	Skin	<i>Citrobacter freundii</i>	93
C15	Gut	<i>Bacillus spp</i>	90
C16	Gill	<i>Morganella morganii</i>	90

210 **Table 5. Zone of inhibition of the bacterial isolates strains from *Clarias gariepinus***

bacterial isolates	OFL	AUG	NIT	CPR	CAZ	CRX	GEN	CXM
	30%		30%	30%		100%	5(25%	100%
C1	S	R	R	S	I	R	R	R
C2	S	R	I	S	R	R	S	R
C3	R	R	R	R	R	R	R	R
C4	S	R	S	S	R	R	S	R
C5	I	R	S	I	R	R	R	R
C6	R	R	R	R	R	R	R	R
C7	S	R	S	S	R	R	S	R
C8	R	R	R	R	R	R	R	R
C9	R	R	R	R	R	R	R	R
C10	R	R	R	R	R	R	R	R
C11	R	R	R	R	R	R	R	R
C12	R	R	R	R	R	R	R	R
C13	R	R	R	R	R	R	R	R
C14	R	R	R	R	R	R	R	R
C15	R	R	R	R	R	R	R	R
C16	R	R	S	R	R	R	R	R
C17	R	R	I	S	R	R	I	R
C18	S	R	R	R	R	R	R	R
C19	S	S	S	S	S	R	S	R
C20	R	R	S	I	R	R	S	R

211 S = Sensitivity 16 and above, I= intermediate 9-15, R= Resistance 0-8

212 Legend

213 C1- C16 are the identified bacterial strains

214 C17- C20 are unidentified strains.

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216 The result after BLAST on NCBI revealed that the selected isolate of bacteria have their homology (%),C1
217 from the gill as *Aeromonas hydrophilla* (KJ806459.1) with 96%, C2 from the skin *Pantoea anthphila*
218 (JX993784.1) with 94%, C3 from the skin *Cronobacter sakazakii*(HQ880374.1)89%. C4 from the gill *Serratia*

219 *marcescens* (JQ315926.1) 87%. C5 from the gut *Shigella boydii* (JQ315926.1) 87%. C6 from the skin
220 *Pseudomonas* sp(KJ534480.1) 82%. C7 from the skin *Citrobacter freundii* (DQ294287.1) 90%. C8 from the
221 skin *Citrobacter freundii*(AB680434.1) 94%. C9 from the gill *Pseudomonas gessardii* (KJ726608.1) 94%. C10
222 from the skin *Citrobacter freundii* (JQ7958145.1) 89%
223 C11 from the gut *Serratia liquefaciense* (Gu586145.1) 80%. C12 from the skin *Citrobacter freundii*
224 (KC2108467.1) 80%. C13 from the skin *Escherichia coli* (KC165787.1) 94%. C14 from the skin *Aeromonas*
225 *hydrophila* (AB473035.1). C15 from the gut *Bacillus* sp (AY626828.1) 80%. C16 from the gill *Morganella*
226 *morganii* (JX875917.1) 82%
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NOTE: A motor disc antibiotic has 8 different classes of antibiotics incorporated with it. Listed below are the antibiotics:
Ceftazidime(CAZ)30mg,Cefuroxime(CRX)30mg,Gentamicin(GEN)10mg,Cefixime(CXM)5mg,Ofloxacin(OFL)5mg,Augumentin(AUG)30mg,Nitrofurantoin(NIT)300mg,Ciprofloxacin(CPR)mg

232 **Table 6. Anti microbial percentage sensitivity and resistance of the isolate to antibiotics**

233		Sensitivity		Intermediate		Resistance	
234		N	%	N	%	N	%
235	OFL	6	30.0	1	5.0	13	65.0
236	AUG	1	5.0	0	0.0	19	95.0
237	NIT	6	30.0	2	10.0	12	60.0
238	CPR	6	30.0	2	10.0	12	60.0
239	CAZ	1	5.0	1	5.0	18	90.0
240	CRX	0	0.0	0	0.00	20	100.0
241	GEN	5	25.0	1	5.0	14	70.0
242	CXM	0	0.00	0	0.00	20	100.0

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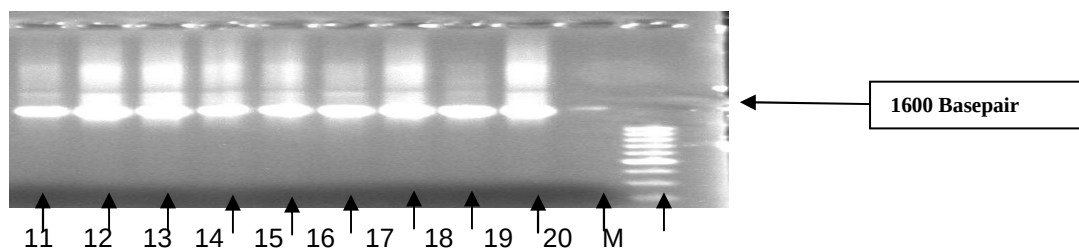
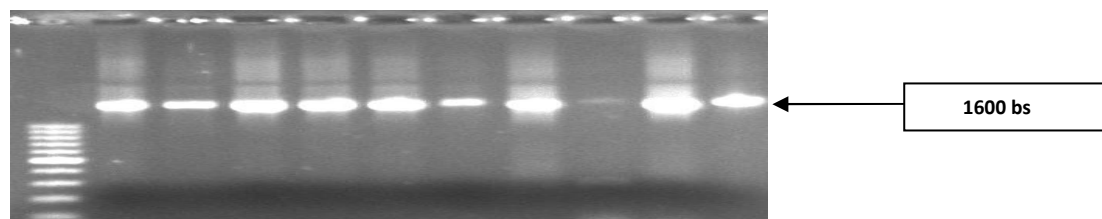


Plate 1. Gel electrophoresis for the 16S primer

Discussion

The various water quality parameters recorded at the river in which it is favorable for fish growth and culture except the pH which is read 8.1 which is little bit high and still favourable for fish culture and growth and the parameters fell within the standard range that has already been documented. The temperature of 29.8°C was recorded and with this range it was supported and recorded by (5), (6) and (7) for the range of temperature.

The pH value which is recorded in this is high due to the location that is near to brackish water but the fish were able to survive and breed in this environment in which there would have been mixture during the process, pH value of 6.5 to 9.0 is documented and agreed upon by (8) and (9) as value most suitable for fish production and maximum growth.

Table 2 shows the morphometric characteristics of the 10 *Clarias gariepinus* gotten from Ebute Igbo-oro River, the total length falls within the range of 40-52cm and the standard length falls within the range of 35.5-46cm while the head length falls within the range of 9.0-11.00. The weight in grams ranged from 252-965. Due to the bacterial infections the head length varied in the smaller fish compared to the bigger ones.

The bacteria isolate (16 isolates) *Citrobacter freundii* (4 isolate), *Pseudomonas* sp (2 isolate), *Aeromonas hydrophila* (2 isolate), *Serratia* sp (2 isolate) while the rest *Pantoeanthopilla*, *Cronobacter sakazakii*, *Sigella boydii*, *Escherichia coli*, *Bacillus* sp, and *Morganella morganii* had single occurrence. This indicates that bacterial belonging to *Citrobacter* and *Pseudomonas* group were more prevalent in healthy *C. gariepinus* of this water body. This finding is in line with the study of (10) (11) and (12) where several bacterial were identified and considered as bacillus, *Pseudomonas*, *Morganella morganii*, and *Citrobacter freundii* was dominant.

The finding is corroborated by the work of (13) where the 17 bacteria selected belong to *Bacillus* spp and their homology(%) ranges from 96-99% with their various accession numbers

In the study, it was observed that ofloxacin(5mg), Nitrofurantion (300mg) ciprofloxacin(5mg) are antibiotics to which the bacterial isolates present on the skin of with *Clarias gariepinus* are highly sensitive and can be used to combart or treat them in the event of an infection.

The different bacterial isolates showed varying degree of resistance to the different antibiotics. Thirty percent (30 %) of the bacterial isolates namely C1, C2, C4, C7, C18 and C19 were sensitive to Ofloxacin (OFX). C4, C5, C7, C16, C19 and C20, representing 30 % of the bacterial isolates were similarly sensitive to Nitrofurantoin (NIT). All the isolates were sensitive to Cefuroxime (CRX) and Cefixime (CXM).

CONCLUSION

This work provides information on the BLASTING of gene sequence of the bacterial isolates from the gill, gut and skin of samples swabbed from *Clarias gariepinus* in Ebute-Igbo-oro landing site of Yewa river. This study confirms the existence of different species of bacteria identified from the skin, gills and gut of sixteen (16) bacterial isolates. After BLASTING to the species level using genetic method to identify them, the finding

confirm that C1 from the gill has *Aeromonas hydrophilla*, C2 from the skin *Pantoea anthphila*, C3 from the skin *Cronobacter sakazakii*, C4 from the gill *Serratia marcescens*, C5 from the gut *Shigella boydii*, C6 from the skin *Pseudomonas sp*, C7 from the skin *Citrobacter freundii*, C8 from the skin *Citrobacter freundii*, C9 from the gill *Pseudomonas gessardii*, C10 from the skin *Citrobacter freundii*, C11 from the gut *serratia liquefaciense*, C12 from the skin *Citrobacter freundii*, C13 from the skin *Eshericha coli*, C14 from the skin *Aeromona hydrophila*, C15 from the gut *Bacillus sp*, C16 from the gill *Morganella morganii* only were swabbed from samples of *Clarias gariepinus* at Ebute-igbo-oro landing site of Yewa river

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