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Isolation and Identification of Bacteria Found in the Milt of Cultured *Clarias gariepinus*

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ABSTRACT

This study investigated bacteria that are associated with milt in cultured samples of African catfish - *Clarias gariepinus*. Male broodstocks of *Clarias gariepinus* were collected from private Fish Farm in Ogun State, Nigeria, while the fish's milt was collected and analyzed for microbial load at laboratory of the Department of Microbiology, Federal University of Agriculture, Abeokuta, Ogun State. The molecular characterization of the amplicon was done at International Institute of Tropical Agriculture, Ibadan. Bio-edit was used for importing and mining nucleotide sequences into gene bank. As revealed by the results, bacterial organisms that were present in the milt of *Clarias gariepinus* are *Aeromonas caviae*, *Proteus mirabilis*, *Serratia rubidaea*, *Pseudomonas mosselii*, *Acinetobacter soli* and *Klebsiella variicola*. The Basic Local Alignment Search Tools revealed the percentage similarity ranging from 86%-97.04% and their accession numbers. These bacteria indicated high levels of faecal contamination in the environment. In conclusion, bacteria were found in the milt of cultured catfish and are capable of being pathogenic to humans and may increase the vertical transfer to fry during breeding and rearing stage. Therefore, fish farmers should maintain a hygiene and serene environment during breeding and culturing of catfish.

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1. Introduction

One of the most common Nigerian fresh water fish species is *Clarias gariepinus* of the family *Clariidae*. The development of catfish farming in Nigeria was driven by socioeconomic objectives which include generation of additional family income, nutrition improvement of rural community, as well as creation of employment [1]. Although aquaculture production in Nigeria has experienced rapid growth in recent years, the industry is still constrained by some factors such as inadequate cooperative financial intervention on the growth of catfish aquaculture value chain, inadequate feed supply, and issues of fish diseases [2]. Studies have shown that tremendous high mortality rates and economic losses have been reported in catfish aquaculture due to infectious pathogens such as bacteria, viruses, fungi and parasites [3]. In the past, fish farming industry in Nigeria had been more focused towards the quality of eggs and larvae rather than that of sperm. However, in recent time, various authors, in an attempt to produce fingerlings of good quality, gave more attention towards obtaining fish milt with high quality [4]. Ultimately, the quality of milt is a measure of the ability of sperm to successfully fertilize an egg. Through the process of natural selection, the characteristics of sperm will be optimized so as to maximize the fitness of the individual male in relation with the specific reproductive strategy of each species [5]. Some findings have shown that milt of fish could be infected with a wide range of microbes present in the water body [6]. The microbes such as bacteria that are present on the body or internal organs of fish indicate the extent of pollution of the water environment [6]. However information on sperm-related microbiota might divulge the impacts of bacteria in the aggregation of sperm and sperm mortality. Such information might also be useful to identify probiotic bacteria for the improvement of sperm quality. Hence, this research work evaluates bacteria associated with milt in cultured samples of African catfish.

2. Materials and Methods

2.1 Sample Collection and Identification

A total number of eight (8) male broodstock were used. The cultured fish were purchased from the Broodstock Section of a private Fish Farm, Mowe, Ogun State of Nigeria. They were transported live to the laboratory in a large plastic container s filled up to half of its capacity with water and subjected to clinical examinations. Through the features of the redness of the genital papilla,

the matured male samples were identified.

2.2 Milt Collection

It is necessary to sacrifice male brood fish or surgically removed part of their testes in order to obtain the fish spermatozoa. Thus, male broodstocks of African Catfish, *Clarias gariepinus* collected for the study were dissected via the abdomen and the gonads were removed using standard laboratory method. However, blood clots and other tissues were rinsed away. The gonads were placed in the buffer solution prior to maceration to maintain its potency. Gonads were macerated in Petri dish and semen was transferred into freshly labeled sample bottles.



Plate 1. The milt or gonads of *Clarias gariepinus*

2.3 Isolation of Bacteria and Purification

Isolation of bacteria from the collected semen of the cultured broodstocks was carried out aseptically using different media. These media included tryptic soya broth at 25 °C and at 37 °C for 18-24 hours, then followed by pouring it onto tryptic soya agar, blood agar, Rimler-Shoots agar, Thiosulfate Citrate Bilesalt Sucrose agar (TCBS), and finally incubate at the same time and temperature. The isolated bacterial strains were purified in accordance to the documented guideline in literature [7].

2.4 Characterization of Bacterial Isolates

Bacterial isolates were characterized by their morphological/macrosopic and microscopic characteristics and

identification using molecular means.

2.5 RAPD-PCR Analysis and DNA Extraction

Isolation of DNA from broth culture of bacteria at log phase was done by following procedure described by Murray and Thompson [8] with little modifications. The modified protocol without the use of proteinase K has given good result, yielding quality DNA of approximately 20 µg from a 2 mL bacterial culture.

2.5.1 Primers and PCR Amplification and Resolution of RAPD Markers

For PCR amplification of bacterial DNA template, a panel of 2 numbers of decamer random primers was used. The PCR cocktail mix consisted of 1 µL of 25 mM MgCl₂, 1 µL each of forward primer and reverse primer, 2.5 µL of 10x PCR buffer, 1 µL of DMSO, 2 µL of 2.5 mM dNTPs, 0.1 µL of 5 u/µL Taq DNA polymerase, and 3 µL of 10 ng/µL DNA. However, using 13.4 µL Nuclease free water, the total reaction volume was made up to 25 µL.

2.5.2 Identification by 16S rRNA

Primers used in the present study targeted the variable regions of 16S rRNA of bacterial community. However, a pair of universal primers (17F and 1525R) succeeded to amplify the 16S rRNA gene in the PCR reaction and the resulting sequences covered variable regions 1 (V1) to 7 (V7) of 16S rRNA in bacterial isolates in order to accurately identify the bacterial species. Comparing the nucleotide sequences of 16S rRNA gene using BLAST.

2.5.3 Polymerase Chain Reaction Cycling Parameter

Initial denaturation was done at 94 °C for 5 mins. Immediately after the initial denature, 36 cycles of denaturation at 94 °C for 30 secs, annealing at 56 °C for 30 secs and elongation at 72 °C for 45 secs respectively the follows. Thereafter, a final elongation of step at 72 °C for 7 minutes and hold temperature at 10 °C forever was

attained. Amplified fragment were observed on ethidium bromide-stained 1.5% agarose electrophoresis gels. The size of the amplicon is about 1500 bp and the DNA ladder is 1 kb from NEB. The sequencing was performed using genetic analyzer ABI 3500 from Thermo Fisher.

2.6 Statistical Analyses of Data

Data were analyzed using descriptive statistics. The bacteria nucleotides from sequencing were run using Bioedit software on data base and were placed on National centre for Biotechnology information (NCBI) data base with the aid of Basic Local Alignment Search Tools (BLAST).

3. Results

3.1 Molecular Bacteria Isolates and Their Characterization

Table 1 depicts the molecular bacteria isolates and characterization. Bacteriological examination of the Milt of eight (8) broodstocks of *Clarias gariepinus* (African catfish) revealed the different bacteria with their accession number, number of nucleotide sequence, % similarity and the bacteria. The organisms were *Aeromonas caviae*, *Proteus mirabilis*, *Serratia rubidaea*, *Acinetobacter soli*, *Pseudomonas mosselii*, *Klebsiella variicola* and *Acinetobacter gernerii*. The lowest % similarity of 86% was found in *Aeromonas caviae* while the highest % similarity of 97.04% was associated with *Klebsiella variicola*.

3.2 Genomic DNA Band of the Sequenced Bacteria Isolates

The amplification products of two Random Amplified Polymorphic DNA (RAPD), product in the isolates, types of amplified DNA bands and number of amplified DNA bands generated by these primers. Primers (27F and 1525R) were used. In Plate 2, the molecular base pair

Table 1. Molecular bacteria isolates and their characterization

Fish Samples	Accession number	Number of nucleotide sequence	% similarity	Genomic identification
1	MK598335.1	1383	86	<i>Aeromonas caviae</i> strain ACDMC1235
2	MH396745.1	1322	96.94	<i>Proteus mirabilis</i> strain WWv278
3	AB860302.1	1081	92.71	<i>Acinetobacter gernerii</i>
4	NA	NA	NA	NA
5	LR590463.1	1337	96.78	<i>Serratia rubidaea</i> strain NCTC12971
6	MG757398.1	966	88.56	<i>Pseudomonas mosselii</i> strain CIPMRG-3
7	MT394056.1	1149	95.99	<i>Acinetobacter soli</i> strain OsEp-Plm-30P2
8	CP050958.1	1181	97.04	<i>Klebsiella variicola</i> strain FDAARGOS-628

NA: No nucleotide sequence found and it did not blast for molecular characterization

of the PCR products generated by these primers ranged from 1500 bp to 300 bp. Fifty one polymorphic bands were generated by the two primers. The primer 1525R generated 26 unique bands while 27F produced 25 unique bands. The primer 27F divulged clear variations in RAPD products between the studied bacterial isolates.

Table 2. The primer code and its sequences

Primer code	Sequence
27F	AGAGTTTGATCMGGCTCAG
1525R	AAGGAGGTGWTCCARCCGCA

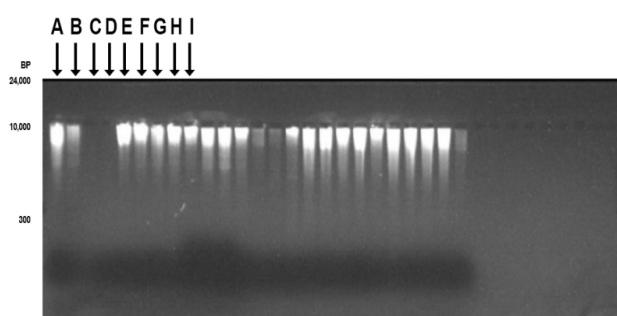


Plate 2. Genomic DNA amplicon observed under ultraviolet light showing the bands

Index: A = Control Ladder; B= Sample 1; C= Sample 2; D= Sample 3; E= Sample 4; F= Sample 5; G= Sample 6; H= Sample 7, I= Sample 8.

4. Discussion

Microorganisms isolated in the Milts used for the study were *Aeromonas caviae*, *Proteus mirabilis*, *Acinetobacter gernerii*, *Serratia rubidaea*, *Pseudomonas mosselii*, *Acinetobacter soli*, and *Klebsiella variicola* species. According to Gennari and Dragotto^[9], organisms isolated were classified into various forms; some are spoilage bacteria such as (*Proteus mirabilis*, *Acinetobacter* species and *Pseudomonas mosselii*), two enteric pathogenic organisms (*Serratia rubidaea* and *Aeromonas caviae*) and one opportunistic pathogen (*Klebsiella pneumoniae*).

The reasons for this load of bacteria associated with *C. gariepinus* from earthen pond may be due to contamination as a result of indiscriminate deposition of waste materials into the ponds through run offs, animal excreta and other environmental wastes. Free roaming animals can also contribute to faecal contamination of fish ponds.

Akinyemi^[10] in his work isolated some bacteria from

milt of broodstock in hatchery and these were *Salmonella* sp, *Esherichia coli*, *Proteus* sp, *Staphylococcus aureus*, *Vibrio* sp, *Shigella* sp, *Providencia rettgeri* and *Staphylococcus epidermidis*. Awe^[11] isolated various bacteria from parts of African Catfish (skin, gills and intestine). The bacteria isolated were *Pseudomonas aeruginosa*, *Aeromonas veronii*, *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecium*. The bacteria found in the milt for this present were not similar to bacteria^[10,11] in the milt of *Clarias gariepinus* broodstocks in the hatchery of farms in Southwestern Nigeria.

The major pathogens associated with the degradation of milt quality as reported by Gram and Huss^[12] indicated that bacteria observed in the present study were the major causes of microbial spoilage of fresh fish after capture and the microbial count on the different media. However the putative detrimental effect of bacteria on milt quality is still controversial while some studies have revealed that bacteria most frequently isolated from the milt or genitourinary tracts have no effect on semen quality^[13].

Information on milt flora is not readily available and limited; studies involving the digestive system reported the influence of ingested food on bacteria community found in the alimentary canal. The mere presence of these bacteria in the milt of *C. gariepinus* broodstock is of potential pathogenicity. However, the presence of bacteria in fish milt may cause a transfer of these bacteria to the resulting fish seed via vertical transfer to the progeny. Microbial counts in the fish milt samples may also be attributable to transporting and quarantining as well as handling by fisheries personnel. Olufemi^[14] reported that bacteria were responsible for many fish diseases especially those associated with environmental stress such as poor handling and transferring. Bacteria Genera and species were aerobes or facultative anaerobes. This explains why they could be found in the spermatozoa of the broodstock fish which is also in agreement with Bairagi *et al.*^[15] who isolated similar bacteria from fish gut. It also suggests that these bacteria are systemic. However, a similar work by the author on fish hematological bacteriology showed no significant difference in both infected and non-infected broodstock milt^[10].

Although, the total bacterial count on fish milt could rarely indicate the quality of the semen, however, it could give an indication of the risk of low capacitation^[12]. These bacteria have the capability of reducing trimethylamine and produce hydrogen sulphide from sodium thiosulphate which represent a secondary metabolite that constitutes fresh fish spoilage. *Klebsiella variicola* being present in the milt of fish samples in this study indicated that the

water used for culturing was faecally contaminated, and this may be due to the faecal waste from surrounding water through run-off.

Findings have shown that *Pseudomonas mosselii* was the sixth most common bacteria isolated. However, the observed numbers of *P. mosselii* in the present study was low compared to previous reports^[12]. The reason for low occurrence could be due to the fact that during storage at 0 °C they had fewer isolates. Therefore, *P. mosselii* is the major pathogen associated with fresh fish spoilage during refrigeration as divulged^[12]. The genus *Aeromonas* observed in this study is known to contain a number of opportunistic pathogens causing diseases of aquatic and terrestrial animals, including human beings^[16]. Herein, *A. caviae* bacterium was isolated from diseased *C. gariepinus*. The presence of *Aeromonas* spp. in fish milt may be due to environmental stress factors, such as high organic load, overcrowding, and sub-lethal oxygen levels, consequently causing infection after host injury or stress response^[17]. Another bacteria recorded in this study is *Proteus mirabilis* which has been identified as commensal in warm bodied animals^[18]. Therefore, its present in the milt of the sampled *C. gariepinus* might be due to faecal contamination released by the fishes in the pond. *P. mirabilis* is also an opportunistic pathogen that could primarily affect an individual whose immune system is compromised.

Presence of *Serratia rubidaea* in this study may be due to fish wound from peer bite in the pond which subsequently lead to an abscess in an open fracture. In addition, it is also possible that the bacteria were transmitted to the fish via water, since poor water quality condition may also affect the development of the disease. *Serratia rubidaea* is an *enterobacterium* described as a new species of *Serratia* and was first isolated from water and soil^[19]. This bacterium was recognized to be a human pathogen. It was reported that *S. rubidaea* has an extensive distribution in aquatic environments but no report yet in fish^[20].

Two different species of *Acinetobacter* were isolated in the milt of *Clarias gariepinus* used in this present study. The isolation of these pathogens from the milt might be due to contamination coming from waste materials present in the pond. However, the role of *A. baumannii* for channel catfish, *Channa striatus*, *Ictalurus punctatus*, and snakehead, as the fish pathogen has been well documented but studies on *A. gernerii* and *A. soli* in fish milt are yet to be reported^[21]. The potential spreading and persistence of *Acinetobacter* species in the environment are well known. It is obvious that *Acinetobacter* spp. is often isolated from healthy or diseased fish as the component of mixed

bacterial flora because most bacteria prevailing in water environment colonize gills, skin and digestive tract of the aquatic animals.

5. Conclusions

This study has brought to light, the bacterial species associated with milt of *Clarias gariepinus* cultured in the pond. The presence of these microbes in large population indicates high levels of faecal contamination which might have been contracted from the pond and from the environment.

Conflict of Interest

There is no conflict of interest.

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