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Genetic diversity studies of bacteria isolated from *Clarias gariepinus* along Yewa river in Nigeria using Random Amplified Polymorphic DNA (RAPD) techniques and their antibiotic resistance profile

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Abstract

Pathogenic bacteria are responsible for heavy mortality in both wild and cultured fish. Molecular characterization was carried out using Random Amplified Polymorphic DNA-Polymerase Chain Reaction technique (RAPD-PCR), sensitivity to antibiotics of bacteria in *Clarias gariepinus* post juveniles sampled. Bacteria were isolated from the gut, gills and skin of the fish. Identification was done using the conventional culture-based method. Thirty bacteria isolates were selected and the DNAs were extracted using CTAB method, PCR amplification of the isolates was carried out using RAPD primer and five primers were used. Data collected were subjected to descriptive (mean and standard deviation) statistics. There were 63 polymorphic and 14 monomorphic markers generated from the five RAPD markers. The primers generated 77 alleles altogether. Out of the 10 antibiotics used, Cephalexin recorded the highest inhibition zone (33 mm) on one sample, Gentamicin on second sample had (30 mm). The least inhibition zone was recorded in Cotrimoxazole on the second sample with (8 mm), 70.5 % bacteria strains were susceptible to Gentamicin while two samples displayed 100 % resistance to all the antibiotics. This study concluded that there are pathogenic and opportunistic bacteria species in *C. gariepinus* which could be zoonotic.

Key words: bacteria isolates, genetic diversity, RAPD-PCR, DNA extraction, sensitivity, *Clarias gariepinus* Polymorphism, Antiboitic

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Introduction

Fish is a vital food source for people; it is the most important single source of high-quality protein, providing approximately 16 % of the animal protein consumed by the world's population, according to (FAO, 1997). It is a particularly important protein source in regions where livestock is relatively scarce, fish supplies less than 10 % of animal protein consumed in North America and Europe, but 17 % in Africa, 26 % in Asia and 22 % in China (FAO, 2000). FAO estimates that about one billion people worldwide rely on fish as their primary source of animal protein (FAO, 2000). Fish also has economic and substantial social importance. The value of fish traded internationally is estimated to be US $ 51 billion per annum and over 36 million people are employed directly through fishing and aquaculture (FAO, 2000).

One of the major threats to fish life is disease which is an illness of fish body caused by micro-organisms creating infection or internal disorder. It is a complex interaction between a susceptible host, the environment and the pathogen. In the presence of a causative agent in an effective number, a susceptible host will suffer infection in adverse conditions. Diseases conditions manifest in various ways for the impairment of the normal physiology in the host. Diseases may lead to a tremendous death rate in

the aquatic ecosystem (Karsi et. al., 2002). Fish are extremely susceptible to contamination by microbes as a result of their aquatic environment and soft tissues. According to (Olaoye et. al., 2013), millions of bacteria, many of which are potential spoilers are present in the guts, in the gills and in the surface slime covering the skin of fish, though the skin is the first layer of defense, and is involved in both physical and immune defenses against pathogenic bacteria, viruses and parasites. The flesh of the fish is normally sterile, bacterial invasion and growth on the fish are prevented by the body's natural defense system when the fish is alive but the defense system breaks down after death and bacteria multiply and invade the flesh (Clucas and Ward, 1996).

This research was carried out to develop a baseline data on bacteria associated with C. gariepinus from Ajilete location on Yewa River as a result of the very few research works that have been carried out on this river. The main objective of this study is to characterize and identify the bacteria associated with *C. gariepinus* from Ajilete location on Yewa River using the RAPD-PCR method and also to evaluate the bacteria susceptibility to antibiotics.

Materials and Methods

Sample Collection and Morphometric charateristics

The study was done at Ajilete on Yewa River which lies within longitudes 2° 50'E and latitudes 6° 22'N. Bacteria samples were taken from the gill, skin and gut of *C. gariepinus* post juveniles 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. The samples collected were later cultured to get the pure culture of the bacteria. Water samples were also collected for examination in the laboratory. Twenty post juveniles with approximately 0.5 g of *C. gariepinus* were randomly sampled and swabs were taken from the fish for the examination of microbial load count. Morphometric characteristics of the fish such as the standard length, total length and head length were also determined using a centimeter ruler. The weight of each individual fish was also determined and physico-chemical parameters such as pH, Dissolved Oxygen and Temperature were taken.

Bacteria Isolation and Extraction of DNA

The media was weighed and prepared according to the specification. Nutrient agar of 0.8 g was measured and dissolved in 100 ml of distilled water in a conical flask and covered with foil paper. The agar was placed in an autoclave to sterilize it for 15 minutes at 121° C. After sterilization, the flask was allowed to cool down to room temperature and 5 ml of nutrient broth was aseptically pipette into sterile labeled McCartney bottles. The bacteria isolate was transferred into specified McCartney bottles labeled with 9 ml of nutrient broth with the aid of a sterile inoculating wire loop; the broth culture was then incubated at 37° C for 24 hours. Pure colonies of the bacteria were grown overnight in the broth, Bacteria isolates were transferred to eppendorf tube and it was spun down at 14,000 rpm for 2 mins, the supernatant was discarded and extraction of DNA was done using CTAB method (Akinyemi and Oyelakin, 2014).

PCR amplification using RAPD primers

DNA samples were diluted to 20 – 40 ng/µl, RAPD-PCR analysis was done with 5 operon primers. The PCR mix comprises of 1µl of 10X buffer, 0.4µl of 50mM $MgCl_2$, 0.5µl of 2.5 mM dNTPs, 0.5 µl 5 mM primer, 0.05 µl of 5 units/µl Taq with 2µl of 20 ng/µl template DNA and 5.05 µl of distilled water to make-up 10 µl reaction mix. PCR amplification was done using MJ Research Thermal Cycler (PTC-200 model), the primer used includes OPB-07, OPH-08, OPH-12, OPT-03 and OPT-14. The thermocycler used has an initial denaturation temperature of 94°C for 3 mins, followed by 45 cycles of 94°C for 20 seconds, 37° C for 40 seconds, 72°C for 40 seconds and the final extension temperature of 72°C for 5mins and the 10°C hold. The PCR amplicon electrophoresis was done on 1.2% agarose gel.

Sensitivity Test

Antimicrobial susceptibility was determined by the disk diffusion method as described by Kirby-Bauer in (Bauer et. al., 1996), in accordance with the guidelines of the Clinical and laboratory standards institute (CSLI, 2007). The isolates were tested against 10 antibiotics which included: amoxicillin (30 µg), erythromycin (10 µg), ofloxacin (30 µg), chloramphenicol (30 µg), cephalexin (30 µg), pefloxacin (30 µg), gentamicin (20 µg), streptomycin (10 µg), ceftriaxone (30 µg), and cotrimoxazole (30 µg). Each set (10 discs) was imbedded in a plate of pure isolates with specific bacteria and observed for action after incubating for 24 hours at room temperature. After about 30 minutes, the diameter of inhibition zones which appears as distinctly clear zones around the antibiotic disc were measured and interpreted as sensitive, resistant or partially sensitive using a common disc diameter value as 2.0 mm.

Statistical Analysis

The numerical data obtained for the length and weight recorded was subjected to Analysis of Variance (ANOVA) using SPSS (Duncan Multiple Range Test was used to separate the means). The bands obtained from the gels was transferred to numerical values 1 represents the presence of a band and 0 represents absence of a band, the data then subjected to analysis using NTSYS software to draw the dendogram for the bacteria isolates.

Results

Physico-chemical parameters and Morphometric characteristics

The Physico-chemical parameters recorded at the study site are temperature, pH and Dissolved oxygen with value range of 28.7-29.4^{0}C, 6.6, and 3.15 mg/l respectively (Table 1) The average total length, standard length, head length and average weight of the fish sampled at point A were 28.35±0.716 cm, 23.57±0.711 cm, 5.03±0.111 cm and 52.42±0.945 g respectively while that recorded at point B were 26.77±0.292 cm, 21.79±0.332 cm, 4.98±0.103 cm and 50.17±0.695 g respectively and point C had 26.787±0.398 cm, 22.15±0.318 cm, 4.07±0.110 cm and 48.13±0.313 g respectively, Table 2 showed the result whereby the total length, standard length of fish sampled in point A were significantly different from the other two points. The head length of fish sampled at point C was significantly different from point A and B, while the average weight recorded from point A; point B and C are significantly different from one another.

Morphology of Bacteria Isolated and Bacteria Count

The morphology of the bacteria isolated is presented in Table 3. The total bacterial count in the guts, gills and skin of advanced C. gariepinus juveniles was recorded and gut has the highest bacteria count (4.72×10^4), the next is the skin with 3.5×10^4 while the gill recorded the least count of 2.53×10^4.

PCR amplification using RAPD markers

There were 63 polymorphic markers generated from the five RAPD markers. There were also 14 monomorphic markers from the primers. A total number of 77 markers were generated.

Nineteen alleles were generated from the first primer (OPT-03, Plate: 1), 18 alleles from the second primer (OPH-12) and 17 alleles from the third primer (OPH-08, Plate: 2) while there were 14 and 9 alleles from the fourth and fifth primers respectively as represented on Table 4. Figure 1 showed the clustering analysis for the 30 strains. At coefficient point 67.8, two groups were formed with *Lysinibacillus sphaericus* strain being separated from the other 29 strains, at similarity coefficient point 69.5, the 29 strains formed another two groups with *Escherichia coli* strain, *Comamonas jiangduensis* strain, *Pseudomonas putida* strain, *Proteus mirabilis* strain forming a group different from the other 25 strains. At point 71.5, the 25 strains form two subgroups with *Brevibacillus agri* strain being separated from the others. At point 71.9, *Proteus hauseri* strain was separated from the others while the 24 strains form a different group. At similarity coefficient score of 76.2 an unidentified bacterium was separated from the other remaining strains forming another subgroup. The cluster analysis showed that *Bacillus cereus* strain and *Comamonas kerstersii* strain are more related with a similarity coefficient of 97.6 when compared with *Proteus vulgaris* strain and *Comamonas kerstersii* strain with similarity coefficient of 76.6.

Table 1: Physico-chemical parameters of the river at the location.

Parameter	Values	WHO (2006)
Dissolved Oxygen	3.15	5
Temperature	28.7-29.4	< 40
pH	6.6-6.9	6.5-9.5

Keys: WHO=world health organization,
Temperature measured in ^{0}C, degree Celsius
DO = measured in mg/L

Table 2: Morphometric characteristics of the fish sampled (P<0.05).

	Point A	Point B	Point C
Total length	28.35±0.716	26.77±0.292	26.787±0.398
Standard Length	23.57±0.711	21.79±0.332	22.15±0.318
Head length	5.03±0.111	4.98±0.103	4.07±0.110
Average Weight	52.42±0.945	50.17±0.695	48.13±0.313

Length in centimeter, weight in grams

Table 3: Morphology of Isolated bacteria

Bacteria Organism	Motility	Opacity	Elevation	Edges	Consistency	Color
Enterobacter spp	+	Opaque	Flatten	smooth	wet	White
Pseudomonas fluorescens	+	Opaque	flat	smooth	wet	Grey
Klebsiella spp	-	Opaque	round	smooth	wet	White
Proteus spp	+	Opaque	round	smooth	wet	White
E. coli	+	Opaque	Flatten	smooth	wet	White
Staphylococcus saprophyticus	-	Opaque	Flatten	smooth	wet	Yellow/golden
Bacillus subtilis	+	Opaque	Flatten	rough	dry	Yellow
Streptococcus spp	-	Translucent	flat	smooth	wet	Creamy white

Table 4: Primer Sequences and percentage polymorphism

S/N	Primer name	Primer Sequences	No of Monomorphic markers	No of Polymorphic markers	Total no of markers	Percentage Polymorphism (%)
1	OPB 07	GGTGACGCAG	02	07	09	78
2	OPH 08	GAAACACCCC	02	15	17	88
3	OPH 12	ACGCGCATGT	05	13	18	72
4	OPT 03	TCCACTCCTG	02	17	19	89
5	OPT 14	AATGCCGCAG	03	11	14	79
	Total		14	63	77	82

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

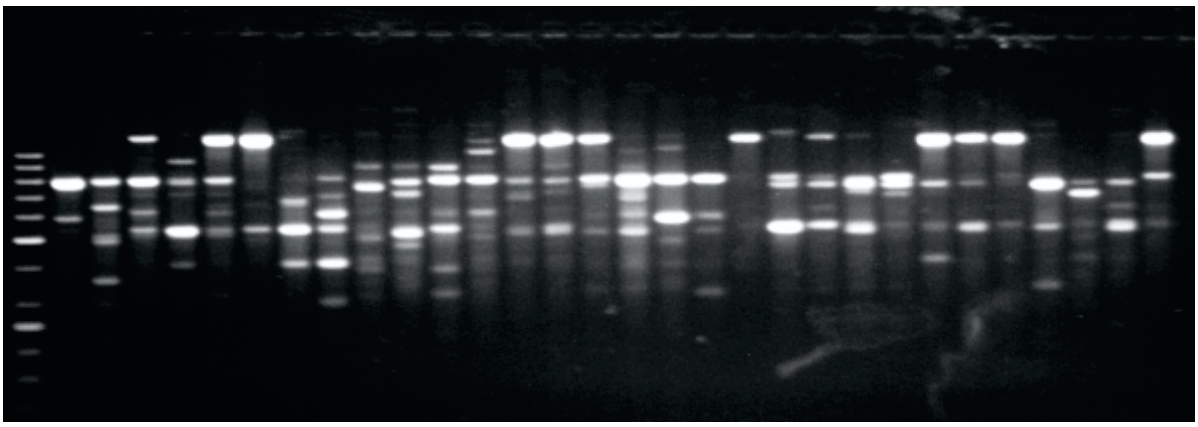


Plate 1: Electrophoresis gel for RAPD primer (OPT-O3)

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

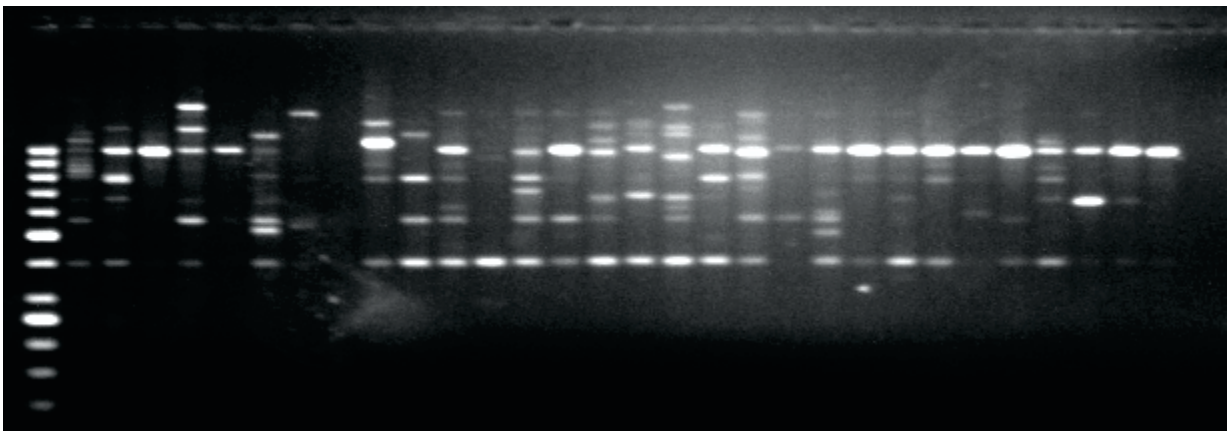
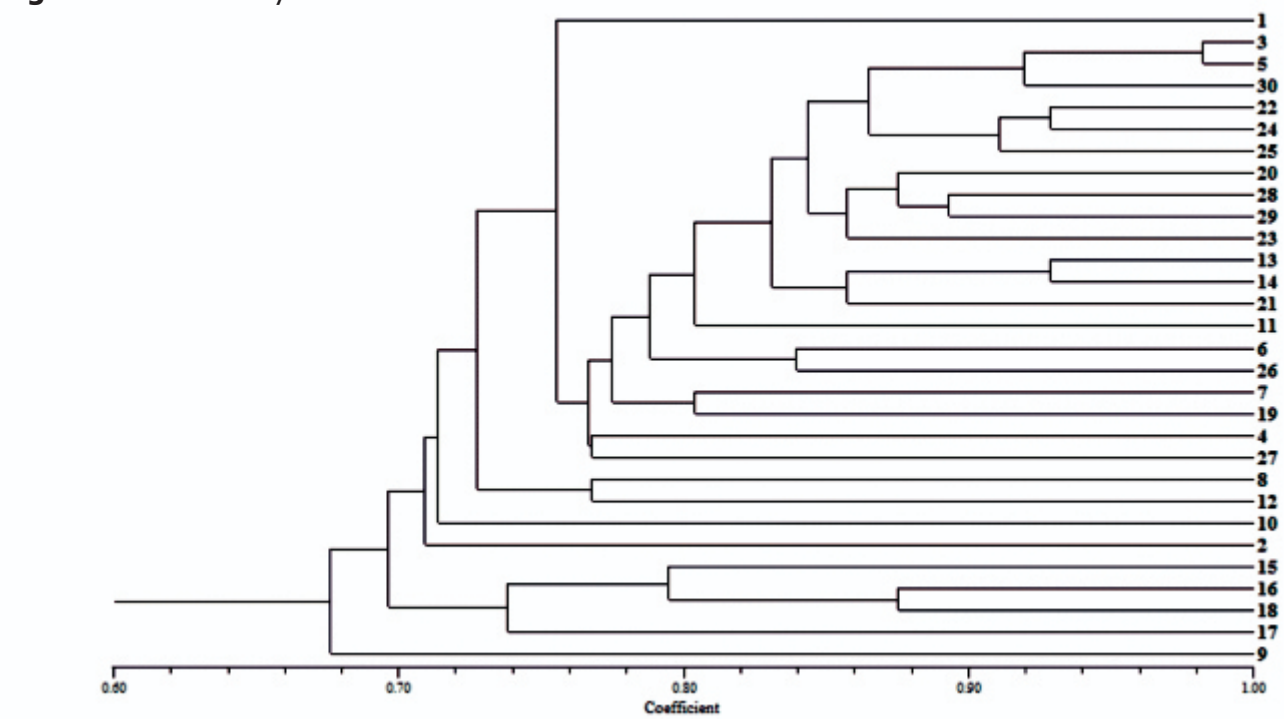


Plate 2: Electrophoresis gel for RAPD primer (OPH-08)

Figure I: Cluster analysis of the 30 selected isolates



Antibiotic Susceptibility Test

The antibiotic susceptibility test was performed according to Kirby-Bauer disk diffusion method. The antibiotics tested are; Erythromycin, Amoxicillin, Cephalexin, Ofloxacin, Streptomycin, Chloramphenicol, Ceftriaxone, Gentamicin, Pefloxacin, Cotrimoxazole. *B. cereus* strain was susceptible to Cephalexin (24 mm), Ofloxacin (14mm), Gentamicin (9mm) and Pefloxacin (15 mm) but was resistant to Erythromycin, Amoxicillin, Streptomycin, Chloramphenicol, Ceftriaxone and Cotrimoxazole. I. indica strain was susceptible to Erythromycin (15 mm), Ofloxacin (10 mm), Streptomycin (18mm), Chloramphenicol (20 mm), Ceftriaxone (14 mm), Gentamicin (17 mm) and was resistant to Amoxicillin, Cephalexin, Pefloxacin and Cotrimoxazole. *C. kerstersii* strain was susceptible to Cephalexin (24mm), Ofloxacin (21 mm), Gentamicin (10 mm), Pefloxacin (14 mm), however it was resistant to Erythromycin, Amoxicillin, Streptomycin, Chloramphenicol, Ceftriaxone, Cotrimoxazole. *P. vulgaris* strain QC51 was only susceptible to Gentamicin with inhibition zone of 21 mm while it was resistant to all other antibiotics. *L. sphaericus* strain was susceptible to Erythromycin (22 mm), Gentamicin (15 mm) and was resistant to all other antibiotics. *P. hauseri* strain was resistant to all the antibiotics except Amoxicillin (10 mm), Gentamicin (16.5 mm) and Pefloxacin (15 mm). *C. bifermentans* strain was susceptible to Erythromycin (23 mm), Chloramphenicol (15 mm), Ceftriaxone (10 mm), while it was resistant to the other antibiotics.

P. mirabilis strain was susceptible to Cephalexin (17 mm), Ceftriaxone (11 mm) and Pefloxacin (14.5 mm), and was resistant to the others. *P. putida* strain was susceptible to Amoxicillin (12.5 mm), Cephalexin (14.5 mm), Ofloxacin (12 mm), Gentamicin (16.5 mm), Pefloxacin (18 mm), but was resistant to the others. *E. coli* strain was susceptible to Erythromycin (10 mm), Chloramphenicol (11 mm), Ceftriaxone (14 mm), Gentamicin (30 mm), Cotrimoxazole (8 mm), while it was resistant to the other antibiotics. *C. jiangduensis* strain was resistant to majority of the antibiotics except Amoxicillin (12 mm), Cephalexin (20 mm), Ofloxacin (13 mm), Gentamicin (12 mm) and Pefloxacin (11 mm). E. cloacae strain was susceptible to Erythromycin (28 mm), Cephalexin (26 mm), Ofloxacin (16 mm), Streptomycin (12 mm), Chloramphenicol (15 mm), Gentamicin (12 mm) but was resistant to others. *S. marcescens* strain displayed inhibitory zone of 28 mm, 16 mm, 16 mm, 15 mm, 13 mm with Cephalexin, Ofloxacin, Streptomycin, Gentamicin and Pefloxacin respectively while it was resistant to the other antibiotics. *A. faecalis* strain was susceptible to Cephalexin (33 mm), Ofloxacin (19 mm), and Pefloxacin (16 mm), while it was 100 % resistant to the other antibiotics. *L. fusiformis* strain was susceptible to Cephalexin (26 mm), Ofloxacin (15 mm), Gentamicin (10 mm), Pefloxacin (17 mm) while it was resistant to others. *M. odoratimimus* strain displayed a perfect resistance to all the antibiotics (Table: 5).

Table: 5: Sensitivity Test of Bacteria Isolated using 10 different Antibiotics

Bacteria	ERY	AMX	CPX	OFL	STR	CHL	CRO	GEN	PFX	COT
Brevibacillus agri strain	-	-	-	-	-	-	-	-	-	-
Bacillus cereus strain	-	-	24	14	-	-	-	9	15	-
Ignatzschineria indica strain	15	-	-	10	18	20	14	17	-	-
Comamonas kerstersii strain	-	-	24	21	-	-	-	10	14	-
Proteus vulgaris strain	-	-	-	-	-	-	-	21	-	-
Lysinibacillus sphaericus strain	22	-	-	-	-	-	-	15	-	-
Proteus hauseri strain	-	10	-	-	-	-	-	16.5	15	-
Clostridium bifermentans strain	23	-	-	-	-	15	10	-	-	-
Proteus mirabilis strain	-	-	17	-	-	-	11	-	14.5	-
Pseudomonas putida strain	-	12.5	14.5	12	-	-	-	16.5	18	-
Escherichia coli strain	10	-	-	-	-	11	14	30	-	8
Comamonas jiangduensis strain	-	12	20	13	-	-	-	12	11	-
Enterobacter cloacae strain	28	-	26	16	12	15	-	12	-	-
Serratia marcescens strain	-	-	28	16	16	-	-	15	13	-
Alcaligenes faecalis strain	-	-	33	19	-	-	-	-	16	-
Lysinibacillus fusiformis strain	-	-	26	15	-	-	-	10	17	-
Myroides odoratimimus strain	-	-	-	-	-	-	-	-	-	-

KEYS: ERY- Erythromycin, AMX- Amoxicillin, CPX- Cephalexin, OFL- Ofloxacin, STR-Streptomycin, CHL- Chloramphenicol, CRO- Ceftriaxone , GEN- Gentamicin, PFX- Pefloxacin, COT- Cotrimoxazole.

Discussion

This study was carried out to access and characterize the bacteria associated with advanced *Clarias gariepinus* isolated from Ajilete location on Yewa River in Ogun state, Nigeria. Physico-chemical parameters were recorded at the location, Water quality determines how habitable the water will be for fish. There are ranges of physical, biological and chemical components that affect the Quality of water. These variables cause water pollution, whereas others enable a direct tracking of the sources of pollution (Olatunji et. al., 2011).

The temperature recorded ranges from 28.7^0C-29.4^0C, the value recorded was similar to that of (Akan et. al., 2010) and (Taiwo et. al., 2014) who recorded temperature value range of 26.8^0-27^0C, (Saidu and Musa, 2012), the result is within (WHO, 2006) standard. The pH is within the range of 6.5-9.5 (WHO standard) Aquatic organisms are affected heavily by pH because virtually all their metabolic activities are dependent on pH (Ewa et. al., 2011). This value obtained is however normal for aquatic lives and hence it had minimal effect on acidity (Akan et. al., 2010).

The study has shown that there were high bacterial counts found in the organs of the fish (advanced *Clarias gariepinus* juveniles) in the guts, gills and skin. The gut recorded the highest value (4.72×10^4), while the lowest bacterial count was recorded in the gill (2.53×10^4) of the fish. It was reported by (Ekundayo et. al., 2013) that the bacterial load associated with the gills are constantly maintained at low level, this implies that fish probably has a working mechanism which makes it possible to keep the bacteria number low thereby affording it an appreciable degree of protection against invasion of bacteria by gill micro flora (Ezeri 2001). This is in accordance with (Ekundayo et. al., 2013), who both reported lower counts in the range of 10^4-10^5 for *Clarias* sp. and Tivkaa Joseph, who also reported 1.2×10^4-2.16×10^5cfu. However, this result is significantly low when compared with the result of (Egbere et. al., 2010), who reported counts of 10^8 in their study of *Clarias gariepinus* and also with the findings of Adedeji et. al., (2012) who reported bacterial counts in the range of 10^{12}-10^{13} CFU.

There is a direct link between bacterial community of river water and the micro flora commensal of fish that dwell there. Bacteria present in aquatic environment can be ingested by fish and this can modify the dominant bacteria in its body (Donkeng et. al., 2011, Surgita et. al., 1996, Apun et. al., 1999 and De Sousa & Silva-Souza, 2001). The results from the molecular characterization for the 30 selected isolates identified seventeen bacterial strains. The organisms isolated in this study are generally those associated with tropical freshwater environment and have been isolated from soils,

sediments, invertebrates, planktons and digestive tracts of many animals in the aquatic environment (Austin and Austin, 2007). The microorganisms include *Pseudomonas, Escherichia* and *Enterobacter* are periodically implicated as being pathogenic to fish (Loch et. al., 2012, Starliper 2001, Akoachere et. al., 2009 and Brenkman et. al., 2008) reports of infections caused by other isolated bacteria on fish have been minimal. The isolation of these pathogens from fish samples is worrisome because of their potential in causing ill health in human. Stress and consequently immune suppression is probably the commonest underlying cause of disease in fish.

It can be deduced from this study that cephalexin is a good antibiotics for *A. faecalis* strain. Bacteria share common environment with fish in their natural habitat and thus become resident fish flora, but they can cause epizootics under stressful conditions. These stressful conditions may be brought about by chemical, physical and or biotic factors (Ekundayo et. al., 2014). (Okonko et. al., 2009) reported that apart from disease occurrence in fish, the potential of water body harboring pathogenic microbes that can be zoonotic is well documented for both developing and developed nations. As a result of this, foods from aquatic sources should be handled with great care in order to avoid food poisoning (Adebayo-tayo et. al., 2012), as water related diseases continue to be one of the major health issues worldwide.

Conclusion

This study indicates that there are various bacteria species present in the gills, gut and skin of the fish. This could cause diseases in fish and also they could be involved in the transmission of such diseases to man. Fish and fish products have been reported severally as a means of food-borne bacterial infections in man. RAPD-PCR analysis of bacteria DNA used showed that it could be used to genotype the bacteria associated with advanced *C. gariepinus* juveniles.

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