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ORIGINAL RESEARCH

Investigation of Bacterial Organisms Associated with Eggs of Cultured Broodstock Catfish *Clarias gariepinus* (Burchell, 1822) from Ogun State, Nigeria

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Abstract:

Introduction: The gene bank on fish diseases is usually enriched via information on the existing and emerging microorganisms.

Aim: This study evaluated the bacterial organisms associated with eggs of cultured Catfish broodstock collected from a private Fish Farm in Ogun State, Nigeria.

Materials and Methods: Fish samples were taken to the Microbiology laboratory, Federal University of Agriculture, Abeokuta Ogun State, for egg microbial analyses. The amplicon was taken to International Institute of Tropical Agriculture, Ibadan, for molecular characterization while Bio-edit was used for importing and mining nucleotide sequences into gene bank.

Results: The results revealed that the following bacterial organisms were present in the eggs of *Clarias gariepinus*: *Pseudomonas stutzeri, Acinetobacter gerneri, Enterobacter cloacae, Acinetobacter haemolyticus* and *Aeromonas caviae.* Based on the similarity percentage of sequence generated and matched on NCBI database, *Enterobacter cloacae* had 98.36% similarity, with *Acinetobacter haemolyticus* of 97.21%, and *Pseudomonas stutzeri* with 80.48%. *Acinetobacter gerneri* and *Aeromonas caviae* had 88.08% and 93.75% similarity percentage respectively.

Conclusion: Bacteria were found in the eggs of cultured catfish, although some of them are free-living water bacteria and may not pose any threat.

Key words: Ova**,** *Clarias gariepinus*, Bacteria, Molecular method.

All co-authors agreed to have their names listed as authors.

1. INTRODUCTION

Fish is an important source of animal protein for many households as it contributes more than 60% of the world's supply of protein, especially in developing countries [1]. Fish and fishery products represent a very valuable source of protein and essential micronutrients for balanced nutrition and good health. The State of the World Fisheries and Aquaculture [1], has described Aquaculture as the farming of aquatic animals, including finfish, crustaceans, molluscs, etc. and aquatic plants, mostly algae, using or within freshwater, seawater, brackish water and inland saline water. However, aquaculture in Nigeria is practised primarily in inland freshwater. Aquaculture practices as a business venture can bring significant development in the rural and urban areas by improving family income, providing job opportunities and increasing food supply and reducing insecurity of lives [2].

However, world aquaculture production is vulnerable, and an increase in disease outbreaks has been reported due to culture intensification, resulting in partial or loss of production [3]. Overcrowding, periodic handling, low or high changes in temperature, poor water quality and poor nutritional status also contribute to physiological changes in fish, such as stress or immune suppression, thus heightening susceptibility to infection. Bacteria exist as a micro-flora in the environment of fish and are found on the skin, intestines and gills of fish. Bacterial diseases are widespread and could be of particular importance in fish farming. They have been identified to cause significant mortality in both wild and cultured fish [4].

The following group (genus) of pathogens have been isolated from fish cultured at various times and different locations: *Aeromonas, Pseudomonas, Corynebacteria, Enterobacteria, Mycobacterium, Nocardia, Myxobacteria, Streptomyces and Vibrio* [5]. Fish populations, both farmed and wild, depend upon producing good-quality eggs; thus, poor egg quality is one of the major constraints in the expansion of aquaculture of both marine and freshwater fish species. In the fish farming industry, good quality eggs have been defined as those exhibiting low mortalities at fertilisation, hatching and first feeding [6]. Fish egg quality is defined as the egg's ability to be fertilised and subsequently develop into a normal embryo [7], while Coban *et al* [8] define egg quality as the potential of an egg to hatch into a viable larva. Egg quality is significant for producing high quality fish larvae that guarantees the profitability of hatcheries. In fish culture, egg quality control is necessary in species recently introduced in fish culture [9]. However, bacteria have been found in eggs of fish as reported by researchers such as Akinyemi [10] on cultured *Clarias gariepinus* broodstock in fish hatchery systems in southwest Nigeria. The presence of bacteria in the eggs of fish could be transferred to the swim-up fry and reduce the quality of fry and fingerlings in general. This study aimed to evaluate the microorganisms that are found in the eggs of cultured *Clarias gariepinus.*

2. MATERIAL AND METHODS

2.1 Collection of Fish Sample

Six (6) adult African catfish (*Clariasgariepinus)*, weighing 900g-1kg, were used for this research. They were collected from a private fish farm in Mowe, Ogun state, and transported in jerry cans with 5 litres of water. They were transferred alive to the Microbiology laboratory, Federal University of Agriculture, Abeokuta OgunState in a large plastic container of 20 litres but filled up to 10 litres ofwater and subjected to clinical and bacteriological examinations.

2.2 Collection of Eggs

Stripping of the female spawners was done by gently pressing their abdomen with a thumb from the pectoral fin towards the genital papilla. Ovulated eggs flow out easily. The eggswere more or less transparent flattened and a gram of egg contains approximately 600 eggs. All procedure was carried out under aseptic conditions using sterile materials such as conical flask (250ml), Sterile Petri dishes, Measuring cylinder, sterile syringes, Beakers, Mac Cartney bottles, and Cover slip. The image of the eggs of *Clarias gariepinus* in their sac is as shown in **Plate 1.**

Plate 1: Image of the eggs of *Clarias gariepinus* in their sac (Mg 810 x 1080)

2.2.1 Isolation of bacteria and purification

Bacteria was isolated aseptically from collected eggs of the cultured broodstocks. They were streakedon different media for the isolation of bacteria as tryptic soy broth at 25°C and at 37°C for 18-24 hours, then poured onto tryptic soya agar, blood agar, Rimler-Shoots agar, Thiosulfate Citrate Bilesalt Sucrose agar (TCBS), and incubate at the same time and temperature. Purification of the isolated bacterial strains was performed according to the procedure of Austin and Austin [11].

2.3 DNA Extraction

The nutrient broth of samples collected were subjected to direct removal of DNA from the solution [12] using the silica gel procedure, in which DNA is selectively bound to a solid support medium via chemical interactions. DNA is eluted and collected for analysis after washing the DNA-bound support to remove unwanted materials [13]. The DNA extraction kits (QIAampMini Kit (250) cat no 51306)based on the affinity approach were used for the extraction.

The genotyping protocol for the kit and the steps involved in DNA extraction include the following:

i. Buffer AE was placed into 70^{o}C water bath
ii. 180µlof ATL was added to the isolate
iii. 20µl of proteinase K was added
iv. Sample incubated at 56^{o}C until completely lysed, and shaking heat block at 500rpm lysis usually complete in 2-3hours.

2.4 Polymerase Chain Reaction (PCR)

After DNA extraction, the PCR cocktail mix includes 2.5µl of 10x PCR buffer, 1ul of 25mM $MgCl_2$, 1µl each of forward primer and reverse primer, 1µl of DMSO, 2µl of 2.5mMDNTPs, 0.1µl of 5u/µlTaq DNA polymerase, and 3µl of 10ng/µl DNA. The total volume was 25µl using 13.4 µl Nuclease-free water.

2.4.1 Primer Sequence for Bacteria

27F: AGAGTTTGATCMTGGCTCAG
1525R: AAGGAGGTGWTCCARCCGCA

2.4.2 PCR cycling parameters

Initial denaturation at 94^{o}C for 5 minutes, followed by 36 cycles of denaturation at 94^{o}C for 30seconds, annealing at 56^{o}C for 30seconds and elongation at 72^{o}C for 45seconds. This is followed by a final elongation step at 72^{o}C for 7minutes, and the temperature holds at 10^{o}C forever. Amplified fragments were visualised on ethidium bromide-stained 1.5% agarose electrophoresis gels. The size of the amplicon is about 1500bp, and the DNA ladder used is 50bp from New England Biolabs (NEB). The sequencing was done using genetic analyser ABI 3500 from Thermo Fisher.

2.5 Statistical Analysis

Bio Edit software was used for importing and mining nucleotide sequences into Gene Bank, and BLAST was carried out on the NCBI website. Data are presented using tables.

3. RESULTS

3.1 Molecular sCharacterisation of Bacteria Isolates

The molecular examination of the eggs of six (6) broodstocks of *Clarias gariepinus* showed the presence of different bacterial species. Bacterial species identified were *Pseudomonas stutzeri, Acinetobacter*

gerneri, Enterobacter cloacae, Acinetobacter haemolyticus and *Aeromonas caviae.* Based on percentage of similarity on result of this study, 98.36% *Enterobacter cloacae* closely matches the sequence generated from the egg of fish sample while 97.21% of *Acinetobacter haemolyticus* matches the sequence generated and *Pseudomonas stutzeri* with 80.48%. *Acinetobacter gerneri* and *Aeromonas caviae* had the matches of sequence generated similarity of 88.08% and 93.75% respectively. In addition, no nucleotide was found in the egg of one fish sample that was used for this study, which may be due to inadequate DNA extract from the sample

Table 1: The molecular bacteria isolate in the egg of *Clarias gariepinus*

Fish Samples	Accession number	No of nucleotide sequence	% similarity	Genomic identification
1	MT568614.1	1365	80.48	*Pseudomonas stutzeri*
2	AB860302.1	1112	88.08	*Acinetobacter gerneris*
3	NA	-	-	-
4	MT576693.1	1135	98.36	*Enterobacter clocae*
5	CP018260.1	1148	97.21	*Acinetobacter haemolyticus*
6	AP019195.1	1027	93.75	Aeromonas cavaie

NA= nucleotide sequence is absent

3.2 DNA amplicon from the isolated bacteria strains separated on the agarose gel (M-maker)

Plate 2 illustrates the amplification products of two RAPD products in the isolates, as well as the numbers and types of amplified DNA bands generated by these primers. The molecular size of the PCR products generated by these primers ranged from 1500 to 300 bp.The two primers generated fifty-one polymorphic bands. The primer 1525R was found to be more potent, generating 26 unique bands, while the latter primer produced 25 unique bands. The primer 27F revealed clear variations in RAPD products between the studied bacteria isolates.

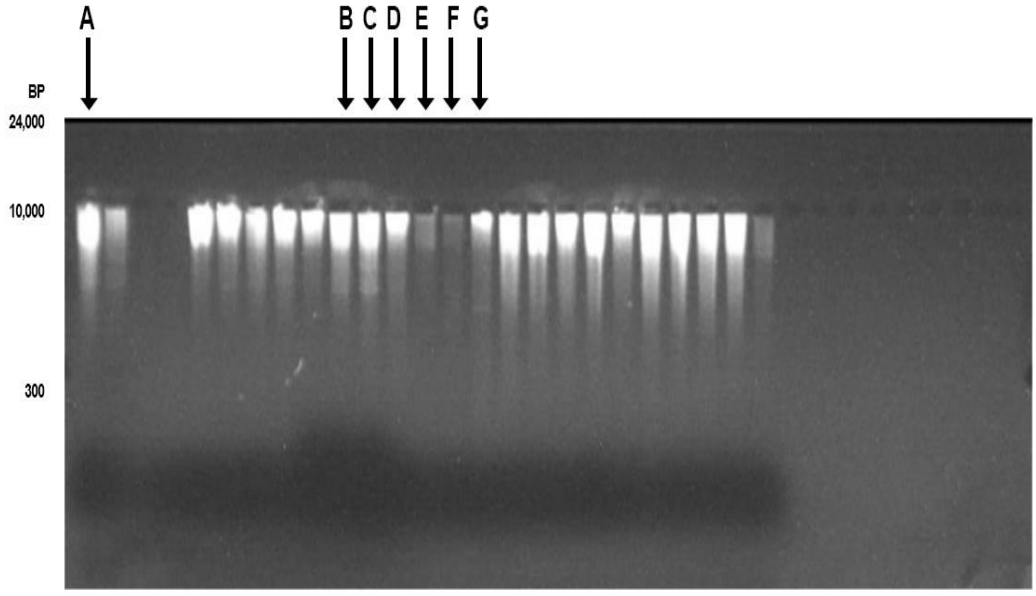

Plate 2: DNA amplicon from isolated bacteria strains separated on the agrose gel (M-maker)
A= Control ladder, B = Sample 1, C= Sample 2, D = Sample 3, E=Sample 4, F=Sample 5, G=Sample 6

4. DISCUSSION

From the molecular characterisation carried out from this study, the bacteria isolated were *Pseudomonas stutzeri, Acinetobacter gerneri, Enterobacter caviae, Acinetobacter haemolyticus and Aeromonas caviae.*

However, Akinyemi [10] using the Biochemical method, identified these bacterial organisms- *Salmonella spp, Escherichia coli, Proteus spp, Staphylococcus aureus, Virbrio spp, Shigella spp, Providencia rettgeri, Staphylococcus epidermidis.* Furthermore, Akinyemi [10] in his study, discovered that similar bacteria species were found in all the production cycles in the three farms from research work on microorganisms associated with eggs of cultured *Clarias gariepinus* brood stock in fish hatchery systems in South-western Nigeria he worked on..

Awe [14-15] isolated *Aeromonas veronii, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus agalactiae and Enterococcus faecium* from three different parts (skin, intestine and gills) of African catfish (*Clarias gariepinus)* collected from private fish farmers along Uren river in Odogbolu, Odogbolu local Government of Ogun state, Nigeria. Similarly [15] identified a new strain of *Aeronomonas*,*Aeromonas veronii,* and thus has added to the strain of bacteria found on African catfish *Clarias gariepinus,*especially in the gills and skin of the fish reared in earthen ponds. Though, two genera of bacteria *Aeromonas* and *Pseudomonas* found in Awe [14] were also found in this work.

According to Hansen and Olafsen[16] members of the genera *Pseudomonas, Alteromonas, Aeromonas, and Flavobacterium* were the major microfloras on both cod and halibut eggs. From report of Austin [17], on eggs of different *Oncorhynchus sp,Cryophaga, Flavobacterium, and Pseudomonas spp* were the dominant bacteria. In his study, *Acinetobacter calcoaceticus* and *Photobacterium phosphoreum* were the dominated bacteria in a marine fish-rearing unit, as well as *Vibrios spp.,* members of the cytophaga-Flexibacter and Cytophaga-flavobacterium groups. Sugita *et al*[18] discovered that *Flavobacterium spp., Ahydrophila, Pseudomonas spp., Micrococcuis spp., and A. punetata*were predominant in goldfish *(C. auratus) eggs. Acinetobacter gerneri, Enterobacter caviae* and *Acinetobacter haemolyticus* were the other bacteria found that were different from other previous works.

The reasons for these may be because most fish hatcheries and farms source their broodstock from the wild and, thereby introduce new species or genera of bacteria to the environment apart from *Aeromonas* and *Pseudomonas* that were commonly found in the hatchery. Akinyemi,[10] employed biochemical tests method for identifying the bacterial organisms while Awe[14-15] used biochemical and molecular methods of identification of bacteria in his research work. Previous research work by Awe[14] aligns with the method used in this study.

5. CONCLUSION

Bacteria were found in *Clarias gariepinus* eggs purchased from private farms in Ogun State, Nigeria. Bacteria like *Pseudomonas stutzeri* and *Aeromonas caviae* recorded in this study are free-living water bacteria. They may not pose any threat, while *Acinetobacter gerneri, Enterobacter cloacae* and *Acinetobacter haemolyticus* may be a source of food spoilage.

COMPETING INTERESTS

We declare that there are no competing interests

AUTHORS' CONTRIBUTIONS

Folalu Adekunle AWE designed the study. Gabriel Olarinde MEKULEYI[1], Lateef Akorede BADMOS[1], and Hammed Kolawole SHITTU managed the literature searches and wrote the first draft of the manuscript while Folalu Adekunle AWE and Gabriel Olarinde MEKULEYI reviewed the first draft before submission.

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