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Investigation of bacterial organisms associated with milt of wild broodstock *Clarias gariepinus* (Burchell, 1822) Caught from Ogun River

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ABSTRACT: This study was carried out to isolate and identify the bacteria species that are found in wild *Clarias gariepinus.* Identification of bacterial organisms from catfish provides updated information on emerging and existing organisms thereby enriching the gene bank on fish disease. Catfish broodstock was collected from fishermen of Ogun River located in the heart of Abeokuta, Ogun State, Nigeria. Fish samples were taken to the Microbiology Laboratory, Federal University of Agriculture, Abeokuta Ogun State for milt microbial analyses, amplicons were taken to the International Institute of Tropical Agriculture, Ibadan for molecular characterization. Bio-edit was used for importing and mining nucleotide sequences into the gene bank. The result revealed that the following bacterial organisms were present in the milt of *Clarias gariepinus: Bacillus subtilis, Aeromonas caviae* and *Aeromonas veronii.* The Basic Local Alignment Search Tools revealed the percentage similarity ranging from 78.60 to 98.81% and their accession numbers. These bacteria indicate high levels of faecal contamination in the environment. In conclusion, bacteria were found in the milt of wild catfish.

Keywords: Bacteria, molecular characterization, wild catfish.

INTRODUCTION

The fish farming industry has been more focused on the quality of eggs or larvae rather than that of sperm, even though the quality of both gametes may affect fertilization success and larval survival. Sperm quality in farmed fish may be affected by different components of broodstock husbandry; collection and storage of sperm prior to fertilization or the fertilization procedure. The quality of sperm is highly variable and depends on various external factors such as the feeding regime, the quality of the feed and the rearing temperature of the fish (Billard *et al*., 1995). According to Rurangwa *et al.* (2004), any quantifiable physical parameter that directly correlates with the fertilization capacity of sperm could be potentially used as a measure of sperm quality. Different approaches for quantification of sperm quality have been suggested but motility is most commonly used since high motility is a prerequisite for fertilization and correlates strongly with fertilisation success (Rurangwa *et al*., 2004). Sperm

motility is the most evaluated criteria for sperm quality assessment in fish due to its correlation with fertility (Rurangwa *et al*., 2001; Adewumi *et al*., 2005) and it is affected by several parameters like temperature, pH, ions and their concentrations, dilution and the parasites affecting their environment.

The recruitment of wild fish as well as the controlled production in aquaculture is a biological event strongly linked to reproductive success and in particular, to the fertilization of mature oocytes (Karoui *et al*., 2011). The use of high-quality gametes is of great importance for ensuring the production of valuable offspring for aquaculture (Kjørsvik *et al*., 1990; Bromage and Roberts, 1995).

Milt quality is a key feature for a successful breeding process, especially in cases where spermatozoa have suffered physical changes by cooling. Studies on fish semen have shown some individual variations in parameters like sperm motility and fertilizing ability. Moreover, the spermatic indexes may vary among males or in the same individual (Rana, 2005).

The milt of fish is usually infected with a wide range of microbes present in the water body. These bacteria are often found in the scales, gills, gut and alimentary tract of the fish. The bacteria present on the body of the internal organs of fish indicate the extent of pollution of the water environment hence the bacteria flora of the fish depicts the bacteria flora of the water environment (Obiajuru, 1991).

In some species, the presence of bacteria in milt microbiota affects the sperm during spermatogenesis or sperm maturation. The role of intracellular bacteria in the process of spermatogenesis or its effect on sperm quality in fish is still unclear, therefore increasing the importance of this study. Unlike the case for gut microbiota, there are few studies on the detection of bacteria in sperm/spermatophores in aquatic animals, even though bacteria are closely related to all life stages of marine organisms (Bergh, 2000). Akinyemi (2009) isolated *Salmonella* sp, *Escherichia coli*, *Proteus* sp, *Staphylococcus aureus*, *Vibrio* sp, *Shigella* sp, *Providencia rettgeri,* and *Staphylococcus epidermidis* from Milt of broodstock raised in a hatchery, while Awe *et al.* (2021) isolated *Pseudomonas aeruginosa*, *Aeromonas veronii*, *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecium* from the skin, gills and intestine of Africa catfish. Furthermore, microorganisms isolated in the Milts of *C. gariepinus* by Awe *et al.* (2022) were *Aeromonas caviae*, *Proteus mirabilis*, *Acinetobacter generic*, *Serratia rubidaea*, *Pseudomonas mousseline*, *Acinetobacter soli*, and *Klebsiella variicola* species.

In 2019, Awe isolated the following bacterial organisms from three different parts of the fish skin, intestine, and gills: The African catfish *Claris gariepinus* (Burchell 1822) from a private fish farm along the Uren River in Odogbolu, Odogbolu local government of Ogun state, Nigeria. They were *Aeromonas veronii, Bacillus subtilis*, *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus agalactiae,* and *Enterococcus faecium.*

Although studies about the effect of bacteria on sperm quality have not yet been reported, however, information on sperm-related microbiota might reveal the role of bacteria in the aggregation of sperm and sperm mortality. This information might also be useful to identify probiotic bacteria for the improvement of sperm quality. Therefore, this study evaluates the bacteria associated with the milt of wild African mud catfish (*Clarias gariepinus*).

MATERIAL AND METHODS

Sample collection and identification

A total number of 4 male broodstock were used. The wild fish were obtained from fishermen at Ogun River located in the heart of Abeokuta Ogun State and thereafter were transferred alive to the laboratory in a large plastic container filled up to half of its capacity with water and subjected to clinical and bacteriological examinations. Matured male samples were identified using redness of the genital papilla as a guide.

Milt collection

The male broodstocks of African Catfish, *Clarias gariepinus* populations were sacrificed by using a standard laboratory method called percussive stunning, after which the abdomen was dissected and the gonads were removed. 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 a Petri dish and semen was transferred into freshly labeled sample bottles.

Post collection examination

The collected gonads of *C. gariepinus* were evaluated for gonad weight using a sensitive weighing balance. The pH of the semen was determined using a pH colour paper. The Sperm volume and microbial assessment were determined.

Volume determination

The semen volume was determined using a calibrated syringe after gentle maceration of the milt sac and the semen was taken up slowly.

Serial dilution/plating

Serial dilution was carried out by using the sperm sample

of wild broodstocks by crushing. This was done by mixing 10 g of each sperm sample in 9 ml of distilled water. 1 ml of the semen was pipette into 9 ml of peptone water already dispensed in six Mac cartney bottles. The first bottle gave a dilution of 10^{-1} from the semen solution. The procedure was carried out repeatedly until a dilution of 10^{-2} was obtained. One ml of the last diluent was each poured aseptically into sterile dishes containing the nutrient agar for bacterial growth and put in the incubator for 24 hours.

Isolation of bacteria and purification

Isolation of bacteria was carried out aseptically from collected semen of the wild broodstocks. They were cultivated on different media for the isolation of bacteria as tryptic soya 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 Bile salt Sucrose agar (TCBS), and incubate at the same time and temperature. The purification of the isolated bacterial strains was performed according to Austin and Austin (2007).

Molecular analysis and characterization of bacterial isolates

Bacterial isolates were characterized using molecular methods.

RAPD-PCR analysis DNA extraction

The protocol developed by Murray and Thompson (1980) with modifications was used for the isolation of DNA from broth culture of bacteria at the log phase. The modified protocol without the use of proteinase K has given good results, yielding quality DNA of approximately 20 µg from a 2 ml bacterial culture.

PRIMERS AND PCR amplification and resolution of RAPD markers

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

Identification using 16S rRNA

Primers used in the present study targeted the variable regions of 16S rRNA of the bacterial community. However, a pair of universal primers (17F and 1525R) succeeded in amplifying 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 the 16S rRNA gene using BLAST, showed the similarity of studied different bacterial isolates and their accession numbers.

Polymerase chain reaction cycling parameter

Initial denaturation at 94ºC for 5 minutes, followed by 36 cycles of denaturation at 94ºC for 30 seconds, annealing at 56ºC for 30 seconds and elongation at 72ºC for 45 seconds. This is followed by a final elongation of step at 72ºC for 7 minutes and holding temperature at 10ºC forever. Amplified fragments were visualized on ethidium bromide-stained 1.5% agarose electrophoresis gels. The size of the amplicon is about 1500bp and the DNA ladder is 1kb from NEB. The sequencing was performed using genetic analyzer ABI 3500 from Thermo Fisher.

Statistical analysis of data

Data were analyzed using descriptive statistics. The bacteria nucleotides from sequencing were run using Bio-edit software on the NCBI database and were placed on the National Centre for Biotechnology Information (NCBI) database with the aid of Basic Local Alignment Search Tools (BLAST).

RESULTS

Molecular bacteria Isolates and its characterization

Bacteriological examination in the Milt of four (4) wild broodstocks of *Clarias gariepinus* (African catfish) revealed the isolation of different bacterial isolates, while all bacteria isolates were preliminarily differentiated according to their morphological and cultural methods before the molecular results. Based on the frequency of occurrence on results of this study, *Bacillus subtilis* was the most predominant bacterial species isolated from milt of fish samples with an incidence of 98.81%. *Aeromonas caviae* with 93.75% closely followed while the least was recorded in *Aeromonas veronii* (78.60%). In addition, no bacteria isolates were detected in the milt of one fish sample (Table 1).

Genomic DNA Band of the sequenced bacteria isolates

Table 2 illustrates the amplification products of two Random Amplified Polymorphic DNA (RAPD) products in

Table 1. Molecular bacteria isolate and its characterization.

Fish samples	Accession number	Number of nucleotide sequence	% similarity	Genomic identification
1	MH187652.1	1070	98.81	*Bacillus subtilis strain BPA24*35
2	MH396745.1	1027	93.75	*Aeromonas caviae GSH8M-1*
3	AB860302.1	1314	78.60	*Aeromonas veronii strain CHS102*
4	NA	NA	NA	NA

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

Table 2. The primer code and its sequence.

Primer code	Sequence
27F	AGAGTTTGATCMGGCTCAG
1525R	AAGGAGGTGWTCCARCCGCA

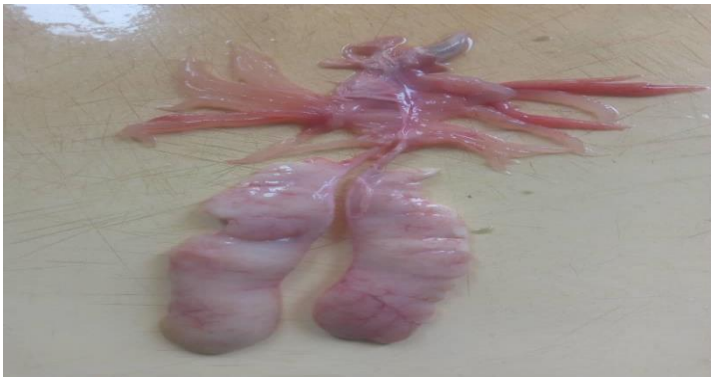

Plate 1. The male testicles containing milt of *Clarias gariepinus.*

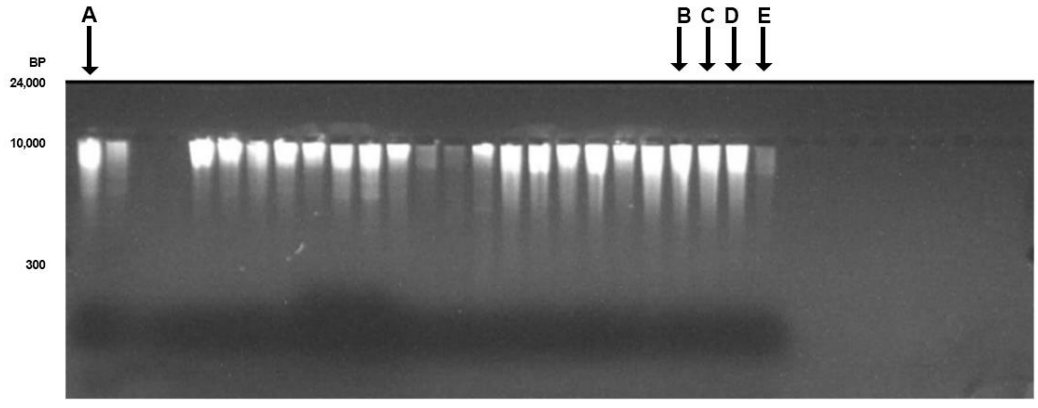

Plate 2. DNA amplicon from the isolated bacteria strains separated on the agarose gels (M-Marker). Index: A = Control Ladder, B= Sample 1, C = Sample 2, D = Sample 3, E = Sample 4.

the isolates as well as the number and types of amplified DNA bands generated by these primers. Primers (27F and 1525R) were used. In Plates 1 and 2, the molecular base pair of the PCR products generated by these primers ranged from 1500 to 30,000bp. Fifty-one polymorphic bands were generated by the two primers. The primer

1525R was found to be the more potent in generating 26 unique bands while the latter primer produced 25 unique bands. The primer 27F revealed clear variations in RAPD products between the studied bacterial isolates.

DISCUSSION

Fish are in constant interaction with a wide range of pathogenic and non-pathogenic microorganisms, in the aquatic environment, so fish suffer from various types of diseases and pathogens play an important role in affecting fish health, if the pathogenic load increases, it leads to disease. Fish are susceptible to a wide variety of bacterial pathogens causing large mortality in aquaculture. Microorganisms isolated in the Milts used for the study were *Bacillus subtilis, Aeromonas caviae* and *Aeromonas veronii.*

Akinyemi (2009) isolated the following bacteria from milt of broodstock in the hatchery and they were *Salmonella* sp, *Esherichia coli*, *Proteus* sp, *Staphylococcus aureus*, *Vibrio* sp, *Shigella* sp, *Providencia rettgeri* and *Staphylococcus epidermidis.* Awe (2019) and Awe *et al*. (2021) 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*. Akinyemi (2009) identified the bacteria using bio-chemical methods while Awe (2019) used both bio-chemical and molecular methods. This research also used the molecular method of characterizing the bacteria. All the bacteria identified by Akinyemi (2009) on bacteria in the milt of *Clarias gariepinus* brood stocks in the hatchery of farms in Southwestern Nigeria were not found in this study.

Information on milt flora is not readily available and limited; studies involving the digestive system reported the influence of ingested food on the 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 (1998) also reported that bacteria were responsible for many fish diseases, especially those associated with environmental stress such as poor handling and transferring. This could account for why the bacteria isolated were not significantly different on humans, soil and other sources (Gosh *et al*., 2002).

The presence of *Bacillus subtilis* in the milt of fish samples is an indication that the water where the broodstock was fished was faecally contaminated, this may be due to the faecal waste from the surrounding populace.

Bacillus subtilis is one of the best characterized bacteria and is used as a model organism for Gram-positive bacteria. *Bacillus subtilis* is a rod-shaped bacterium, which produces endospores that allow the survival of extreme environmental conditions including heat and desiccation. The presence of *Bacillus subtilis* may be due to highly toxic factors resulting from human waste activities (i.e faecal waste) in the river and other inland water bodies.

The genus *Aeromonas* contains a number of opportunistic pathogens causing diseases of aquatic and terrestrial animals, including human beings (Van der Marel *et al*., 2008). Herein, *A. caviae* bacterium was isolated from diseased *C. gariepinus*. The presence of *Aeromonas* spp. in fish milt may be due to high organic load.

Conclusion

In conclusion, this research has brought to light, the bacterial species associated with milt of *Clarias gariepinus* obtained from the wild. The presence of these microbes indicates high levels of faecal contamination which might have been released by the populace around the water body. The bacteria present in the internal organs of fish indicate the extent of pollution of the water environment hence the bacteria flora of the fish depicts the bacteria flora of the water environment and in some species, the presence of bacteria in milt microbiota affects the sperm during spermatogenesis or sperm maturation. The intracellular bacteria has effects in the process of spermatogenesis by interfering and multiplying thereby reducing the quality of milt produced.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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