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Molecular Detection of Tetracycline Types (A) and (B) Resistant Genes in Bacteria Associated with *Chrysichtys nigrodigitatus* from Abeokuta, Nigeria

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Abstract
The study aimed at isolating and using molecular tools to characterize the bacteria found in the gills intestine and skin using Tetracycline types A and B resistant markers. A total of forty four bacteria samples were isolated from the gills, skin and intestines of fifteen *Chrysichtys nigrodigitatus* and their antibiotic susceptibility and resistance to tetracycline type A was determined. The bacteria isolates collected were grown on Eosin Methylene Blue (EMB) and Salmonella-Shigella Agar. The pure colony of the bacteria was inoculated on Nutrient Broth overnight at 37^0C and DNA extraction was done along with PCR analysis using Tetracycline resistance genes type A and B markers. The antibiotic sensitivity test was also conducted using the Maxi disc high profile (-ve) diffusion method. From the result, the mean values of the body weight, total length, and standard length of the fish samples were 147.99 ± 66.4g 126.55 ± 5.55g and 20.05 ± 4.13g respectively. The result of the physical and chemical properties of Ogun River showed temperature (29.0°C), pH (7.8), Dissolved Oxygen (4.9mg/l) and Ammonia (2.3ug/dL). The total mean bacteria count in the gill samples was $1.06 \pm 0.20 \times 10^6$CFU/g, while in the intestine samples, it was $1.36 \pm 0.27 \times 10^6$ CFU/g and in the skin samples is $0.77 \pm 0.21 \times 10^6$ CFU/g. The 45 samples gave 135 isolates and in total, there were five bacteria found in the gills, intestine and skin of the *Chrysichtys nigrodigitatus.* The five bacteria isolates included *Escherichia coli, Staphylococcus aureus, Bacillus spp, Salmonella spp and Pseudomonas auregenosa.* It was revealed that the isolates were sensitive to Ciprofloxacin, Pelfloxacin, and Tarivid. It was also indicated that all the bacterial isolates were susceptible to Ciprofloxacin and Sparfloxacin. The result on *Escherichia coli* showed susceptibility to Pefloxacin, Ciprofloxacin and Tarivid while it equally showed resistance to Streptomycin, Augmentin and Amoxacillin, Gentamycin, Septrin and Chloranphenicol. The molecular detection shows that *Escherichia coli* and *Salmonella spp* is susceptible to Tetracycline Type A while other are resistant to it, so therefore tetracycline type A can only be used to treat infection with *Escherichia coli* and Salmonella spp.

Keywords: Bacteria, PCR analysis, Antibiotics, Tetracycline gene, Chrysichtys nigrodigitatus

Introduction

Fish is a well-traded food commodity worldwide (Allison, 2011) and a major source of animal protein in many countries, including Nigeria (Bènè *et al*., 2015). Fish accounts for approximately 17% of the global animal protein intake (FAO, 2014). Fish is a delicacy with demands that cut across all socio-economic, religious, educational, or age groups (Adebayo-Tayo *et al*., 2008). It is one of the most important animal protein sources that is widely consumed by all races and classes of people (Abolude and Abdullahi, 2005). Its harvesting, handling, processing and distribution provide a livelihood for millions of people. Fish protein has been found to be rich in essential amino acids which are suitable for complementing high-carbohydrate diets. They are also rich in minerals such as thiamine, riboflavin and precursor vitamins A and D (Akanni, 2010). Infectious diseases in fish occur when susceptible fish are exposed to virulent pathogens under certain environmental stress conditions. Bacteria occur almost everywhere in nature. The bacteria in fish intestines are somewhat dependent on the food being consumed but normally contain Vibrio, A chromobacter, Pseudomonas and Peromonas in addition to smaller numbers of Gram-positive bacteria including Clostridium. The bacteria of fish are mostly Psychrophilic, growing between 0°C and about 30°C with some strains growing as low as -75°C. The

discovery of antibiotics and their clinical potential in treating bacterial infections in fish represented an extremely important milestone in the field of fish microbiology and pathology. Bacteria cause a high percentage of fish diseases and infections in fish farming (Bondad *et al*., 2005). It was observed that water-borne pathogens spread faster compared with terrestrial pathogens (Emmanuel *et al*., 2014). According to FAO (2014), Aquaculture provides more than half of the fish that is consumed globally. With the increasing demand and commercialization of aquaculture production, there has been an increase in fish losses due to disease outbreaks (Bondad *et al*., 2005). This outbreak is due to pathogens such as bacteria, fungi, and viruses present in the environment (Cristea *et al*., 2012). Cultivated fish have become more susceptible to both pathogenic and opportunistic bacteria. Different antibiotics are commonly adopted in the treatment of diseases and prophylaxis in intensive aquaculture; among these antibiotics is Tetracycline and Amoxicillin. The objectives of this study are to identify bacteria found in the gills, intestine and skin using biochemical tests to determine the sensitivity of the bacteria to synthetic antibiotics and use molecular tools to characterize the bacteria found in the gills intestine, and skin using Tetracycline types A and B resistance markers.

Methodology

Study area

The study was carried out in the lower part of the Ogun River, along the Akomoje area in Abeokuta, Nigeria. The river location lies between longitude 3°21'S and latitude 7°21'E North of Abeokuta with a size of 1000 hectares. Ogun River is a perennial river in Nigeria, which has a coordinate of 3°28'E and 8°41'N from its source in Oyo State to 3°25'E and 6°35'N in Lagos State where it enters Lagos Lagoon.

Samples Collection and Morphometric Analysis

Fish of approximately 400±100g *Chrysichtys nigrodigitatus* were collected from a fish pond in Abeokuta, Nigeria. A total of 16 fish were obtained from five different earthly ponds. The fish were kept in a sterile plastic container. Fish samples were weighed and dissected using a medical kit so as to obtain bacteria from the gills skin and intestine. The water quality parameters such as Temperature, Ammonia, Dissolved Oxygen and pH were evaluated using standard reagent methods. The standard length and total length in centimeters (cm) of the fish were measured and recorded after weighing the fish samples in grams (g). Media for microbiological analysis were prepared. Eosin Methylene Blue Agar (EMB) and Salmonella-Shigella Agar (SSA) were weighed and prepared following the manufacturer's description. The bacteria sample obtained from the gills, intestine and skin were serially diluted to 10 and 1ml aliquots and spread on Salmonella Shigella Agar (SSA) and Eosin Methylene Blue (EMB) agar using the pour plate method, and they were then incubated for 24 hours at 37°C. The Agar plates were observed for bacterial growth. Total bacterial counts were determined by counting the number of colonies on the surface of the Agar plates and expressed as colony-forming units per gram (CFU/g).

Diffusion Test of Antibiotics

Antimicrobial susceptibility test was done using classes of antibiotics; the test for the bacteria isolates was performed by placing Bauer Kirby Disc diffusion methods on Mueller Hinton Agar. The following antibiotics discs were used: Septrin (30ug), Chloramphenicol (30ug), Sparfloxacin (10ug), Ciprofloxacin (30ug), Amoxicillin (30ug), Augmentin (10ug), Gentamycin (30ug), Pefloxacin (30ug), Tarivid (10ug) and Streptomycin (30ug). A pure culture of 0.5 McFarland was spread on Mueller Hinton Agar and allowed to dry at room temperature. Each antibiotic disc was placed on the inoculated agar and incubated at 37°C for 24 hours. The diameters of the zone of inhibition were measured and recorded. The antibiotic susceptibility of each isolate was interpreted as Susceptible (S), Intermediate (I) and Resistant (R) according to Clinical and Laboratory Standard Institute guidelines (CLSI, 2012).

DNA Extraction, PCR Analysis, and Gel Electrophoresis

Pure bacterial isolates were subcultured in 3mls of Nutrients Broth at 37°C for 18hrs. The genomic DNA was extracted from the cultured Isolates using a DNA extraction kit (Norgen Biotek Corporation Canada) following the manufacturer's specifications. The concentration and purity of DNA extracted from each isolate were determined using a Nanodrop machine. The integrity of the DNA was also quantified on an agarose gel according to Akinyemi and Oyelakin, (2014). DNA samples were diluted so as to have a final concentration of 20-50ng/µl. The reaction mix was carried out in 20µl final volume containing 60-80ng genomic DNA and 0.1 µM of the Forward and Reverse primers of Tetracycline types A and B, (Inqaba, West Africa) 2mM $MgCl_2$, 125µM of each dNTP, and 1 unit of Taq DNA polymerase (Table: 1). The PCR analysis was done using the MJR 200 Thermal Cycler (USA), and the PCR profile used was an initial denaturation temperature of 94°C for 3 minutes, followed by 35 cycles of denaturation temperature of 94°C for 40 seconds, annealing temperature of 50°C for 50 seconds and extension temperature of 72°C for 60 seconds, followed by final extension temperature of 72°C for 5 minutes. PCR amplicon electrophoresis was carried out by size fractionation on 1.4% agarose gels. Electrophoresis was done at 120 volts for 2 hours. The DNA was visualized digitally using a Gel image system (Hercules, CA, USA).

Data Analysis

The Morphometric data obtained were subjected to descriptive statistics using SPSS (Statistical Package for Social Scientist) version 2010 to compute the frequency, percentage frequency, mean and standard deviation of bacteria occurrences in the skin, gills and intestine of *Chrysichthys nigrodigitatus.*

Results and Discussion

Water Quality and Morphometric values

The physical and chemical parameters of the river were observed and the water temperature was (29.0°C), pH (7.8), Dissolved Oxygen (4.9mg/l) and Ammonia (2.3ug/dL) and the morphometric characteristics of the fish used were total length, standard length and body weight Table: 2.

Cultural method

The cultural method of determining bacteria isolates shows that three of the isolates were *Salmonella* Spp., *Escherichia coli*, and *Pseudomonas aeruginosa,* while the other two are *Staphylococcus aureus a*nd *Bacillus spp.* The total mean bacteria count in the gill samples was 1.06 ± 0.20 X 10^6CFU/g, while in the intestine samples is1.36 ± 0.27 X 10^6CFU/g and in the skin samples it is 0.77±0.21 X 10^6CFU/g (Table 3). The lowest bacterial counts were recorded on the skin while the highest bacterial were recorded on the intestine. The 45 samples gave 135 isolates: 28 (15.3%) were *Escherichia coli*, 30 (22.2%) *Staphylococcus aureus,* 34 (25.2%) *Bacillus sp,* 30 (22.2%) *Salmonella sp,* 13 (9.6%) *Pseudomonas auregenosa* (Table: 4).

Antibiotics susceptibility

The Maxi disc high profile (-ve) diffusion method against the bacteria isolates shows that all the bacteria were susceptible to Ciprofloxacin and Sparfloxacin. *Escherichia coli* were also susceptible to Pefloxacin, Ciprofloxacin and Tarivid. *E.coli* was also resistant to Streptomycin, Augmentin Amoxicillin, Gentamycin, Septrin and Chloranphenicol.

Amplification of Tetracycline Types A and B

The amplification with tetracycline A shows that sample 1 has a single band with a 1,700base pair and sample 2 has a single band as well but with 1,050 basepair (Figure:1). Samples 3 and 4 have no amplification while the fifth sample has a single band with1,600 basepair (Table: 5). Amplification with tetracycline B show that all the samples have the resistant gene with a band at position 1,500 basepair, except sample 5 with 1,550 basepair. There are additional bands on sample 3 with 3,500 base pairs, and sample 5 also has 3,600 base pairs (Figure: 2). Therefore, bacteria in the samples are all resistant to Tetracycline type B antibiotics. Oyelakin *et al*., (2021) reported that all five bacteria obtained from C. gariepinus were resistant to tetracycline B which is in line with this research work.

The use of antibiotics as prophylaxis therapeutants and growth promoters has resulted in the deposition of their residues in food animals such as meat, milk, eggs and fish (Ottinger *et al*., 2015). This deposition in food fish has been traced to the transfer of antibiotic resistance to humans, reproductive disorders, carcinogenicity, etc. (Emmanuel *et al*., 2014). This work showed that the intestine had the highest concentration of bacteria count at 1.36 ± 0.27 (10^6CFU/g) which disagrees with results obtained by Akinyemi *et al*. (2011). The most prevalent group in the intestine was *Bacillus sp*. The gills also had a high concentration of bacteria 1.06 ± 0.20 (10^6CFU/g). It was dominated by *Salmonella sp* and *Staphylococcus aureus*. The skin had the least amount of bacteria 0.77 ± 0.21 (10^6CFU/g). *E. coli* was isolated from the gut and gills of *Chrysichtys nigrodigitatus* which supports the results obtained by Efuntoye *et al*., (2012) who observed that *E. coli* are found in the gills and intestines of healthy *Chrysichtys nigrodigitatus*. *Salmonella* was obtained from the skin, gills and intestines of *Chrysichtys nigrodigitatus* similar to Budiati, (2013) who reported the presence of *Salmonella* in *Chrysichtys nigrodigitatus* cultured in a pond. *Salmonella* infections remain a major public health concern worldwide, contributing to the economic burden of both industrialized and underdeveloped countries through the costs associated with surveillance, prevention and treatment of diseases Crump *et al*., (2004). Yakub *et al*., (2015) identified *Escherichia coli* and *Salmonella* spp from the chickens in Jos, Nigeria; this reveals that *Escherichia coli* are not only peculiar to fish but also to other animals. Molecular results using a Tetracycline A primer showed that these isolated bacteria are not resistant to the Tetracycline A antibiotics. Tetracycline A has been used for the treatment of bacterial and protozoan infections in clinical and veterinary medicine.

Akinyemi et al., (2016) reported that there were eight bacteria species associated with the gills, gut, and skin of the Clarias gariepinus found along the Idogo and Yewa Rivers in Nigeria. These include Citrobacter spp, Escherichia coli strains, Morganella morganii strains, Alkaligenes feacalis strains, Erwinia tasmaniensis strains, and Proteus penneri strains. Escherichia coli is the only one found in this research work. Ekeleme *et al*. (2016) obtained Pseudomonas spp, Serratia spp and Aeromonas spp, dominantly on the skin of C. gariepinus, and the sensitivity test showed that the three identified bacterial isolates were sensitive to Ofloxacin (5 mg), Nitrofurantoin (300 mg), and Ciprofloxacin (5 mg). The bacterial isolates were 100% resistant to Cefixime (5 mg) and Cefuroxime (30 mg).

Conclusion

The research work has shown that there are five bacteria associated with the skin, gills and intestine of *Chrysichtys nigrodigitatus,* namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Bacillus spp* and *Salmonella spp*. The bacteria obtained are susceptible to Tetracycline type A but resistant to Tetracycline type B. All the isolated bacteria are resistant to the tetracycline type B, and the fish has the ability to resist the effects of the antibiotics. This implies that the tetracycline type B cannot be used to treat infections caused by consuming C. *nigrodigitatus*. There were two antibiotics used in this research work, it is therefore recommended that Tetracycline Type A should be used for the treatment of infection resulting from the following bacteria isolates: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Bacillus spp* and *Salmonella spp.*

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Table 1: Primers name and their sequences

PRIMERS NAME	SEQUENCES
TETRACYCLINE TYPE (A) FW	GCTACATCCTGCTTGCCTTC
TETRACYCLINE TYPE (A) RV	CATAGATCGCCGTGAAGAGG
TETRACYCLINE TYPE (B) FW	TTGGTTAGGGGCAAGTTTTG
TETRACYCLINE TYPE (B) RV	GTAATGGGCCAATAACACCG

Table 2: Morphometric characteristics

	Mean	Std. Deviation
Body Weight	147.9913	66.41130
Total Length	26.5467	5.55104
Standard Length	20.0533	4.12793

Table 3: Mean of Bacteria count from Gills, Intestine and Skin

Sample	Mean± SD (10^6CFU/g)
Gills	1.06 ± 0.20
Intestine	1.36 ± 0.27
Skin	0.77 ± 0.21

Table 4: Numbers of Bacteria Identified on the Gills, Skin and Intestine

Type of Bacteria Isolate	Gill	Skin	Intestine	Total
Escherichia coli	9	8	11	28
Staphylococcus aureus	11	8	11	30
Bacillus spp	10	12	12	34
Salmonella spp	11	8	11	30
Pseudomonas auregenosa	4	4	5	13
	45	**40**	**50**	**135**

Table 5: Band size of the isolates amplified with Tetracycline Type A antibiotics

Sample No	1,700bp	1,600bp	1,050bp
1	+	-	-
2	-	-	+
3	-	-	-
4	-	-	-
5	-	+	-

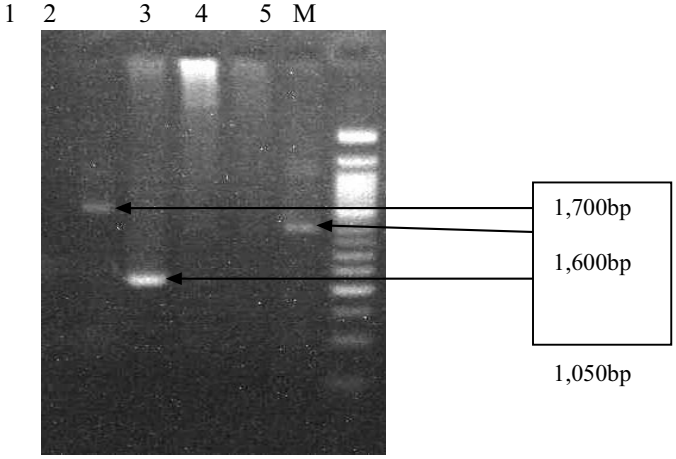

Figure 1: Amplification of Tetracycline Type (A) gene determinants on agarose gel

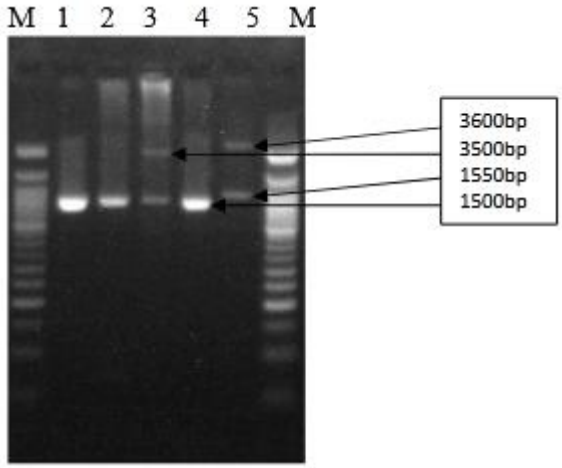

Figure 2: Amplification of Tetracycline Type (B) gene determinants on agarose gel.