

Original Research Article

MOLECULAR CHARACTERIZATION AND HAEMOLYSIS OF BACTERIA ASSOCIATED WITH *Tilapia zilli* FROM IJAKA-OKE LOCATION ON YEWA RIVER

ABSTRACT

Fishes are 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°C for 18 hours. The mean total length of the fishes was 30.00±9.00 (cm), weight 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 22.9⁰C, the pH of the water was 6.1 and Dissolved Oxygen 6.89mg/l. There were 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.

Key words: Bacteria Isolates, RAPD-PCR, Genetic diversity, Haemolysis, *Tilapia zilli*.

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 (Shinkafi and Ukwaja, 2010). 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 (FAO, 2006). There is

an appreciable increase in the demand for fish which is the cheapest source of animal protein (Shinkafi and Ukwaja, 2010). 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, (WHO, 1997) 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 and 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 (Ligia *et al.*, 2003). However, the presence of potential human pathogens suggests the fish consumed raw, undercooked and improperly handled may cause disease to susceptible individuals. According to Douglas (2007), 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 (Douglas, 2007). 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.

Materials and Methods

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 km² and is located within the West African tropical climate. Ijaka-Oke is landing site along Yewa River in Yewa North Local Government area of Ogun State, Nigeria. Bacteria isolates were taken from twenty *Tilapia zilli*. It was 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 Akinyemi (2012). 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 were taken.

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 121⁰C for 15 minutes. The agar media was allowed to cool and was dispensed into Petri plates. 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 plates. The culture media were incubated overnight at 37⁰C. Pure colonies were prepared from the original culture using Nutrient Agar and Mac Conkey Agar. Pure colonies bacteria isolates were grown overnight in liquid broth, it was transferred to eppendorf tube and it was spun down at 14,000rpm for 2mins, the supernatant was discarded and the extraction of DNA was done using CTAB method Akinyemi and Oyelakin 2014. The DNA was later resuspended in 100µl of sterile distilled water. DNA concentration of the samples were measured and the genomic purity were determined. The DNA was further check on 1.0 % agarose gel and was visualised on UV light source.

RAPD-PCR amplification and Gel electrophoresis

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 40ng - 80ng genomic DNA, 0.1 µM of the primers, 2mM MgCl₂, 125µM of each dNTP and 1 unit of Taq DNA polymerase. The thermocycler profiles has an initial denaturation temperature for 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 40seconds, followed by final extension temperature at 72⁰C 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.

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 37⁰C for 18 hours and was observed for haemolysis. After sometimes a clear zone of incubation was observed and the diameter was measured.

Statistical Analysis

The bands obtained from the gel was transferred to numerical figures, 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 dendrogram for the bacteria isolates.

Results and Discussions

Physico-Chemical parameters and Morphometric Characteristics

The Physico-chemical parameters recorded at the study location are temperature, pH and Dissolved Oxygen with values of 22.1⁰C-22.9⁰C, 6.6mg/l, and 6.89mg/l respectively (Table 1). The mean total length, mean standard length, mean head length, mean weight of the fish was 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 between 22.1⁰C-22.9⁰C is within UNEP standard and is similar to Saidu and Musa (2012), Akan *et al.*, (2010). 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 (Ewa *et al.*, 2011, Akan *et al.*, 2010). Dissolved Oxygen (DO) is relatively high with value of 6.85mg/l (Taiwo *et al.*, 2014),

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). Figure 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.

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 bacteria strains identified, 5 of it exhibited partial haemolysis (83.05%), 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.

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Table1: Water quality parameters of Ijaka-Oke Location on Yewa River.

Parameter	Values	WHO (2006)
Dissolved Oxygen	6.89 mg/L	5mg/L
Temperature	22.1-22.9 ⁰ C	<40 ⁰ C

pH 6.1-6.9 6.5-9.5

125 **Keys:** WHO=world health organization, °C=degree Celsius.

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127 **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

128 **Weight in grams, Length in centimeter**

129 **Table 3: Primer Sequences and percentage polymorphism**

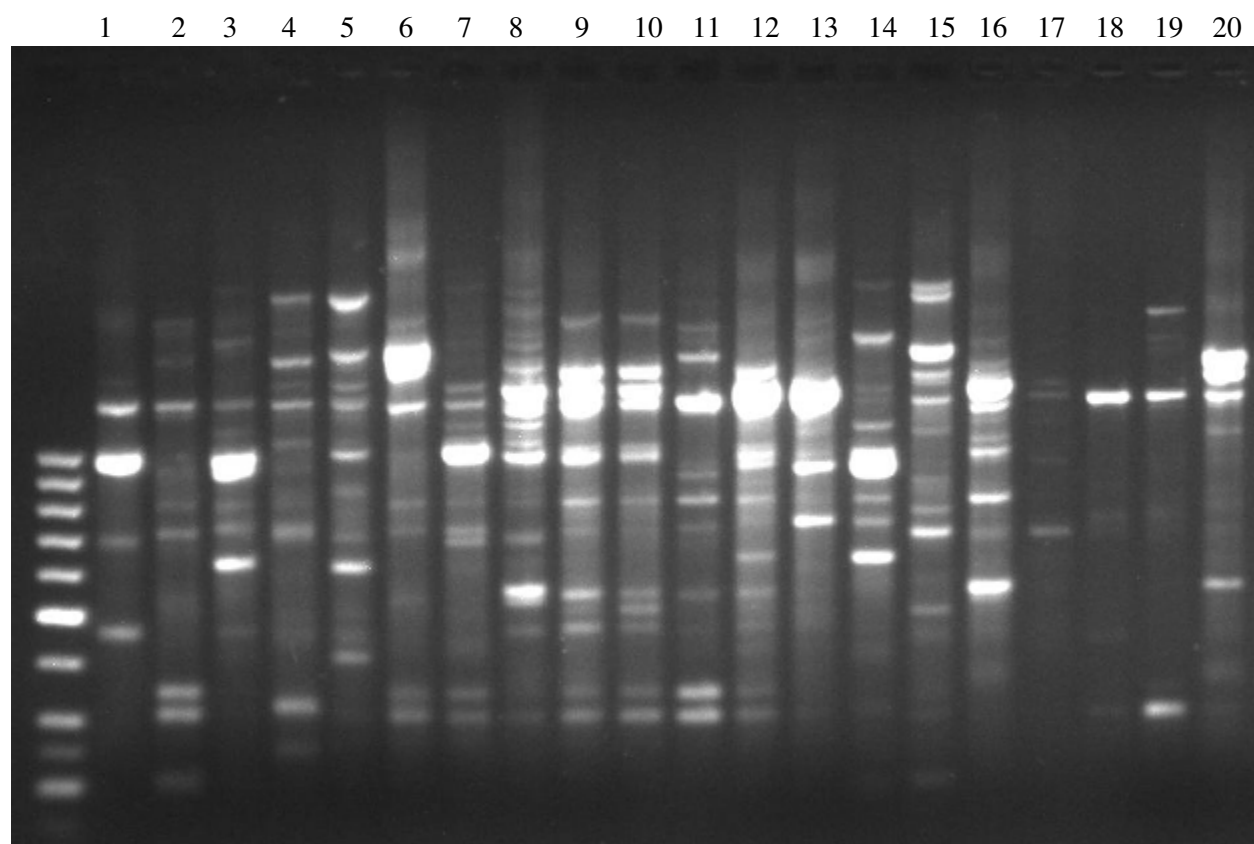
S/N	Primer Name	Primer Sequence	No of Monomorphic markers	No of Polymorphic markers	Total no of markers	Percentage Polymorphism
1	OPB 12	CCTTGACGCA	02	14	16	85
2	OPB 20	GGACCCTTAC	04	10	14	74
3	OPH 08	GAAACACCCC	01	16	17	94
4	OPH 12	ACGCGCATGT	03	05	08	63
5	OPH 19	CTGACCAGCC	05	07	12	58
	Total		15	52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

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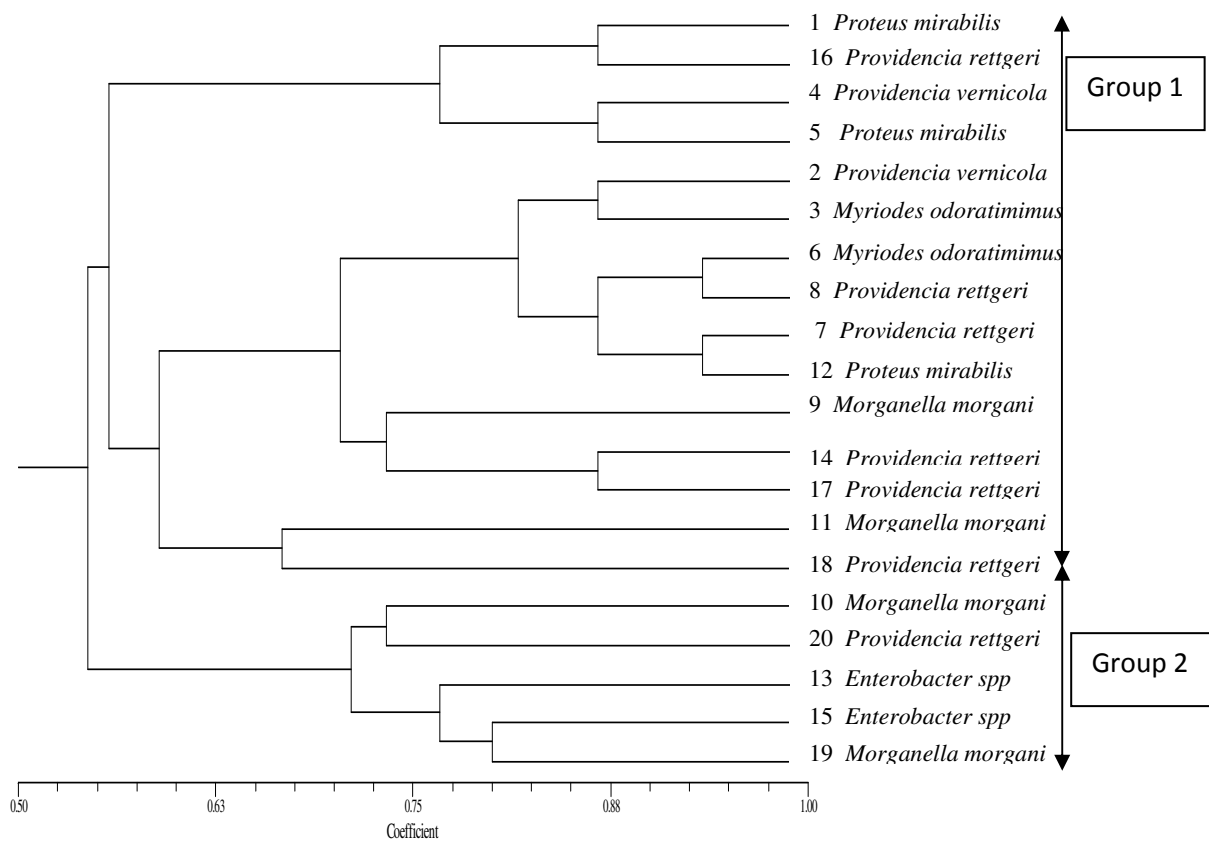
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135 **Plate 2: Electrophoresis gel for RAPD primer OPB 12**

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138 **Figure 1: Cluster analysis of the 20 selected isolates**



141 **Table 4: Haemolytic pattern of bacteria isolated.**

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

Keys: α =partial haemolysis, γ =no haemolysis.

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145 **Discussion**

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

148 Physico-chemical parameters were recorded at the location. The parameters recorded include
149 temperature, dissolved oxygen and pH. The temperature recorded at the location ranges between
150 22.1⁰C-22.9⁰C, this result is within UNEP standard and it is similar to Saidu and Musa (2012),
151 Akan *et al.*, (2010). The pH is within the range 6.5-9.5 (UNEP standard). Aquatic organisms are
152 affected heavily by water pH because virtually all their activities are dependent on pH (Ewa *et*
153 *al.*, 2011). This value recorded is however normal for aquatic lives and hence it minimal effect
154 on acidity (Akan *et al.*, 2010). Dissolved Oxygen (DO) is relatively high with value of 6.85mg/l.
155 Dissolved Oxygen is usually used to measure the degree of pollution by organic matters, the
156 organic substances destruction as well as the self-purification capacity of the water body (Taiwo
157 *et al.*, 2014), the amount of dissolved oxygen present in water is highly dependent on the water
158 temperature (warmer water carries less dissolved oxygen than colder water). The Dissolved
159 oxygen value recorded is slightly above the UNEP (2006) standard limit of 5mg/l for sustenance
160 of aquatic life, any value below the standard will affect aquatic life adversely, having a
161 concentration below 2mg/l will eventually lead to death of most fishes (Taiwo *et al.*, 2014).

162 There are a lot of molecular typing methods for accessing the differences between bacteria
163 isolates; however, the main criteria to choose the appropriate typing methods are their cost, type,
164 simplicity, constancy of performance and reproducibility. RAPD-PCR is quick, cheap, relatively
165 simple to achieve and was used in this study to differentiate the 20 bacteria isolates DNA. The
166 bacteria isolated include that belonging to the genera *Proteus*, *Providencia*, *Myriodes*,
167 *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 (Chen *et al.* 1995), 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 2 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 morgana*, 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 Bardkci and Skibinski (1994) who stated that the patterns of similarities and differences between population showed broad agreement across primers and the overall similarity level varied between primers.

Haemolytic proteins are isolated from pathogenic bacteria and β -haemolysin is a very important bacterial virulence factors, 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 view of this result, Wang *et al.*, (1996) 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 (Facklam & Washington, 1991). According to Rowe and Welch, (1994), 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.

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 they 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.

Base 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.

215 **References**

- 216 Akan, J. C., AbdulRahman, F. I. and Yussuf, E. (2010). Physical and chemical parameters in
 217 Abattoir waste water sample. *Pacific Journal of Science and Technology* 11 (1):
 218 640-648.
- 219 Akinyemi, A.A. (2012). Plant Extracts as Alternative Treatment for *Pseudomonas aeruginosa*
 220 Occurrence in *Clarias gariepinus* (BURCHELL, 1822) juveniles. *Journal of*
 221 *Science a Multidisciplinary Research*. Vol 4.
- 222 Babu, P.S., 2000. Ichtyozoonoses. *Fish Farmer International*, 14: 14-17.
- 223 Bardakci, F. and Skibinski, D. O. F. (1994). Applications of the RAPD technique in tilapia fish:
 224 species and subspecies identification. *Heredity* 73: 117- 133.
- 225 Chen, j., Nye, H. E., Kelz, M. B., Hiroi, N., Nakabeppu, Y., Hope, B. T., Nestler, E. J. (1995).
 226 Regulation of delta FosB and FosB-like proteins by electroconvulsive seizure and
 227 cocaine treatments. *Mol Pharmacol*. 48: 880-889.
- 228 Douglas, D. (2007). Identifying fresh water Aquarium fish disease. Available on line at
 229 <http://fishsuite101.com/article.cfm/identifyingfishdiseases>.
- 230 Ellis, A.E. (1999): Immunity to bacteria in fish. *Fish Shellfish Immunol*. 9: 291–308.
- 231 Ewa, E. E., Iwara, A.I., Adeyemi, J. A., Eja, E.I., Ajake, A. O. and Out, C.A. (2011).Impact of
 232 industrial activities on water quality of omoku creek sachá *Journal of*
 233 *Environmental Studies* 1(2): 8-16.
- 234 Ezeri, G.N.O., (2001). Haematological response of clarias gariepinus to bacterial infection and
 235 prophylactic treatment. With antibiotics, *Journal of Aquatic Science*; 16:22-24.

- 236 Facklam, R. R. and Washington, J. A. (1991). Streptococcus and related catalase-negative Gram-
237 positive cocci. In: Manual of Clinical Microbiology. American society for
238 Microbiology, Washington DC, pp. 238- 257.
- 239 FAO, (2006). La situation mondiale des pêches et de l'aquaculture 180p.
- 240 Ligia, M., (2003). Bacterial microflora in the gastrointestinal tract of Nile tilapia, Oreochromis
241 niloticus, cultured in a semi-intensive system.
- 242 Rowe, J. E. and Welch, R. A. (1994). Assay of haemolytic toxin. *Methods Enzymology* 235, 657-
243 667.
- 244 Saidu, M. and Musa, J. J. (2012). Impact of Abattoir Effluent on river Lanza, Bida, Nigeria.
245 *Journal of chemical, biological and physical sciences* 2(1):132-136.
- 246 Shinkafi, S.A. and Ukwaja, V.C. (2010). Bacteria Associated with Fresh Tilapia Fish
247 (Oreochromis niloticus) Sold At Sokoto Central Market in Sokoto, Nigeria.
248 *Nigerian Journal of Basic and Applied Science*, 18(2): 217-221.
- 249 Sowunmi., A.A., M.A. Okunubi and M.O. Efuntoye, (2008). Occurrence of bacteria in gill and
250 buccal cavity of Clarias gariepinus (Burchell, 1822) and Tilapia zilli (Gervais) from
251 Lekki lagoon, Southwest Nigeria.
- 252 Taiwo, A. G., Adewunmi, A. R., Ajayi, J. O., Oseni, O. A. and Lanre-Iyanda, Y. A. (2014).
253 Physico-Chemical and Microbial Analysis of the Impact of Abattoir Effluents on
254 Ogun River Course. *International Journal of ChemTech Research CODEN (USA):*
255 *IJCRGG*. Vol.6, No.5, pp 3083-3090.
- 256 Tiamiyu A. M., Emikpe B. O. and Adedeji O. B. 2011. Isolation and Identification of Aerobic
257 Bacteria Flora of the Skin and Stomach of Wild and Cultured Clarias Gariepinus

- 258 and Oreochromis Niloticus from Ibadan, Southwest Nigeria. *Journal of Applied*
259 *Sciences Research*, 7(7): 1047-1051.

- 260 UNEP & Gems Water Programme, (2006), *Water Quality for Ecosystem and Human Health*,
261 Ontario Canada

- 262 Wang C. Y., Mayo M. W., Baldwin A. S., (1996) JR TNF-and cancer therapy-induced apoptosis:
263 potentiation by inhibition of NF-κB. *Science*. 274:784–787.

- 264 Wedemeyer, G. A. (1996): Physiology of Fish in Intensive Culture Systems. Chapman & Hall,
265 New York. Pp 232.

- 266 WHO., (1997). Prevention and Control of enterohaemorrhagic Escherichia coli (EHEC)
267 Infections. Report of a WHO Consultation, Geneva, Switzerland, 28(1).