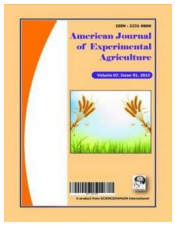

American Journal of Experimental Agriculture
11(6): 1-7, 2016, Article no.AJEA.19984
ISSN: 2231-0606

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Molecular Characterization and Haemolysis of Bacteria Associated with *Tilapia zilli* from Ijaka-Oke Location on Yewa River

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Authors' contributions

This study was conducted in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJEA/2016/19984
Editor(s):
(1) Lanzhuang Chen, Laboratory of Plant Biotechnology, Faculty of Environment and Horticulture, Minami Kyushu University, Miyazaki, Japan.
Reviewers:
(1) Maria del Carmen Bermudez Almada, Center for Food Research and Development, Mexico.
(2) Graciela Castro Escarpulli, National Polytechnic Institute, Mexico.
Complete Peer review History: http://sciencedomain.org/review-history/13754

Original Research Article

Received 6th July 2015
Accepted 16th January 2016
Published 18th March 2016

ABSTRACT

Fishes are a source of animal protein, rich in minerals, amino acids, and essential fatty acids. Fishes can be confronted with microbial contamination in their natural habitat and their transformation products can be a source of microbial infections and food poisoning. In the natural habitat, microorganisms are usually found on the entire outer surface (skin and gills) and in the intestines of fishes. Genetic diversity study of the bacteria isolates was done using RAPD technique. Bacteria were isolated from the gills, gut and skin of *Tilapia zilli* from Ijaka-Oke location on Yewa River. Morphometric characteristics of the 20 *Tilapia zilli* sampled was done. DNA were extracted from 20 bacteria isolates using CTAB method, PCR amplification of the 20 isolated DNA was carried out using five RAPD primers. Haemolysis of the 20 bacteria was also carried out using 7% of sheep blood in Nutrient Agar and it was used to culture a pure colony of the isolates and incubated at 37℃ for 18 hours. The mean total length of the fishes was 30.00±9.00 (cm), weight

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220±4.3 (g), head length 7.4±2.00 (cm) and standard length 21.30±4.50 (cm). The water quality parameters were also recorded with the water temperature at 22.9^0C, the pH of the water was 6.1 and Dissolved Oxygen 6.89 mg/l. Fifty – two (52) polymorphic markers were generated from the 5 RAPD markers. There were also 15 monomorphic markers from the primers. A total number of 67 markers were generated. The haemolysis showed that none of the bacteria displayed complete haemolysis.

Keywords: Bacteria isolates; RAPD-PCR; genetic diversity; haemolysis; Tilapia zilli.

1. INTRODUCTION

Fish constitute an important part of the daily diet of people in many countries and has been one of the major food sources for human for many centuries [1]. Fishes are a source of animal protein, rich in minerals, amino acids, and essential fatty acids. Therefore, fishes are a valuable contribution to the diet of malnourished populations [2]. There is an appreciable increase in the demand for fish which is the cheapest source of animal protein [1]. However, fishes can be confronted with microbial contamination in their natural habitat and their transformation products can be a source of microbial infections and food poisoning. In the natural habitat, microorganisms are usually found on the entire outer surface (skin and gills) and in the intestines of fishes. Bacterial flora of freshly caught fishes depends on the environment in which they were captured. Furthermore, [3] reported that the level of contamination of a fish at the time of capture, depends largely on the microbiological quality of the water and the environment in which it was harvested. Water body can be extremely polluted by animal and human wastes thereby containing several microorganisms which may contribute to it pollution. The presence of various organisms which are particularly pathogenic to human in fish is only suggestive, its significance in initiation of human disease is unknown [4]. However, the presence of potential human pathogens suggests the fish consumed raw, undercooked and improperly handled may cause disease to susceptible individuals. According to [5], bacterial infections and fish disease are very common and are one of the most difficult health problems to deal with and also they are usually transmitted by fish that have made contact with other diseased fish. Bacteria tend to enter the fish body through the skin or gills or it can stay on the body surface [5]. Therefore the objective of this research work is to characterize and identify the bacteria associated with *Tilapia zilli* and study the genetic relatedness of bacteria associated with this species and also to evaluate the bacteria haemolysis.

2. MATERIALS AND METHODS

2.1 Sample Collection and Morphometric Characteristics

The study was carried out on Yewa River, it lies approximately within latitudes 60° 22′N and longitudes 20° 50′E of the Greenwich Meridian. It has a basin with total catchment area of approximately 5000 km^2 and is located within the West African tropical climate. Ijaka-Oke is a landing site along Yewa River in Yewa North Local Government area of Ogun State, Nigeria. Bacterial isolates were taken from twenty *Tilapia zilli.* These were taken from the gut, gills and skin using swab sticks, the swab sticks were kept in a cooling chest in other to preserve the samples collected according to [6]. Morphometric characteristics of the fish such as the Total Length, Standard Length and Head Length were also determined. The weight of an individual fish was also determined and physico-chemical parameters such as Dissolved Oxygen, Temperature and pH of the water were taken.

2.2 Bacteria Isolation and Extraction of DNA

The media for culture was weighed according to the specification. Nutrient agar was measured and dissolved in appropriate volume of distilled water inside a conical flask. It was covered with aluminum foil and autoclaved at 121℃ for 15 minutes. The agar media was allowed to cool and was dispensed into Petri dishes. The swab sticks used for taking samples of the skin, gut and gill were then streaked on the culture media already placed in the Petri dishes. The culture media were incubated overnight at 37℃. Pure colonies were prepared from the original culture using Nutrient Agar and Mac Conkey Agar. Pure colonies of bacterial isolates were grown overnight in liquid broth and transferred to eppendorf tube. The tube was spun down 14,000 rpm for 2 min, the supernatant was discarded and the extraction of DNA was done using CTAB method [6]. The DNA was later resuspended in

100 µl of sterile distilled water. DNA concentration of the samples were measured and the genomic purity were determined. The DNA was further checked on 1.0% agarose gel and was visualised on UV light source.

2.3 RAPD-PCR Amplification and Electrophoresis

RAPD is the acronym for Rapid Amplified Polymorphic DNA. It is a genetic technique of using neuclotides (8 – 12), to form several arbitrary short primers. These are then amplified by the PCR using large templates of genomic DNA.

PCR amplification was done using MJ Research Thermal Cycler (PTC-200 model). The RAPD primers used for PCR amplification were OPB-12, OPB-20, OPH-08, OPH-12 and OPH-19. The PCR reaction mix was carried out in 20µl final volume containing 40 - 80ng genomic DNA, 0.1 µM of the primers, 2 mM $MgCl_2$, 125 µM of each dNTP and 1 unit of Taq DNA polymerase. The thermocycler profiles has an initial denaturation temperature for 3 min at 94℃, followed by 45 cycles of denaturation temperature at 94^0C for 20 seconds, annealing at 37℃ for 40 seconds and primer extension at 72℃ for 40seconds, followed by final extension temperature at 72℃ for 5 minute was added. PCR amplicon electrophoresis was carried out by size fractionation on 1.2% agarose gels. The gel electrophoresis was done at 100V for 2 hours. The DNA was visualized and photographed on UV light source.

2.4 Haemolysis

The media used was prepared using 7% sheep blood in Nutrient Agar, the media was used to culture a pure colony of the isolate. It was incubated at 37℃ for 18 hours and was observed for haemolysis. After sometime a clear zone of incubation was observed and the diameter was measured.

2.5 Statistical Analysis

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

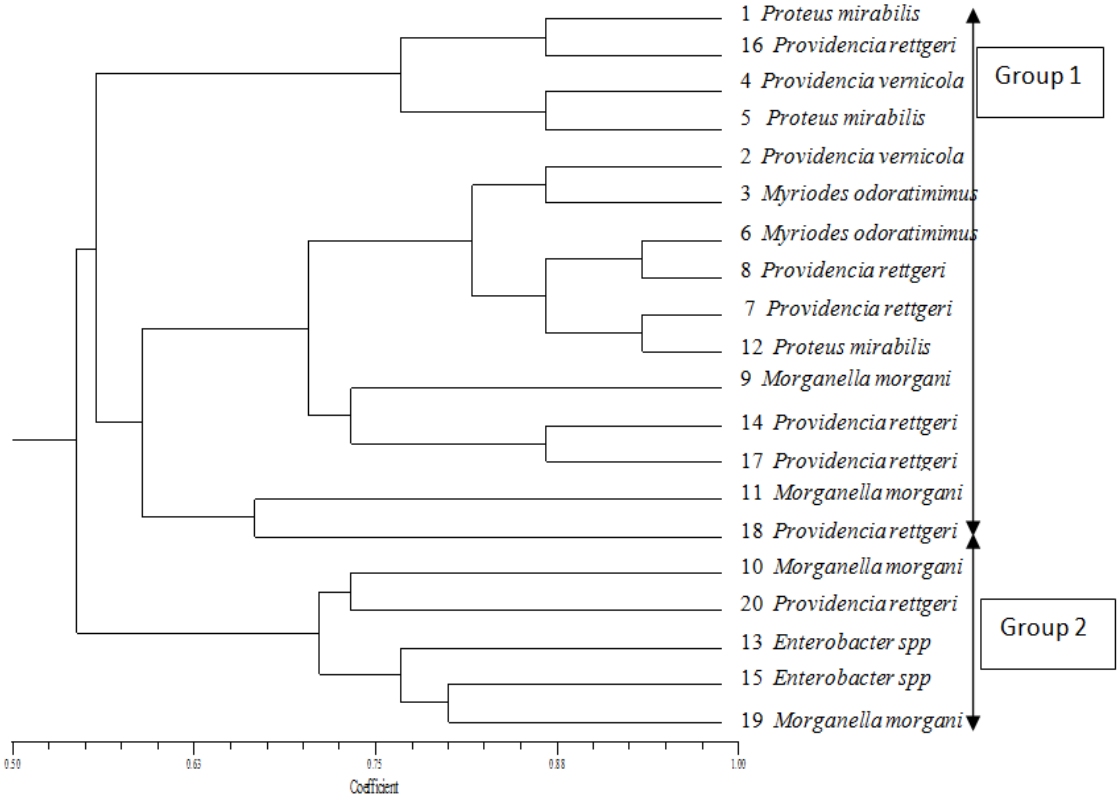

Fig. 1. Cluster analysis of the 20 selected isolates

3. RESULTS AND DISCUSSION

3.1 Physico-chemical Parameters and Morphometric Characteristics

The Physico-chemical parameters recorded at the study location are temperature, pH and Dissolved Oxygen with values of 22.1 - 22.9℃, 6.6 mg/l, and 6.89 mg/l respectively (Table 1). The mean total length, mean standard length, mean head length, mean weight of the fish were determined to be 30.00±9.00 (cm), 21.30±4.50 (cm), 7.47±2.00 (cm), 220±4.3(g) (Table 2). The temperature range of between 22.1℃-22.9℃ is within UNEP standard [7] and is similar to [8] and [9]. The pH is within the range 6.5-9.5 (UNEP standard) [7]. Aquatic organisms are affected heavily by water pH because virtually all their activities are dependent on pH [10,9]. Dissolved Oxygen (DO) is relatively high with value of 6.85 mg/l [11].

Table 1. Water quality parameters of Ijaka-Oke Location on Yewa River

Parameter	Values	WHO (2006)
Dissolved oxygen	6.89 mg/L	5 mg/L
Temperature	22.1-22.9℃	<40℃
pH	6.1-6.9	6.5-9.5

Keys: WHO = World Health Organization, ℃ = Degree Celsius

3.2 PCR Amplification Using RAPD Primers

There were 52 polymorphic markers generated from the five RAPD markers. There were also 15 monomorphic markers from the primers. A total number of 67 markers were generated. Sixteen alleles were generated from the first primer OPB-12, 14 alleles from the second primer OPB-20 and 17 alleles from the third primer OPH-08 (Table 3). Fig. 1 showed the clustering analysis for the 20 strains. At coefficient point 54.8, the isolates formed two groups. Sample 19, 15, 13, 20 and 10 formed a group while the others formed another subgroup. The cluster analysis shows that sample 1 and 16 are closely related, samples 4 and 5 are related, 2 and 3 are closely related, 6 and 8 are related. It can be deduced from the cluster analysis that sample 1 is extremely different from 15 and 19.

Table 2. The morphometric features of the fish sampled

Morphometric features	Mean values ± SE
Total length	30.00±9.00
Standard length	21.30±4.50
Head length	7.47±2.00
Average weight	220±4.3

Weight in grams, Length in centimeter

3.3 Haemolytic Pattern of Bacteria Isolated

Table 4 showed the haemolytic pattern of fish bacteria isolated. None of the bacteria isolated displayed complete haemolysis. *Proteus mirabilis, Myriodes odoratimimus, Providencia rettgeri, Morganella morgani, Enterobacter* spp all displayed partial haemolysis while *Providencia vernicola displayed no haemolysis* out of the 6 bacterial strains identified, 5 exhibited partial haemolysis (83.05%). The five (5) bacterial strains which exhibited haemolysis are *Proteus mirabilis*, *Myriodes odoratimimus, Providencia rettgeri, Morganella morgani* and *Enterobacter spp*; while the sixth (*Providencia vernicola*) was non-haemolytic in Blood Agar.

3.4 Discussion

The aim of this study was to access and characterize the bacteria associated with *Tilapia zilli* isolated from Ijaka-Oke location on Yewa River in Ogun state, Nigeria.

Table 3. Primer sequences and percentage polymorphism

S/N	Primer name	Primer sequence	No of monomorphic markers	No of polymorphic markers	Total no of markers	Percentage polymorphism
1	OPB 12	CCTTGACGCA	02	14	16	85
2	OPB 20	GGACCCTTAC	04	10	14	74
3	OPH 08	GAAACACCCC	01	16	17	94
4	OPH 12	ACGCGCATGT	03	05	08	63
5	OPH 19	CTGACCAGCC	05	07	12	58
	Total		15	52	67	

Table 4. Haemolytic pattern of bacteria isolated

Isolate no.	Isolated organism	Location	Haemolysis
01	*Proteus mirabilis*	Skin	α
02	*Providencia vernicola*	Skin	γ
03	*Myriodes odoratimimus*	Skin	α
04	*Providencia vernicola*	Gill	α
05	*Proteus mirabilis*	Gill	γ
06	*Proteus mirabilis*	Gill	γ
07	*Providencia rettgeri*	Gut	α
08	*Providencia rettgeri*	Gut	α
09	*Morganella morgani*	Gut	α
10	*Morganella morgani*	Gut	α
11	*Morganella morgani*	Gut	γ
12	*Proteus mirabilis*	Skin	γ
13	*Enterobacter spp*	Skin	α
14	*Providencia rettgeri*	Skin	α
15	*Enterobacter spp*	Skin	γ
16	*Providencia rettgeri*	Gut	γ
17	*Providencia rettgeri*	Gill	γ
18	*Providencia rettgeri*	Gill	γ
19	*Morganella morgani*	Gill	γ
20	*Providencia rettgeri*	Skin	γ

Keys: α=partial haemolysis, γ=no haemolysis

Physico-chemical parameters were recorded at the location. The parameters recorded include temperature, dissolved oxygen and pH. The temperature recorded at the location ranged between 22.1 - 22.9℃, this result is within UNEP standard and it is similar to [8,9]. The pH is within the range 6.5-9.5 (UNEP standard). Aquatic organisms are affected heavily by water pH because virtually all their activities are dependent on pH [10]. This value recorded is normal for aquatic lives and hence it minimal effect on acidity [9]. Dissolved Oxygen (DO) is relatively high with a value of 6.85 mg/l. Dissolved Oxygen is usually used to measure the degree of pollution by organic matters. Organic substances sometimes act as agents of destruction as well as self-purification of water bodies [11]. The amount of dissolved oxygen present in water is highly dependent on the water temperature (warmer water contains less dissolved oxygen than colder water). The dissolved oxygen value recorded is slightly above the UNEP standard limit of 5mg/l for sustenance of aquatic life, any value below the standard will affect aquatic life adversely [7].. Having a concentration below 2 mg/l will eventually lead to death of most fishes [11].

There are a lot of molecular typing methods for accessing the differences between bacterial isolates. The main criteria determining the appropriate typing methods are their cost, type, simplicity, constancy of performance and reproducibility. RAPD-PCR is quick, cheap, relatively simple to achieve and was used in this study to differentiate the 20 bacterial isolates DNA. The bacteria isolated include the genera *Proteus, Providencia, Myriodes, Morganella and Enterobacter*. Fish skin, guts and gill are a reliable non-destructive source of DNA. Though there are some difficulties due to consistency and small size of these organs/tissues which can lead to a low amount and poor quality of DNA recovery. As stated by [12], tissues homogenization can be an efficient method in liquid nitrogen to isolate significant DNA on hard consistent tissue. Furthermore, comparing these results, 1.0% agarose gel of DNA samples obtained from fish in Plate 1 from lane 2, 4, 5, 9, total DNA isolated from the skin, gill and gut showed *Providencia vernicola*, well 4, 11, 14 and 15 showed *Morganella morgani*, well 5 showed *Myroides odoratimimus* while well 9, 10, 12, 13, 18, 19, 20 showed *Providencia rettgeri.* These results are in agreement with that of [13], who stated that the patterns of similarities and differences between population showed broad agreement across primers and the overall similarity level varied between primers.

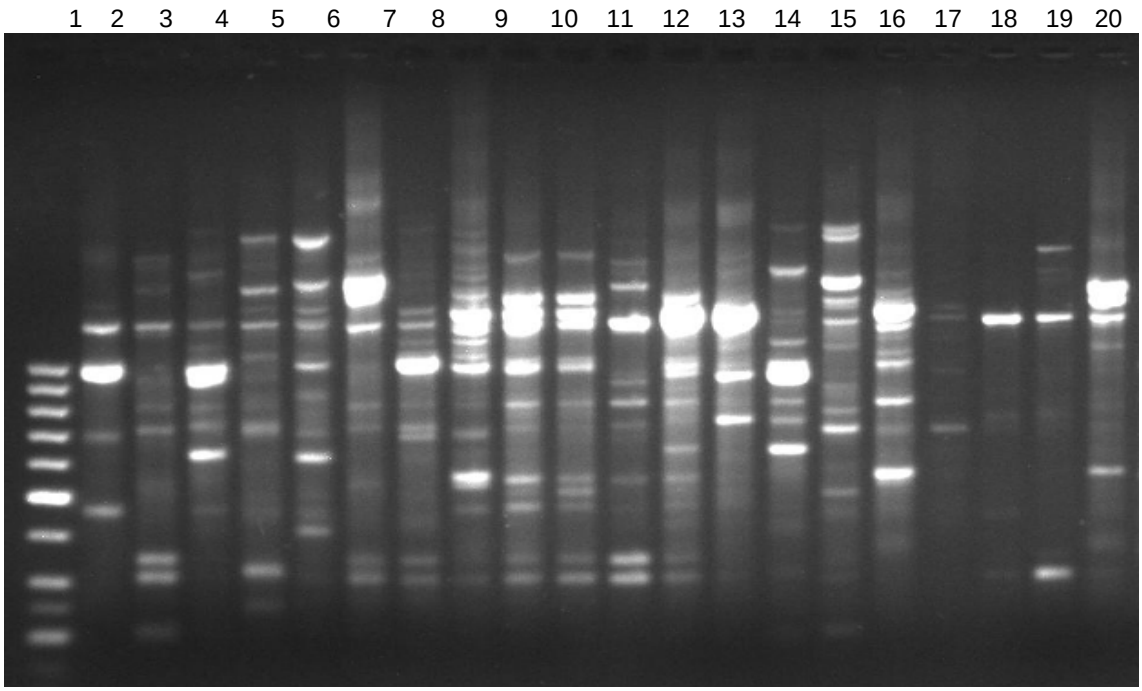

Plate 1. Electrophoresis gel for RAPD primer OPB 12

Haemolytic proteins are isolated from pathogenic bacteria and *β*-haemolysin is a very important bacterial virulence factor, Haemolysin and related proteins containing cystathionine *β* synthase (CBS) domains are bacterial toxins that tend to function by assembling identical subunits in a membrane-spanning pore.

Out of the 6 bacteria strains identified, 5 (83.05%) exhibited partial haemolysis, the five (5) bacteria strains are *Proteus mirabilis*, *Myriodes odoratimimus, Providencia rettgeri, Morganella morgani* and *Enterobacter* spp; while only *Providencia vernicola* was non-haemolytic in blood agar. In line with this result, [14] observed and stated that isolates which phenotypically tested negative for haemolysin production may eventually become positive when another organism Blood Agar is used. Furthermore, the concentration of the blood in the agar may influence the displayed haemolysis; low concentrations may make α- or β-haemolysis difficult to determine while high concentrations may cause β-haemolytic organisms appear as non-haemolytic organisms [15]. According to [16], haemolysin can cause damage to red blood cell and other cell types, such as neutrophils or leukocytes, by two different models of action which involve a phospholipase enzyme or a pore-forming protein.

4. CONCLUSION

This present study showed that the genetic diversity of the bacteria isolated from the gut, gills and skin of *Tilapia zilli* from Yewa River using the molecular approach of RAPD-PCR is very precise, useful and less expensive. The bacteria identified in the guts include strains of *Providencia rettgeri and Morganella morgani*. The bacteria identified in the gills include strains of *Morganella morgani and Providencia rettgeri*. While the strains identified on the skin include *Proteus mirabilis*, *Myriodes odoratimimus, Providencia rettgeri, Morganella morgani, Enterobacter spp* and *Providencia vernicola. Providencia rettgeri* recorded the highest percentage occurrence of 83.5% in the skin, gut and gills.

This study also shows that there are both opportunistic and pathogenic bacteria species in the gills, gut and skin of *Tilapia zilli* at Ijaka-Oke location on Yewa River which could be involved in causing diseases in the fish and also could be involved in the transmission of diseases to man. It can also be deduced from the haemolysis results that the bacteria isolated may not be pathogenic since they exhibits partial haemolysis.

Based on the findings of this study, it is recommended that Hygienic practices should be encouraged when fish are harvested from water body for consumption purpose and also the fish and other edible aquatic organisms should be well cooked in order to curb the transfer of pathogenic bacteria to man and also RAPD-PCR analysis of bacteria DNA described in this work include only that of *Tilapia zilli*, more research work should be done to characterize other fish species in Yewa River and other water bodies of Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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