

Original Research Article

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Genetic Diversity Study of Bacteria Associated with *Clarias gariepinus* from Ebute-Ibooro on Yewa River using Random Amplified Polymorphic DNA

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ABSTRACT

Fish is a relatively inexpensive source of protein and an important source of foreign exchange in many regions of world and water is the physical support in which they carry out their life functions. Genetic study of bacterial isolates found in the guts, gills and skin samples of catfish obtained from Ebutelbooro landing site of Yewa River was carried out using Random Amplified Polymorphic DNA – Polymerase Chain Reaction Techniques. Morphometrics, weight, length of the fishes were recorded and water quality parameters of the water body were also recorded. Bacterial isolates were obtained from samples grown in culture medium using Nutrient agar. Subculturing was done in order to obtain the pure isolates. Five RAPD primers were used, a total of number of 62 markers were generated with 54 being polymorphic and 8 being monomorphic. Two main groups were observed from the dendrogram derived from the RAPD results. Group 1 consists of 1 organism while group 2 consists of 19 organisms. Group 2 were further divided into sub groups with each sub group having its own constituent of organisms. Haemolysis test carried out on this bacteria isolates to determine their pathogenicity showed that 12.5% of the organisms indicated complete haemolysis, 37.5% indicated incomplete haemolysis while the remaining 50% indicated no haemolysis. The results show that RAPD fingerprinting is one of the best molecular tools for identification.

Keywords

Clarias gariepinus,
Bacteria,
RAPD
markers,
Haemolysis.

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Introduction

Fish is an inexpensive source of protein and an important cash crop in many regions of world and water is the physical support in which they carry out their life functions such as breathing, excretion of waste, feeding, maintaining salt balance and reproduction, swimming, breeding, digestion and excretion (Bronmark and Hansson, 2005).

Claridae is the name of the family to which most catfishes belong to. This claridae family is also known as “omnivorous scavengers”. This categories of fish lack scale and they possess barbels (feelers). They also have pectoral fin, head, mouth and respiratory organs. These enable the fish to stay for some time outside water, making

use of atmospheric oxygen (Sydenham, 1975). Catfish is of considerable importance; many of the larger species are farmed and fished for food. Many of the smaller species particularly the genus *Cyprinus* are important in the aquarium hobby (Nelson, 2006).

Aquatic microbes cause infection in fishes. The entry and establishment of pathogenic microbes are facilitated through the injuries caused by parasitic infestation. Fish and Shellfish not only transmit disease to man but are themselves subject to many diseases and capable of transmitting many of the established food borne microbial infections and intoxications (FAO, 1974). *Trichodina* and *Trichodinella* which make up the *Trichodina* complex, *Gyrodactylus* spp, *Edward siellaictaluri*, cysts of *Henneguya* spp are some of the infectious agent of the clariids.

The use of the RAPD analysis is sufficiently discriminatory for the typing of the *E. coli* isolates. Characterization of *Escherichia coli* isolated from cultured catfish by antibiotic resistance and RAPD analysis RAPD-PCR (Samuel, 2011). The number of DNA bands produced for a given primer ranged from 1 to 5 with molecular sizes ranging from 200 bp – 1500 bp. The possible number of RAPD patterns was estimated by the changes of one or more clear bands or band size. There were 9 RAPD patterns (P1-P9) observed using GEN15009 primer. The RAPD-PCR analysis using combination of primers GEN15009 and GEN15010 allowed all the strains of *E. coli* to be differentiated into 13 different genome types, U1 to U13. This indicates that by using combination of primers, the *E. coli* strains can be differentiated into more groups compared to when using these primers individually. Sarkar (2012) while studying Characterization of *Aeromonas hydrophila*

through RAPD-PCR and SDS-PAGE Analysis isolated *Aeromonas hydrophila* from fish (*Labeo rohita*) in pond water, river water, raw meat of chicken and mutton and raw cow milk were characterized through Random Amplified Polymorphic DNA (RAPD) analysis and Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) of cellular proteins. RAPD-PCR amplification of bacterial DNA was done by using ten random primers (OPA-01 to 10) and found some distinct banding pattern on agarose gel. RAPD profile was studied with each isolate and absolute polymorphism indicating its usefulness as an ideal tool for molecular characterization. Other methods like morphological, serological and biochemical characterization gives contradictory results and total cellular protein profiling does not show any significant polymorphism for identification and discrimination.

Molecular characterization of Enterotoxigenic *Bacillus cereus* species isolated from tropical marine fishes using RAPD markers by Prasad (2014) selected some tropical fishes which were shark, anchovy, ribbon fish, sole, mackerel, seer, tuna, snapper, sardine, and pomfret commercially available in Bangalore region. The bacteria were isolated on to marine agar. All the strains were morphologically and biochemically characterized to be *Bacillus cereus*. A total of 10 primers of the OP series were used in the present study. RAPD PCR using primers OPB-08 and OPD-20 generated maximum banding pattern in this study showed high polymorphism between selected *Bacillus cereus* species. Visualizing the physical appearance of hemolysis in cultured blood samples may be used as a tool to determine the species of various Gram-positive bacteria infections. Haemolysis is the

breaking down of the red blood cells. It is one of the major aspects of determining the pathogenicity of an organism. Pathogenicity is the ability of an organism to invade and destroy human cells, tissues and metabolism.

Materials and Methods

Sample Collection

Bacteria samples were collected from gills, gut and skins of Catfish *Clarias gariepinus* in Ebute Ibooro landing site of Yewa River with the aid of swab sticks. 16 adult catfish were collected from the river and swab sticks were used to obtain samples from the fish. Morphological characteristics of the fish such as the head length, total length and standard length were determined. The weight of an individual fish was also determined and the water quality features of the area such as dissolved oxygen, temperature and pH were taken in situ.

DNA extraction and RAPD-PCR

Bacteria isolates grown overnight were transferred to eppendorf tube and it was spun down at 14,000rpm for 2mins. DNA was extracted using the CTAB method Akinyemi and Oyelakin (2014). The quality and integrity of the DNA was fractionalized on 1.0% agarose gels. The reaction mix was carried out in 20 μ l final volume containing 60ng - 80ng genomic DNA, 0.1 μ M of the primers, 2mM MgCl₂, 125 μ M of each dNTP and 1 unit of Taq DNA polymerase. The thermocycler profiles has an initial denaturation temperature of 3mins at 94⁰C, followed by 45 cycles of denaturation temperature at 94⁰C for 20seconds, annealing at 37⁰C for 40 seconds and primer extension at 72⁰C for 40 seconds, followed by final extension temperature at 72⁰C for 5 mins was added. PCR amplicon

electrophoresis was carried out by size fractionation on 2.0% agarose gels.

Statistical Analysis

The bands obtained from the gel was transferred to numerical figure, 1 represents presence of a band while 0 is absence of a band, it was later subjected to analysis using NTSYS software to draw the dendrogram for the bacteria isolates.

Haemolysis

10g of Nutrient agar was weighed and dissolved in 100ml of distilled water and mixed thoroughly. The mixture was autoclaved for sterilization at 121⁰C for 15 minutes. The Nutrient medium was then brought out to cool to the point that it could not burn the skin. Human blood was added

Results and Discussion

Table 1 showed the various standard lengths, total lengths and head lengths with their weights inclusive. The mean total length, mean standard length, mean head length, mean weight of the fish was determined to be 47.00 \pm 12.00(cm), 41.25 \pm 6.50(cm), 10.47 \pm 2.00(cm), 785.50 \pm 713.00(cm) respectively. Figure 2 showed the electrophoresis gel for RAPD primer OPH 08. Plate 1 showed the DNA of the bacteria isolates visualized and photographed on UV light source while plate 2A and 2B showed the PCR amplification visualized and photographed on UV light source. It can be deduced from Table 2 that there were 54 polymorphic markers generated from the five RAPD markers. There were also 8 monomorphic markers from the primers. A total number of 62 markers were generated. Twelve alleles from the first primer OPB-12, 13 alleles from the second primer OPB-20 and 17

alleles from the third primer OPH-08. Also, there were 5 alleles from OPH 12 and 7 alleles from OPH 09. Figure 4 showed the dendrogram for the 20 bacteria isolates. At 50%, the organisms showed a lot of similarities but at 56.5%, there was a difference and the organisms were divided into 2 groups with organism 11 standing in group 2 and the other organisms in group 1. At 69%, the organisms were divided into subgroups with organism 2, 4, 6 and 7 are in a particular group and the rest in another groups. Table 4 showed the result of the ability of the isolated organism to produce Lysins which indicates the pathogenicity of such organisms. 12.5% of the organisms showed complete haemolysis, 37.5% showed incomplete haemolysis while the remaining 50% showed no haemolysis.

The RAPD technique constitutes an efficient tool for the study of the DNA polymorphism. It involves the amplification of random segments of genomic DNA by polymerase chain reaction (PCR) using short single primers of arbitrary sequence. RAPD requires very small quantities of DNA while no cloning, sequencing or hybridization is necessary. For these reasons and with the condition that only a little within-group polymorphism exist, it has a marked advantage over other molecular techniques generally used for genomic studies. Random amplified polymorphic DNA- polymerase chain reaction (RAPD-PCR) was used to discriminate bacteria isolated from fishes in the rivers considering their guts, skins and gills.

There were 54 polymorphic markers generated from the five RAPD markers. 8 monomorphic markers were derived from the primers. A total number of 62 markers were generated. 12 alleles from the first primer OPB-12, 13 alleles from the second primer OPB-20 and 17 alleles from the third

primer OPH-08. Also, there were 5 alleles from OPH-12 and 7 alleles from OPH-09. Percentage polymorphisms of primers OPB-12, OPB-20, OPH-08, OPH-12 and OPH-09 were 100, 100, 89, 63, and 70 respectively. Polymorphism indicates that the alleles are together and that they are coding for the same thing. At 50%, the organisms showed a lot of similarities but at 56.5%, there was a difference and the organisms were divided into 2 groups with organism 11 standing in group 2 and the other organisms in group 1. At 69%, the organisms were divided into subgroups with organism 2,4,6 and 7 are in a particular group and the rest in another groups.

The result above is in agreement with a very similar study by Prasad (2014) in order to determine the molecular characterization of Enterotoxigenic *Bacillus cereus* species isolated from tropical marine fishes using RAPD markers 85.71% and 56.25% polymorphism was observed in OPB-8 and OPD-20 respectively, which indicates high genetic variation among the *Bacillus cereus* strains isolated from tropical marine fishes. According to dendrogram analysis of band pattern generated by Primer OPB-8, *Bacillus cereus* Strain 4 and 3 were closely related whereas strain 4 and Strain 2 were genetically more diverse. On the other hand, dendrogram obtained from OPD-20 Primer RAPD-PCR data showed that *Bacillus cereus* Strain 2 and Strain 4 were genetically more similar whereas Strain 4 and Strain 1 were least similar to each other.

Samuel (2011), in a study to determine the Characterization of *Escherichia coli* isolated from cultured catfish by antibiotic resistance and RAPD analysis RAPD-PCR used primers GEN15009 and GEN 15010. The number of DNA bands produced from a given primer ranged from 1 to 5 with molecular sizes ranging from 200 bp – 1500

bp. The possible number of RAPD patterns was estimated by the changes of one or more clear bands or band size. There were 9 RAPD patterns observed using GEN15009 primer. The RAPD-PCR analysis using combination of primers GEN15009 and GEN15010 allowed all the strains of *E. coli* to be differentiated into 13 different genome types. This indicates that by using combination of primers, the *E. coli* strains can be differentiated into more groups compared to when using these primers individually. Hence, combined primers provide increased sensitivity towards genetic variations. Some of the *E. coli* strains have indistinguishable RAPD fingerprinting profiles which suggest that they may be closely related or have no genetic differences. Certain isolates did not produce band in this analysis mainly because there were no sequence in the bacterial DNA which is complementary to the sequence of the primer. The dendograms generated clearly established the genetic relatedness of the *E. coli* strains, the information that may be useful in epidemiological and population studies.

12.5% of the organisms showed complete haemolysis, 37.5% showed incomplete haemolysis while the remaining 50% showed no haemolysis. One strain of *Aeromonas hydrophila* was tested which showed complete haemolysis. There was also one strain of *Pantoea thophilus*, *Serratia nematodiphila*, *Shigella boydii*, *Bacillus spp.*, *Serratia liquefaciens* and *Morganella morganii* which showed incomplete haemolysis. Two strains of *Pseudomonas spp.* were tested showing complete and no haemolysis. Four strains of

Citrobacter freundii were tested showing 75% no haemolysis and 25% incomplete haemolysis. Complete haemolysis shows that they are pathogenic to the fishes and they can also be pathogenic to humans when consumed. There were differences between strains of certain bacteria over the type of haemolysis they exhibit. This concurs with a similar work by Uaboi-Egbenni *et al.*, (2010) while examining the significance of pathogenic bacteria in the gut of swimming crab, *Callinectes ssp* obtained from Lagos lagoon and market samples stored at freezer temperature (0°C) conducted a blood haemolysis test which showed that the bacterial species isolated from the gut of the swimming crabs are usually found as pathogens associated with the guts of warm-blooded animals

Frazier and Westhoff (1988); Owhe-Ureghe *et al.* (1993) in their studies have implicated *Bacillus cereus* and *Bacillus subtilis* in infections and food poisoning. The pathogenesis of *Escherichia coli* and its occurrence as faecal contaminant has been reported (Frazier and Westhoff, 1988). Other workers have reported virulence and pathogenic *E. coli* strains in different animals. In humans (Reid *et al.*, 2000; Kaper, 2004); in avian (Audouin gulls) (Carmada *et al.*, 2007); in poultry (JanBen *et al.*, 2001). Hussein (2006) reported the prevalence and pathogenicity of shiga toxin-producing *E. coli* in beef and their products. Schmid-Hempel and Frank (2007) observed in their study that in *E. coli* as small as 10 cells are infectious enough to establish disease condition, whereas the dosage can be very high in other pathogens like *Vibrio cholera* (10³-10⁸ cells).

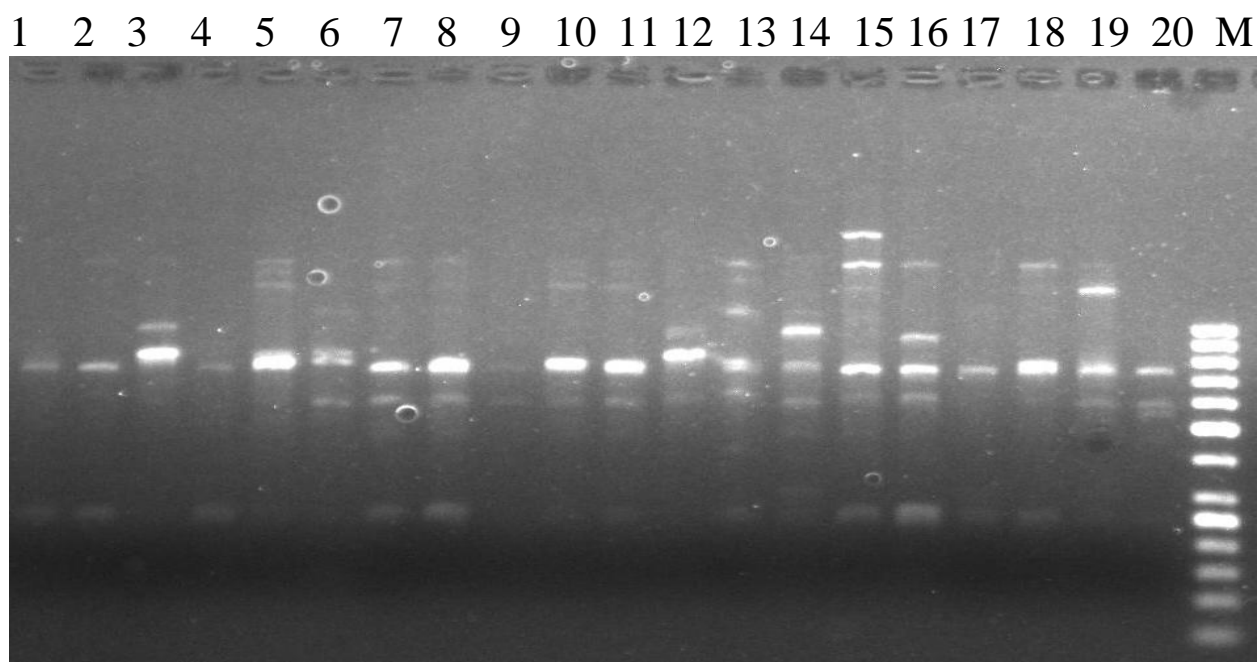


Plate.1 Electrophoresis gel for RAPD primer OPH 08

Table.1 showing the measurement of the morphometric features of the fish

| Parameter | Mean value \pm SE |
|-----------------|-----------------------|
| Mean weight | 785.50 \pm 81.20(g) |
| Total length | 47.00 \pm 0.97(cm) |
| Standard Length | 41.25 \pm 0.84(cm) |
| Head length | 10.47 \pm 0.30(cm) |

Table.2 Primer Sequences and percentage polymorphism

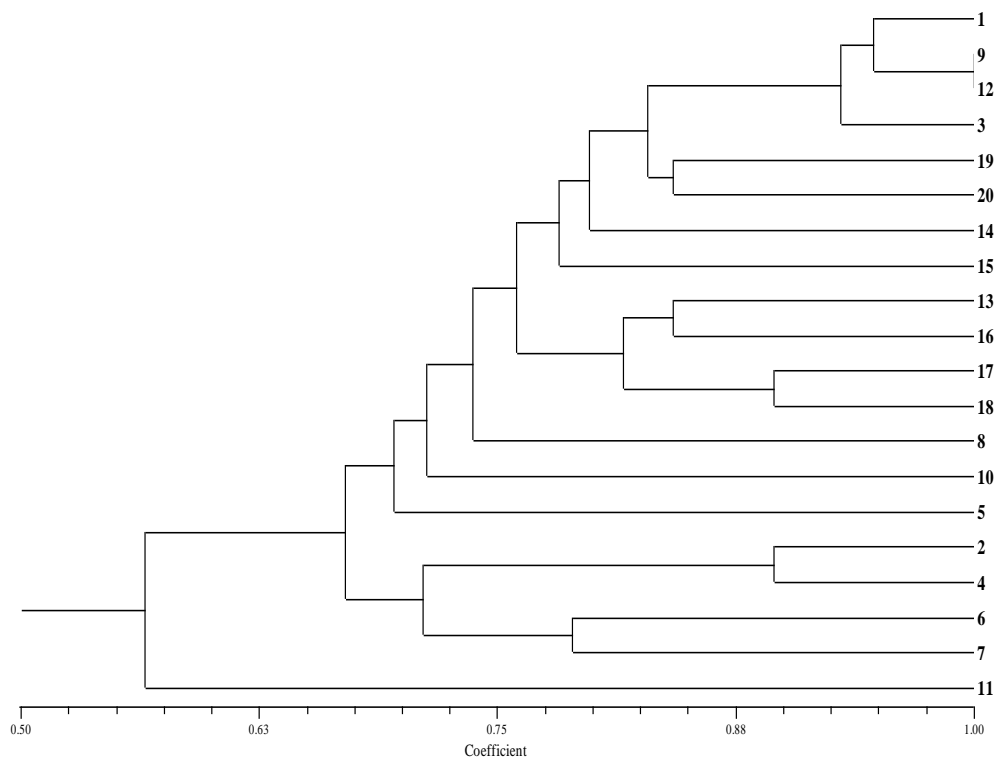
| S/N | Primer name | Primer Sequences | No of Monomorphic markers | No of Polymorphic markers | Total no of markers | Percentage Polymorphism |
|-----|-------------|------------------|---------------------------|---------------------------|---------------------|-------------------------|
| 1 | OPB 12 | CCTTGACGCA | Nil | 12 | 12 | 100 |
| 2 | OPB 20 | GGACCCTTAC | Nil | 13 | 13 | 100 |
| 3 | OPH 08 | GAAACACCCC | 02 | 17 | 19 | 89 |
| 4 | OPH 12 | ACGCGCATGT | 03 | 05 | 08 | 63 |
| 5 | OPH 19 | CTGACCAGCC | 03 | 07 | 10 | 70 |
| | Total | | 08 | 54 | 62 | |

Table.3 Results showing the haemolysis of the isolated bacteria

| Organism | Tissues | Type of Haemolysis |
|-----------|---------|--------------------|
| Sample 1 | Gill | β |
| Sample 2 | Gill | α |
| Sample 3 | Skin | α |
| Sample 4 | Skin | γ |
| Sample 5 | Gills | α |
| Sample 6 | Gut | α |
| Sample 7 | Skin | β |
| Sample 8 | Gut | α |
| Sample 9 | Skin | γ |
| Sample 10 | Skin | γ |
| Sample 11 | Gill | γ |
| Sample 12 | Skin | γ |
| Sample 13 | Gut | α |
| Sample 14 | Skin | α |
| Sample 15 | Gill | β |
| Sample 16 | Skin | γ |
| Sample 17 | Skin | γ |
| Sample 18 | Skin | γ |
| Sample 19 | Gut | α |
| Sample 20 | Gill | α |

KEY: α – Incomplete haemolysis; β – Complete haemo;ysis; γ – No Haemolysis

Fig.1 Dendrogram for the 20 Bacteria Isolates



In conclusion, the presence of these organisms were known to be a major cause of infections in human and animals as it signified a potential threat to farmers who tend to source for their seeds in the wild, fishermen and also consumers through potential transfer of resistance to human and animal pathogens. RAPD marker could be used to study the genetic diversity of bacteria associated with fishes in the wild pointed towards the amplicons generated by OPH-08. The species specificity can be confirmed only by checking these primers with isolates of other similar bacteria. This was also evident in the dendrogram showing the genetic relationship between these organisms. Large number of RAPD genotypes can be an ideal method for species identification (Hadrys *et al.*, 1992). The results show that RAPD fingerprinting is one of the best molecular tools for identification. Nevertheless, RAPD fingerprinting allows the identification of strains but the high variability limits its potential as an aiding method for species identification.

The discrimination of bacteria species can be done by haemolysis tests using blood agar medium. Other than ultramicroscopic, morphological and molecular studies this experiment also conveys the species differentiation due to its lytic activity. Thus, blood agar medium can be a useful medium for pathogenic bacteria survey and investigation. The present review has proved the importance of blood agar utilization in diagnostics and disease analysis.

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