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Molecular Characterization of Bacteria Isolates from Farm-Raised Catfish *Clarias gariepinus* (Burchell, 1822)

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Selected bacterial isolates from skin, gut and gills of Clarias gariepinus were collected from five fish farms at Ijebu Ode. The isolates were assessed using 16S rRNA gene sequencing method to identify them and to construct the phylogenetic relationship. A total of 10 isolates were selected, their colonial morphology determined, thereafter the DNA of the isolates were prepared 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. These sequence data were compared with other gene sequences in GenBank database (NCBI) using a BLAST search to find closely related sequences. 80% of the isolates belonged to different species of Pseudomonas, sharing 92% to 96% 16S ribosomal RNA identity with the respective type-strain, whereas the remaining 2 isolates belonged to Pediococcus acidilactici and Lysinibacillus fusiformis with 96% 16S ribosomal RNA homology.

Keywords: Bacterial isolates; C. gariepinus; DNA extraction; Sequencing; clustering analysis.

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1. INTRODUCTION

Nigeria has enormous inland water mass comprising floodplains, rivers, reservoirs, lakes, pools and ponds, where plenty of fish are available [1]. Though pond offers the cultivation of fish under a controlled condition, it however harbours great number of microorganism which includes probiotics and pathogenic. Several works have shown the presence and occurrence of bacteria flora in pond waters. It was revealed that gram-negative rod shaped bacteria inhabited a cultured population (pond system) [2]. The study further stated that pond water bacteria had a reflection on the bacterial composition of the gills and intestine (gut) of catfish (*Clarias gariepinus).*

Thus there is a need to investigate the microorganism majorly bacteria if only to safeguard public health. The research work done by [3] showed that there is a connections established between fish consumption and public health in terms of communicable diseases, non-communicable diseases, and malnutrition. [4] Further argued that though presence of various organisms which are particularly pathogenic to human in fish is only suggestive; its initiation of human disease is unknown. However, the presences of potential human pathogens suggest that fish improperly handled, undercooked or consumed raw may cause disease to susceptible individual.

In a bid to investigate the microorganism composition in pond water as well as its infection, several identification and characterization could be employed [5]. [6] Stated that, “classical methods for determining viability are time consuming; hence molecular methods have been developed to address this problem”. The classical schemes allow the identification and classification of bacteria strains, but it is unclear whether the difference represents variation between distinct species or between strains of same species. This uncertainty was due to lack of distinctive structures in most bacteria as well as lateral gene transfer between unrelated species. [7] Explained that due to lateral gene transfer, some closely related bacteria can have different morphologies and metabolisms. To overcome this uncertainty, modern bacterial classification emphasizes molecular systematic, using genetics techniques such as guanine-cytosine ratio determination, genome-genome hybridization, as well as sequencing genes transfer such as the16S rRNA gene.

Therefore, the objective of this research work is to identify bacteria flora from gills, guts and skin of *C. gariepinus* through molecular systematic using 16S rRNA gene sequence 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 Ijebu-Ode, Ogun State, Nigeria. It is situated at 6.82º North latitude, and 3.92º East longitude and 68 meters elevation above sea level. Five fish farms were visited which includes; Eriwe Fish Farm Village; Orisun Iye fish Farm; Agoro Fish Farm; Rasak Farm, Ijagun; and Itarin Fish Village. Live fish samples of *Clarias gariepinus* were collected from each of the five fish farms in Ijebu-Ode. Bacteria isolate from each samples were obtained from gills surface, skin, and guts by swabbing method with the use of swab stick aseptically. 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 37ºC.

2.2 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.

2.3 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 Mac Conkey agar. The media were properly weighed and dissolved in conical flasks containing distilled water and boiled gently to dissolve the agar completely. The conical flask was plugged with cotton wool covered with aluminum foil and sterilized in an autoclave at 121ºC for 15 minutes at 1.06mm Hg. Agar media were allowed to cool to 45ºC before pouring aseptically into sterile plastic petri dishes for solidification and finally dry in an incubator. Characteristics colonies from original culture on Nutrient agar and Mac Conkey agar plates was picked by sterile aluminum wire loop and streaked to isolate on sterile Nutrient and Mac Conkey agar plates. These plates were incubated at 37ºC for 24 hours for pure microbial growth. Distinct colonies were Gram stained to know the Gram reaction and recorded. These isolates were placed to their appropriate agar slant labeled and incubated at 37ºC for 24 hours for growth after which they were kept in the refrigerator at 4ºC for identification.

2.4 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 14,000rpm for 2mins, the supernatant was discarded and the DNA was extracted using CTAB method [8]. The DNA was later resuspended in 100µl of sterile distilled water. DNA concentration of the samples were measured and the genomic purity were determined. The DNA was further check 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 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 2.5mMdNTPs, 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 an initial denaturation temperature of 94ºC for 3mins, followed by 30 cycles of 94ºC for 60 seconds, 56ºC for 60 seconds 72ºC for 120 seconds and the final extension temperature of 72ºC for 5 minutes and the 10ºC hold forever. The amplicon 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 96ºC for 1min followed by 25 cycles of Rapid thermal ramp to 96ºC for 10 seconds Rapid thermal ramp to 50ºC for 5 seconds and Rapid thermal ramp to 60ºC for 4 minutes, then followed by Rapid thermal ramp to 4ºC and hold forever. The PCR sequence product was purified before the sequencing using 2M Sodium Acetate washing techniques.The pellet was re-suspend in 5µl 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 Applied Biosystem (AB1 3130xl model) .

2.5 Statistical Analysis

Molecular identification of the bacteria strains was done by blasting the nucleotides sequences on Gene bank database and the statistical analysis to determine the phylogenetic tree was done using CLC software.

3. RESULTS AND DISCUSSIONS

3.1 Colony and Cell Morphologies of Isolates

Table 1 indicated that fish samples from Eriwe fish farm village had the highest mean value in weight, standard length, total length, head length and dorsal length of 1170g, 47.3±8.2cm, 51.7±7.3cm, 10.5±1.4cm and 26.5±3.0cm respectively. The fish samples from Rasak farm Ijagun had the lowest mean value in weight, standard length, total length, head length and dorsal length of 600g, 34.7±2.6cm, 39.3±2.4cm, 8.8±0.49cm and 20.3±2.4cm respectively. Table 2 indicated that Rasak fish farm had the highest temperature, conductivity and pH readings of 30.7ºC, 34.0µs/cm and 6.9 respectively while Orisun iye fish farm ososa had the lowest temperature, conductivity and pH readings of 29.6ºC, 32.0µs/cm and 6.5 respectively. Table 3 indicated that ten isolates of selected bacteria were examined and the colony pigmentation of the isolates were white, grey and creamy. These colonies were dominated by round shapes of different counts 2, 3, and 4; only two isolates had irregular shapes. Colony elevations were raised and flat; edges were smooth and rough, the gram staining showed that 80% were positive and 20% were negative.

3.2 PCR Analysis and DNA Sequencing

The size of the amplified band with 16S universal primer was 1.6Kb for the 10 samples Plate 1. The blasting of the sequence for the isolates shows that there are three types of bacteria species present. The bacteria are eight *Pseudomonas species,* one *Lysinibacillus fusiformis and* one *Pediococcus acidilactici* Table 4. The eight isolates had 96% homology identity with *Pseudomonas*, *Lysinibacillus* (96% homology) whereas *Pediococcus* had 91% homology. (Fig. 1) showed the clustering analysis for the ten strains, *Pseudomonas flluorescens, lysinibacillus fusiformis, Pseudomonas plecoglossicid and, Pseudomonas nitroreducens* are very closely related forming one sub-group. The isolates in the second sub-group are *Pseudomonas flluorescens, Pseudomonas nitroreducens* and *Pediococcus acidilactici* while *Pseudomonas putida* and two *Pseudomonas flluorescens species* stand alone.

Table 1. Summary of morphometric characteristics

Morphometric	A	B	C	D	E
Weight (g)	1170	730	770	600	770
Standard length	47.3±8.2	37.3±2.2	41.3±2.2	34.7±2.6	38.0±3.3
Total length (cm)	51.7±7.3	42.3±2.2	43.0±2.4	39.3±2.4	43.7±3.6
Head length (cm)	10.5±1.4	9.5±0.41	9.2±0.37	8.8 ±0.49	9.7±0.72
Dorsal length (cm)	26.5±3.0	22.7±1.9	23.3±1.9	20.3±2.4	23.3±3.0

A Eriwe fish farm village ; Mean value±SE of parameters; B Orisun iye fish farm Ososa; C Agoro fish farm; D Rasak farms ijagun; E Itanrin fish village

Isolates related to Pseudomonas were represented by *P. fluorescens* (isolates code: ES1A, CT3A, CG3A, AS1D), *P. nitroreducens* (isolates code: AS1B, BG1A), *P. plecoglossicida*

(isolate code: AS3B), *P. putida* (isolate code: AS3A) while *Lysinibacillus fusiformis* (isolate code: BT1A) and *Pediococcus acidilactici* (isolates code: AS1A) Table 4.

Table 2. Summary of water properties

Fish Farm	Temperature (ºC)	Conductivity (µs/cm)	pH
A	30.4	33.0	6.9
B	29.6	32.0	6.5
C	30.0	33.0	6.8
D	30.7	34.0	6.9
E	29.8	33.0	6.7

A Eriwe fish farm village; B Orisun iye fish farm Ososa; C Agoro fish farm; D Rasak farms ijagun; E Itanrin fish village

Table 3. Some characteristics of selected bacteria isolates

ID	Shape	Colour	Consistency	Edges	Elevation	Opacity
BT1A	2 round white	wet	smooth	raised	opaque	+
AS3A	4 round white	wet	smooth	raised	opaque	-
ES1A	3 round grey	wet	smooth	flat	opaque	-
AS3B	2 round creamy	wet	smooth	raised	opaque	-
AS1A	3 round white	dry	smooth	flat	opaque	+
AS1B	4 round white	wet	smooth	raised	opaque	-
AS1D	3 round white	wet	smooth	flat	opaque	-
CG3A	5 irregular grey	dry	rough	flat	opaque	-
CT3A	3 round white	wet	smooth	raised	opaque	-
BG1A	3 irregular white	wet	rough	flat	opaque	-

BT1B – BG1A shows the code of isolate bacteria; A – E represent fish farms; falls at beginning of code S,G, and T located in middle of code represent skin, gill, and gut respectively

Table 4. Nearest relative of various selected isolates of bacteria

Isolates	Substracts	Nearest relatives	Acession numbers	Homology (%)
ES1A	Skin	*Pseudomonas fluorescens* Strain hswX151	JQ236822.1	94
BT1A	Gut	*Lysinibacillus fusiformis* Strain 28XG99	FJ174606.1	96
AS3A	Skin	*Pseudomonas putida* Strain NBA-II GR-1	HM439963.1	96
AS3B	skin	*Pseudomonas plecoglossicida* Strain NBA-II BA-16(2)	HM439959.1	96
AS1A	Skin	*Pediococcus acidilactici* Strain DSPV 358T	FJ751795.1	91
AS1B	Skin	*Pseudomonas nitroreducens* Strain NBRC 1013160	AB681968.1	92
BG1A	Gill	*Pseudomonas nitroreducens* Strain NBRC 1013160	AB680323.1	92
CT3A	Gut	*Pseudomonas fluorescens* Strain WMQ	DQ841246.1	95
AS1D	Skin	*Pseudomonas fluorescens* Strain WMQ B	DQ841246.1	94

ES1A – AS1D shown the isolates code of selected bacteria

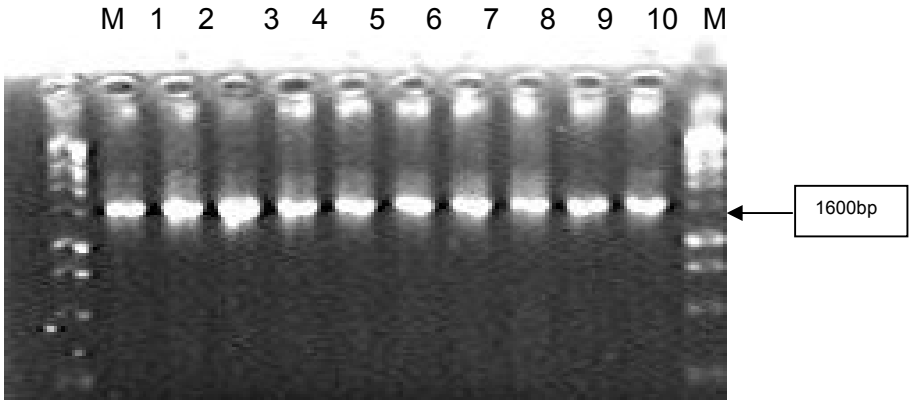

Plate 1. Electrophoresis gel for the isolates using 16S universal primer

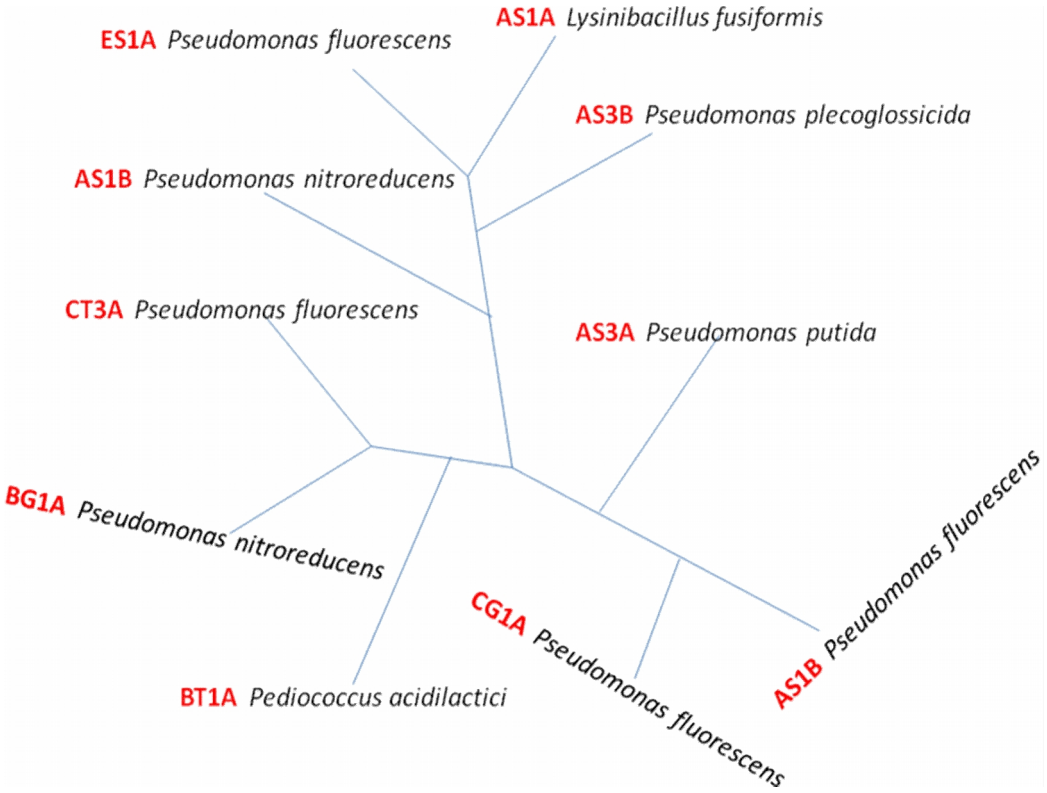

Fig. 1. Clustering Analysis for the Bacteria isolates

The DNA target for amplification using PCR technique was 16S ribosomal RNA in accordance to view of [9] that 16S rRNA has its presence in almost all bacteria, often existing as a multigene family, or operons and the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and the 16S rRNA gene (1,500bp) is large enough for informatics purposes. Fragment of 16S rDNA gene was amplified by PCR using 16S rDNA specific

universal forward and reverse primers, 8F [5'AGAGTTTGATCCTGGCTCAG3'] and 1492R [5'ACGGCTACCTTGTTACGACTT3'] respectively against forward primer (9F; 5'-GAGTTTGATCCTGGCTCAG-3' and reverse primer (1510R; 5'GGCTACCTTGTTACGA-3') by [10] in a similar research on shrimp.

The size of the amplified band in this study was 1,600bp for the 10 bacteria isolates while it was 500bp with the work done by [10]. The BLAST search shows that most selected isolates belong to the Gram negative group which is in line with [2] in which Gram-negative rod shaped bacteria dominated a cultured population of catfish (*Clarias gariepinus).* As the major gram negative bacteria strain found, the dominance of Pseudomonas species in the ponds was also confirmed by [11] which suggested that ponds should be allowed to dry out completely to eliminate or reduce pseudomonas species bacterial pathogens.

4. CONCLUSION

This study has revealed that *Pseudomonas* species are predominantly found in the ponds of population of catfish (*Clarias gariepinus)* with few strains of *Pediococcus acidilactici* and *Lysinibacillus fusiformis. Pseudomonas* species could be found on their skin and in the gut as well as in the gills.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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