# MICROBIAL ISOLATION AND PCR DETECTION OF TETRACYCLINE TYPE-(B) AND SULFONAMIDE (SUL1) RESISTANCE GENES IN BACTERIA ISOLATED FROM *CLARIAS GARIEPINUS*

**[1*]OYELAKIN O. O., [2]OLOYEDE A. R. AND [3]AKINYEMI A. A.**

*[1]Biotechnology Centre, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.*
*[2]Department of Microbiology, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.*
*[3]Department of Fishery and Aquaculture Management, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.*
**Corresponding author:foyelakin@hotmail.com; +2347035770173*

## ABSTRACT

*The study was carried out to isolate and identify the bacteria associated with different parts of Clarias gariepinus and to detect the presence of Tetracycline type B and Sulfonamide-resistant genes in the bacteria as well as determine their antibiotic susceptibility pattern. A total of 48 bacteria isolates from the skin, gill, and intestine of Clarias gariepinus were obtained and characterized microbiologically. The sensitivity test was determined using the Maxi disc high profile (-ve) diffusion method. Pure isolates were cultured in Nutrient broth for DNA extraction, DNA was extracted and PCR analysis was done using Tetracycline type B and Sulfonamide resistance gene primers. All the samples collected showed bacterial growth on Eosin methylene Blue (EMB) and Salmonella-Shigella Agar. Five different bacteria emerged from isolates obtained, namely Escherichia coli, Klebsiella oxytoca, Proteus spp., Salmonella spp, and Shigella spp. The Sensitivity test revealed that the five bacteria are sensitive to ciprofloxacin, pefloxacin, and tarivid. It was indicated that all the bacterial isolates were susceptible to Pefloxacin, Ciprofloxacin, and Tarivid. Escherichia coli were also susceptible to Chloramphenicol and Gentamycin. Escherichia coli was also resistance to Streptomycin, Augmentin and Amoxicillin. Shigella was susceptible to Septrin, Chloramphenicol, Sparfloxacin, Gentamycin, and Streptomycin. Shigella is also resistant to Amoxicillin and Augmentin. Samonella was also susceptible to Septrin, Chloraphenicol, Sparfloxacin, Gentamycin and Streptomycin. PCR analysis of Tetracycline type B and Sulfonamide resistance gene shows that the five bacteria isolates from different parts of the fish were resistant to the two antibiotics used, the five bacteria obtained have 700basepair, 400 basepair and 300basepair for Tetracycline type B and the size of Sulfonamide resistance gene was 400basepair. Therefore, the two antibiotics cannot be used to treat infection as a result of bacteria associated with Tetracycline type B and Sulfonamide*

**Keywords:** ***Clarias gariepinus Bacteria, Antibiotics, Tetracycline, Sulfonamide gene***

## INTRODUCTION

Fish is a major source of protein globally and it accounts for approximately 17% of protein intake (FAO, 2010). In Nigeria, there is an increased demand for fish because it is a cheaper source of animal protein; it is consumed across all socio-economic, religious, educational or age groups (Adebayo-Tayo *et al*., 2008). Fish is eaten fresh, processed, or preserved and fish protein makes up 40-80% of the optimal protein consumed (Adebayo-Tayo *et al*., 2008). It is one of the most important animal protein sources that are widely consumed by all races and classes of people (Abolude and Abdullahi, 2005). Fish production has

provided a livelihood for millions of people. Fish and fishery products are highly nutritious and are excellent sources of other dietary essentials like vitamins and minerals. Fish fat contains a high proportion of polyunsaturated fatty acids which may help to decrease the incidence of atherosclerosis and heart-related diseases (Akande, 2011).

Globally, the production of fish competes with the growth of the world population (FAO, 2010), as the population increases, the production of fish increases. Aquaculture continues to be the fastest-growing animal food-producing sector, and aquaculture accounts for 46% of the total food fish supply (FAO, 2011). Catfishes are very important commercial species that are highly valued (Olokor *et al.*, 2011). In Nigeria, the short supply of animal protein together with the increasing human population has raised the cost of animal protein to a level almost beyond the reach of the low-income group (Ezeri *et al*., 2001). The intensification of aquaculture has led to the promotion of conditions that favor the development of many diseases and problems.

Many pathogens are associated with fish and are found mainly in the gills, guts and skin. Infectious diseases of fishes occur when susceptible fishes are exposed to virulent pathogens under certain environmental stress conditions (Akinyemi and Ajisafe, 2011). Fish diseases are perhaps, the major cause of losses in the aquaculture industry. Bacteria that infect fish belong to three groups: the Gram-negative bacteria (most common), Gram-positive, and acid-fast bacteria, which are obtained from food or the environment (Ducencic and Canda, 2003). Gram-negative bacteria cause most of the diseases in tropical fish.

The fish pathogens are generally found on the skin, gills, water, and surrounding environment of the fish. Infections are more likely to occur in ponds that are heavily stocked since overcrowding provides a favorable situation for the rapid spread of infection among the fish. Bacterial infections are considered the major cause of mortality in aquaculture (Govind *et al.*, 2012). They can infect a single fish and multiply rapidly to cause a substantial fish kill in a few days or weeks. Bacterial diseases are often internal infections and usually require treatment (with antibiotics added to feeds or water). Among the common bacterial infections of fish and causative organisms are Columnaris (*Flexibacter columnaris*), Furunculosis (*Aeromonas salmonicida*), Piscine tuberculosis (*Mycobacterium marinum*) and Vibriosis (*Vibrio* spp). Antibiotics are commonly used in the treatment of bacterial infections of fish. Antibiotics are drugs of natural or synthetic origin that can kill or inhibit the growth of microorganisms. Antibiotics that are sufficiently non-toxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases in humans, animals, and plants (Hernández, 2005).

Tetracycline is a broad-spectrum antibiotic used in animal medicine as well as the aquaculture industry. There are various forms of tetracycline resistance genes (*Tet*) that have been characterized (Roberts, 2005) Resistance to tetracycline is governed by *Tet* genes, which are involved in either active efflux of the drug, ribosomal protection or enzymatic drug modification (Giovanetti, *et al*., 2003). Among the various *Tet genes*, *Tet*(A), *Tet*(B), *Tet*(D), *Tet*(E), and *Tet*(G) are reported in gram-negative bacteria. (Jones, *et al*., 2006). Tetracyclines have been greatly used in aquaculture particularly to control furunculosis in salmonids and oxytetracycline is frequently used in most fish farming industries as a prophylactic agent (Miranda *et al*., 2003). Sulfonamide is

another antibiotic that is used in treating disease in fish and animals in general.

The objectives of the research work are to isolate and identify bacteria associated with the gills, gut, and skin of fish using cultural methods, check sensitivities of the bacteria to the synthetic antibiotics, and characterize the bacteria obtained from the skin, gill, and gut of *Clarias gariepinus* using Tetracycline and Sulfonamide resistance primers.

## MATERIALS AND METHODS

### *Collection of Fish samples and Isolation of Bacteria*

A total of 16 samples of *Clarias gariepinus* fish were collected from Ijebu-Ode, Nigeria, samples were taken from the gill, intestine, and skin of the fish. Serial dilution of the samples was made from $10^{-1}$ to $10^{-6}$ and 1ml aliquot was spread on Salmonella Shigella Agar (SSA) and Eosine Methyl Blue (EMB) agar using the pour plate method, and then incubated for 24 hours at 37°C. The Agar plates were observed for bacterial growth. The colonies were sub-cultured on Nutrient Agar plates for pure culture. 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)

### *Antimicrobial Susceptibility Test*

Bacterial isolates were tested with the Bauer Kirby disc diffusion method on Mueller Hinton agar. The antibiotic discs used include; Septrin (30ug), Chloramphenicol (30ug), Sparfloxacin (10ug), Ciprofloxacin (30ug), Amoxicillin or a different product (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 18 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 Standards Institute guidelines (CLSI, 2012)

### *DNA Extraction, PCR Analysis, and Gel Electrophoresis*

Bacteria isolate was inoculated in 3mls of Nutrients Broth incubated at 37°C for 18hrs. The genomic DNA was extracted from the cultured Isolates according to (Akinyemi and Oyelakin, 2014). The concentration and purity of DNA extracted from each isolate were determined using a Nanodrop Machine. DNA samples were diluted to 20-50ng/µl. The reaction mix was carried out in 20µl final volume containing 60ng - 80ng genomic DNA, 0.1 µM of the forward and reverse primers, of tetracycline type-B and Sulfonamide resistance gene, 2mM $MgCl_2$, 125µM of each dNTP and 1 unit of Taq DNA polymerase Table 1. The PCR was done using an MJR-200 Thermocycler machine with the following PCR profile; initial denaturation temperature of 3 minutes at 94°C, followed by 35 cycles of denaturation temperature of 94°C for 40 seconds, annealing temperature of 55°C for 50 seconds and primer extension temperature of 72°C for 60 seconds, followed by final extension temperature at 72°C for 5 min was added. PCR amplicon electrophoresis was carried out by size fractionation on 1.4% agarose gels with Ethidium Bromide. Electrophoresis was done at 120V for 2 hours. The DNA was visualized on a UV light source.

**Table 1. Sequences of the primer used**

| Primers | Sequences |
|---|---|
| Tet B FW | 5'-TTGGTTAGGGGCAAGTTTTG-3' |
| Tet B RV | 5'-GTAATGGGCCAATAACACCG-3' |
| Sul1 FW | 5'-CGGCGTGGGCTACCTGAACG |
| Sul1 RV | 5'-GCCGATCGCGTGAAGTTCCG |

**Source of the Primer: Inqaba West Africa**

## RESULTS

### *Diversity and Characteristics of Bacteria Isolates*

The 48 bacteria isolates gave 382 counts; 28 (7.32%) *Escherichia coli*, 85 (22.25%) *Klebsiella oxytoca*, 102 (26.70%) *Proteus*, *Salmonella* 120 (31.41%), *Shigella* 47 (12.30%). Table 2 shows the cultural characteristics of the bacteria isolates. It was found that for the Opacity, three of the bacteria isolates were found transparent which are *Shigella* Spp., *Proteus* spp, and *Salmonella* Spp. while the other two are translucent, *Klebsiella* spp. and *Escherichia coli*. Also, the shape/form was recorded and three of the bacteria were found to be round/circular, *Klebsiella* spp., *Proteus* spp., and *Escherichia coli*. While two of the bacteria were found to be rod *Salmonella Spp.* and *Shigella* Spp. For the colouration, two of the bacteria were found colourless which are *Salmonella spp.* and *Shigella spp.*, one was found to be dark-purple, *Klebsiella spp.*, another one was found grey, *Proteus spp* and *Klebsiella spp.* was found pink-purple.

**Table 2. Characteristics of Bacteria Isolates**

| Form/Shape | Surface/Texture | Colour | Elevation | Opacity | Suspected Isolates |
|---|---|---|---|---|---|
| Circular | Mucoid | Pink-Purple | Convex | Translucent | *Klebsiella*spp |
| Rod | Smooth | Colourless | Convex | Transparent | *Shigella*spp |
| Circular | Glistering | Grey | Effuse | Transparent | *Proteus*spp |
| Round | Mucoid | Dark Purple | Flat | Translucent | *Escherichia* coli |
| Rod | Smooth | Colourless | Flat | Transparent | *Salmonella*spp |

### *Antibiotics susceptibility*

The readings were interpreted with the use of the CLSI guide, which says 0-14mm zone is resistance, 14-17mm zone is intermediate, and 17mm and above is susceptible. It shows that all bacterial isolates were susceptible to Pefloxacin, Ciprofloxacin, and Tarivid. *Escherichia coli* were also susceptible to Chloramphenicol and Gentamycin. E. coli was also resistant to Streptomycin, Augmentin, and Amoxicillin. Shigella was susceptible to Septrin, Chloramphenicol, Sparfloxacin, Gentamycin, and Streptomycin. Shigella is also resistant to Amoxicillin and Augmentin. Samonella was also susceptible to Septrin, Chloramphenicol, Sparfloxacin, Gentamycin and Streptomycin. It shows an intermediate response to Amoxicillin and is resistant to Augmentin. Klebsiella was susceptible to Amoxicillin, Augmentin, Gentamycin, and Streptomycin, it also showed intermediate response to Septrin and Sparfloxacin while resistance to Chloramphenicol. Proteus was susceptible to Chloramphenicol, Augmentin, Streptomycin, and Amoxicillin while intermediate to Septrin, Sparfloxacin and Gentamycin are shown in Table 3.

**Table 3. Antibiotics susceptibility patterns of bacteria isolated from gill, intestine and skin of *Clariasgariepinus***

| ISOLATES | SXT | CH | SP | CPX | AM | AU | CN | PEF | OFX | S |
|---|---|---|---|---|---|---|---|---|---|---|
| *Escherichia coli* | I | I | S | S | R | R | S | S | S | R |
| *Proteus mirabilis* | I | S | I | S | S | S | I | S | S | S |
| *Klebsiella aerogenes* | I | R | I | S | S | S | S | S | S | S |
| *Salmonella typhi* | S | S | S | S | I | R | S | S | S | S |
| *Shigella* | S | S | S | S | R | R | S | S | S | S |

**Legend: SXT** Sparfloxacin**, CH** Chloramphenicol, **SP** Streptomycin, **CPX** Ciprofloxacin, **AM** Amoxicillin, **AU** Augmentin, **CN** Tarivid, **PEF** Pefloxacin, **OFX** Gentamycin, **S** Septrin

***PCR analysis of Tetracycline B and Sulfonamide resistance genes***

The size of the amplified band using tetracycline resistance primer ranges from 700 to 300bp for the five bacteria isolates (Plate 1). *Salmonella spp.* has three bands which are 700bp, 400bp, and 300bp, *E. coli* has two bands 700bp and 400bp; *Klebsiella*Spp has three bands 700bp, 700bp, and 300bp. *Proteus spp.* has two bands 700bp and 300bp. *Shigella* Spp has one and with 300bp, All the bacteria have the band with 700bp except *Shigella* Spp. The size of the amplified band using the Sulfonamide resistance gene is 400bp for the five bacteria isolates (Plate 2). The result indicated that all 5 isolates showed positive results with the primer sulfonamide gene. This shows that the isolated bacteria possess the sul1 antibiotics-resistant gene.

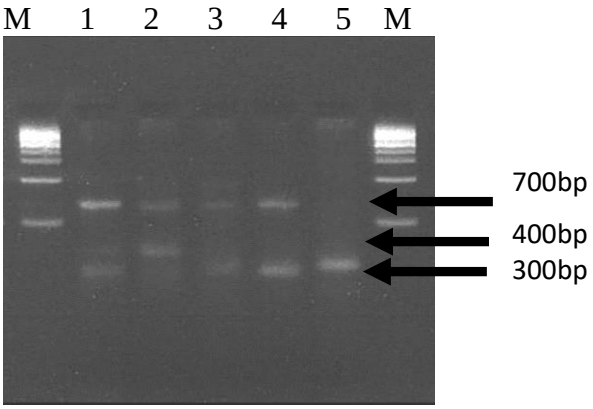


Plate 1. Electrophoresis gel of Tetracycline type(*B*) gene

1. *Escherichia coli* 2. *Klebsiella oxytoca* 3. *Proteus* 4. *Salmonella* 5. *Shigella* M marker

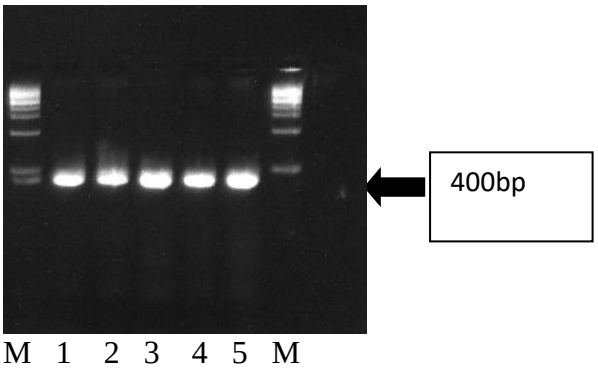


Plate 2. Electrophoresis gel of Sulfonamide (Sul1) gene

## DISCUSSION

The use of antibiotics as prophylaxis, therapeutic, and growth promoters has resulted in the deposition of its residues in food animals; meat, milk, egg and fish (Ottinger *et al.*, 2015). This deposition in food fish has been traced to the transfer of antibiotic resistance to man, reproductive disorders and carcinogenicity (Emmanuel *et al.*, 2014). From this study, gills had the highest concentration of bacteria which corroborates with results obtained by

Akinyemi *et al.,* (2016) and Chuah *et al.,* (2016). The most commonly found in the gut was the *Salmonella* spp. *Proteus* spp are generally found in the gut of *Clarias gariepinus*. *Salmonella* and *Shigella* were also obtained from research work such as Manhodo *et al.* (2018) and Sichew *et al.* (2014). *E. coli* was also isolated from the gut and gills of *Clarias gariepinus* (Efuntoye*et al.,* 2012). *Escherichia coli* was isolated from the gill and buccal cavity of *Clarias gariepinus* in the present research which is in agreement with a study by Sowunmi *et. al.,* (2008), who also reported the presence of *Klebsiella spp*. in the gill and buccal cavity.

*Escherichia coli* was found in the gill, skin, and buccal cavity of *Lutjanusagennes, Pseudotolithus elongates* and *Sphyraena barracuda* from Lagos Lagoon (Akinyemi and Buoro, 2011). *Proteus* spp are found mainly in the skin and gills of *Clarias gariepinus* (Akinyemi *et al.*, 2016). Budiati, (2013) reported that *Salmonella* was isolated from the skin, gill, and gut of *Clarias gariepinus*. The bacteria isolated from piggery and poultry dropping included *Escherichia coli, Proteus* spp., and *Klebsiella* spp. The presence of these bacteria could be due to contamination of the water body as previously reported by Adebayo-Tayo *et al*. (2008). Molecular studies reveal that all the isolates were resistant to the Sulfonamide gene, they all possess the 400basepair band, this antibiotic will not be useful to combat such disease in man when infected fish is eaten. Tetracycline type B does not have any specific band, all the bands shown were not specific so the organisms are susceptible to Tetracycline type and the drug could be used in treating the disease associated with microorganisms.

## CONCLUSION

The persistence of antibiotic-resistant genes in bacteria found on the skin, gill and gut of farmed fish will result in the inefficiency of antibiotics in treating fish diseases. *Salmonella* and *Shigella* which constitute 50% of the bacteria associated with *Clarias gariepinus* are pathogenic and the antibiotic like Sulfonamide cannot be used to treat infection as a result of taking fish that has such organisms. It will also lead to great economic loss on the part of the farmer as larger quantities of the antibiotic will be required to reduce the effect of such bacteria.

### Competing interests

We hereby declare that there was no competing interest among authors.

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