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Use of salt as a disinfectant in the treatments of bacteria found in eggs of cultured *Clarias gariepinus* (Burchell, 1822)

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ABSTRACT: Egg disinfection is considered the most important routine work in hatcheries to avoid bacterial infection of fish eggs. This study assessed the use of sodium chloride (common salt) in the treatment of fish pathogens isolated from the eggs of *Clarias gariepinus*. Nine brood stocks of *Clarias gariepinus* were used. Their eggs were collected and taken to the International Institute of Tropical Agriculture (IITA) Nigeria for culturing, microbial analysis, DNA extraction of bacteria from the nutrient broth, and amplicon testing for molecular characterization. Bio-edit software was used for importing and mining nucleotide sequences into the gene bank. The molecular examination of *Clarias gariepinus* eggs showed the presence of four bacterial organisms namely: *Staphylococcus succinus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Klebsiella pneumonia.* Prior to common salt treatment, *Staphylococcus succinus* was the most predominant bacterial species isolated from the eggs of the fish sample with 84.88% similarity, *Staphylococcus epidermidis* followed closely with 81.66%. After the use of common salt, only *Bacillus* species and *Klebsiella pneumonia* were detected, with a similarity of 98.08% and 82.67%, respectively. Therefore, the common salt contributed immensely towards reducing the microbes and their nucleic acid contents in the fish eggs.

Keywords: Abeokuta, broodstock eggs, cultured catfish, molecular characterization, Ogun State, salt.

INTRODUCTION

The African catfish *Clarias gariepinus* of the family *Claridae* is highly appreciated as a good aquaculture species because of its resistance to diseases, ability to tolerate a wide range of environmental parameters and high stocking densities under culture conditions, relatively fast growth rate, and good quality (Rahman *et al*., 2019). The aquaculture industry is the fastest-growing food-producing sector which accounts for approximately 50% of global fish production (Omeje *et al*., 2020). With the increased demand for fish, pisciculture has also increased to a great extent. However, due to the rise in fish culture, there has also been a rise in fish diseases (World Fish, 2018). Studies have shown high mortality rates and economic losses in catfish aquaculture due to infectious pathogens from bacteria (Adelakun *et al*., 2012). For oviparous animals, the survival of fertilized eggs is a critical component of reproductive success. Egg survival, in turn, can be strongly affected by various factors. Fish eggs, for example, are susceptible to predation (Van West, 2006) and pathogens such as bacteria (Phillips *et al.*, 2008). The

development and survival of eggs are also heavily dependent on abiotic physicochemical parameters, especially temperature and salinity (Nissling and Dahlman, 2010), as well as gas exchange with the surrounding water (Rombough, 1988). Besides the direct effects on egg metabolism and development, abiotic factors can indirectly affect egg survival by influencing the growth and infection capacity of egg pathogens. In fish farming industry, good quality eggs have been defined as those exhibiting low mortalities at fertilization, hatching (Bromage *et al.,* 1992). Different microorganisms have been isolated from eggs and milts of cultured *Clarias gariepinus*. For instance, Gennari and Dragotto (1986) isolated spoilage bacteria such as (*Proteus mirabilis*, *Acinetobacter* species and *Pseudomonas mousseline*), enteric pathogenic organisms (*Serratia rubidaea* and *Aeromonas caviae*), and opportunistic pathogen (*Klebsiella pneumonia*) from eggs of *C. gariepinus.* 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.

Researchers are looking for alternative anti-infective techniques in light of the global rise in antibiotic resistance, in the hopes that these will prevent the development of resistant microbes. In addition to researching new techniques to eradicate pathogenic bacteria, the focus is on how to prevent the development of, and if feasible, eliminate multidrug resistance. The effects of salt on fish are determined both by salt concentration and duration of exposure (Francis Floyd, 1995). Salt is one of the most commonly used drugs in aquaculture. In fact, it is sometimes referred to as the aspirin of aquaculture. Salt, or sodium chloride (NaCl) in its chemical form, is a drug of low regulatory priority for the United States Food and Drug Administration and requires no withdrawal time before marketing. Many forms of salt are used, including table, meat-curing, pickling, and rock salt. Of these, the most commonly used and least expensive form is the mea-tcuring variety. When used properly, salt can treat many external parasites, including Costia, Epistylis, Tricodinia, Chilodonella, and the flukes Dactyogyrus and Gyrodactylus. Salt is used to relieve stress during handling and transport. Egg disinfection against bacteria is an important standard practice in artificial hatcheries. Egg disinfection prevents the transfer of pathogens from brood stock to eggs. This study assessed the use of salt as a disinfectant in the treatment of bacteria found in eggs of cultured *Clarias gariepinus*.

In 2018, 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 faccium.* Akinyemi (2009) concluded that the bacteria flora of *Clarias gariepinus* eggs were not significantly different in the three intensive fish farms examined.

Awe *et al.* (2021) identified a new strain of *Aeromonas,* which is *Aeromonas veronii,* and added it to the strain of bacteria in Nigeria found on African catfish, *Clarias gariepinus,* in the gills and the skin from the earthen fish ponds. Awe (2019) reported that bacteria broth culture was prepared after the DNA was extracted using the CTAB method followed by DNA electrophoresis and the DNA band was visualized and photographed under an ultraviolet light source.

The amplicon was later purified and sequenced using primers for forward and backward reactions. Later, the genomic band was visualized and photographed under an ultraviolet light source, and the sequence of nucleotides for proper identification was carried out on Bio-editsoftware for importing and mining nucleotide sequences into gene banks, and blasting of the sequences was carried out on the National Center for Biotechnology Information (NCBI) website.

Awe (2019) uses the basic local alignment search tool (BLAST) to identify the bacteria found by placing the nucleotides from the genomic sequence of the bacteria for forward and reverse reaction on the website of the National Center for Biotechnology Information (NCBI) and the corresponding answer gotten revealing the accession number and percentage of similarity of the nucleotides that match the bacteria on the database.

Therefore, the objective of this study is to evaluate the use of salt as disinfectants in the treatment of bacteria found in eggs of cultured *Clarias gariepinus*

MATERIALS AND METHODS

Study area

Preliminary work for this study was carried out at the Fish farm and Hatchery of Lagos State University, Ojo and Microbiology Laboratory. Others were the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo State, and the Microbiology Laboratory, Federal University of Agriculture, Alabata, Abeokuta, Ogun State.

Sample collection

Nine specimens of adult *Claris gariepinus*, whose weights

were between 900 g and 1kg, were used for this research. They were collected in a Jerry can, in a 5 litre of water from a private fish Farm at Badagry, Lagos State, and were transferred to Lagos State University Laboratory for further analyses.

Morphometric measurements

Morphometric differentiation among groups was also assessed and documented, including body weight (BW) and total length (TL). The fish were cleaned, and using a measuring board and total length (cm) and standard length (cm) measurements, each fish sample was measured from the tip of the snout (mouth closed) to the expanded tip of the caudal fin. After removing extra water from the fish's body and emptying the water from its buccal cavity, their respective body weights were also measured for each fish to the closest gram. The fish's gonads were taken out during dissection. The gonads of each specimen were examined to determine its sex.

The total length (TL)

To acquire the most accurate measurement of the specimen's full length, each fish was placed on a meter rule and stretched in that manner. From the anterior region's tip of the caudal fin to the posterior region's tip of the mouth part, a measurement was taken.

The standard length (SL)

The measurement was taken from the tip of the caudal peduncle in the anterior region to the tip of the mouthpart in the posterior region.

The total body weights

It was obtained by using an electric meter balance model PM400. Each specimen was weighed one after the other, and the respective weight was recorded.

Egg collection

Clarias gariepinus broodstock was injected with synthetic hormones of 1.4, 0.6, and 0.4 ml, respectively in three batches and allowed to stay for 9–15 hours to complete the latency period prior to stripping. The stripping of female spawners was done by gently pressing the abdomen with a thumb from the pectoral fin towards the genital papilla. Ovulated eggs flowed out effortlessly. The ovulated eggs were more or less transparent and flattened, and a gram of egg contains approximately 600 eggs. The average weight of eggs extracted for brood stock A, B, and C in three batches were 332, 177, and 116 g, respectively.

All procedures were carried out under aseptic conditions using sterile materials such as a comical flask (250 ml), sterile Petri dishes, a measuring cylinder, sterile syringes, beakers, a MacCartney bottle, and a coverslip.

Isolation of bacteria and purification

Isolation of bacteria was carried out aseptically from eggs collected from the female broodstocks. They were cultured on different media for the isolation of bacteria, such as tryptic soya broth at 25°C and 37°C for 18–24 hours, then poured into soya agar, blood sugar, Rimler-Shoots Agar, and Thiosulfate Citrate Bilesalt Sucrose Agar (TCBS) and incubated at the same time and temperature. Purification of the isolated bacterial strains was performed according to Austin (2007).

Molecular analysis and characterization of bacterial isolates

Bacterial isolates were characterized using the molecular method of RAPD Polymerase chain reaction (PCR) Analysis.

DNA extraction

To isolate DNA from bacterial broth cultures that were in the log phase, the Murray and Thompson (1980) procedure was modified. A 2 mL bacteria culture produced quality DNA amounting to about 20 g using the modified protocol without the use of proteinase K.

Primers and PCR Amplification band resolution of RAPD markers

A panel of two decamer random primers was used for PCR amplification of the bacteria's DNA template. The PCR cocktail mix consists of 2.5 µl of 10 PCR buffer, 1 µl of 25 mM $MgCl_2$, 1 µl each of forward primer and reverse primer, and 3 µl of 10 ng/µl DNA. Utilizing 13.4 litres of nuclease-free water, a 25-litre total reaction volume was created Atienzar and Jha, 2006).

PCR (Polymerase Chain Reaction)

After DNA extraction, the PCR cocktail mix consists of 2.5

Table 1. Morphometric features of the broodstock.

Category of brood stocks	Avg. Weight fish(kg)	Total length (cm)	Standard length (cm)	Head length (cm)	Dosage ovaprim (ml)	Weight of egg extracted (g)
Broodstock 1	2.90	76.5	67.0	15.0	1.4	332.0
Broodstock 2	1.20	53.0	45.0	11.0	0.6	177.0
Broodstock 3	0.96	48.0	43.0	9.0	0.4	166.0
Mean±STD	1.69±0.65	59.17±3.12	51.67±2.09	11.67±1.32	0.8±0.08	208.33±5.83

µl of 10 PCR buffer, 1 µl of 25mm MgCl2, 1 µl each of forward primer and reverse primer, 1 µl of Dmso, 2 µl of 2.5 mm DNTPS, 0.1 µl of 5 µ/µltaq DNA polymerase, and 3 µl of 10 ng/µl DNA. The total reaction volume was 25 µl using 13.4 µl of nuclease-free water.

Primer sequence for bacterial identification

27F: AGAGTTTGATCCTGGCTCAG
1429R: GGTTACCTTGTTACGACTT

PCR cycling parameters

Initial denaturation took place at 94°C for 5 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 45 seconds. The temperature is maintained at 10°C indefinitely after a final elongation stage that lasts 7 minutes at 72°C. Amplified fragments were visualized on ethidium bromide-strained 1.5% agarose electrophoresis gels. The size of the amplicon is about 1500 bp, and the DNA ladder used is 1 kbp from New England Biolabs (NEB). The sequencing was done using the genetic analyzer ABI 3500 from Thermo Fisher. While 96 well plates for cycle sequencing and the products were purified using the Ethanol/EDTA precipitation method and 25 ng of the PCR product was used to perform cycle sequencing.

Analysis of cells

After being exposed to salt, the eggs were swabbed, and the bacteria that survived were collected. The bacteria were then placed onto tryptic soy agar (TSA; Oxoid) with 200 lg/mL. Rif using an automated plate spreader (Whitley Automatic spiral plater, Don Whitley Scientific Ltd., West Yorkshire, UK), where they were then incubated for an overnight period at 378°C. The number of colonies was determined using an automatic plate reader. Because Rif's strains were used, the background flora on the eggs was negligible.

Statistical analysis

Bio Edit software was used for importing and mining nucleotide sequences into the Gene Bank while the blasting of sequences was carried out on the NCBI website.

RESULTS

Morphometric features of the sampled brood stocks

The morphometric characteristics (body weight, total length, standard length, and head length), dosage of ovaprim, and weight of eggs extracted from the sampled broodstocks of *Clarias gariepinus* were presented in Table 1. Broodstock 1 had the highest values for all the parameters listed in Table 1. While broodstock 3 had the least values. However, the mean body weight and the total weight and length were 1.6± 0.75 kg and 59±3.12 cm, respectively. The mean standard length was 51.67±2.09 cm, while the mean head length was 11.67±1.32 cm. While the average weight of eggs extracted was 208.335.83 g, the mean ovaprim dosage applied to the broodstock was 0.8±0.08 ml.

Molecular characterization of bacteria isolates

The molecular examination of eggs from brood stocks of *Clarias gariepinus* revealed the presence of four different bacterial species (Table 2). The bacterial species identified were *Bacillus* species, *Klebsiellapneumoniae, Staphylococcus epidermidis,* and *Staphylococcus succinus*. Prior to salt treatment, based on percentage similarity, *Staphylococcus succinus* was the most predominant bacterial species isolated from the eggs of the fish sample, with 84.84%. *Staphylococcus epidermidis* followed closely with 81.66%. After the use of common salt, only *Bacillus* species and *Klebsiella pneumonia* were detected, with a similarity of 98.08% and 82.67%, respectively.

DNA amplicon from the isolated bacteria strain separated on the agarose gel

Plate 1 showed the amplification products of two RAPD

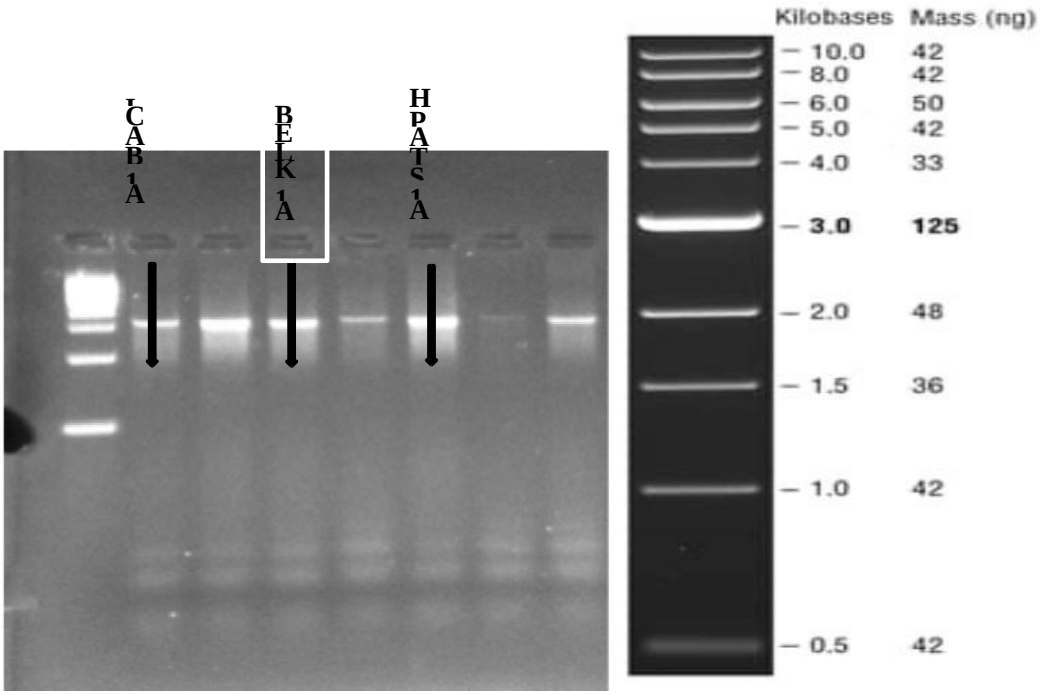

Plate 1. DNA amplicon from isolated bacteria strains separated on the agarose gel (M-maker).

Table 2. Molecular characterization of organisms found in the eggs of *Clarias gariepinus.*

Fish specimen	Biotypes	Similarity (%)	Accession	Strain characterization
_B4	Staphylococcus species	84.84	MH518234.1	Staphylococcus sp.strain CLC-M12
_B9	Staphylococcus species	81.66	KP236244.1	Staphylococcus epidermis strain 0109
_B15	Bacillus species	98.08	KP183926.1	Bacillus sp. BAB-4642
_B19	Klebsiella species	82.67	GU126803.1	Klebsiellapneumoniae strain 2R3-4

Table 3. The nucleic acids found in eggs of *Clarias gariepinus* and their absorbent values.

Sample ID	Nucleic acid (ng/µl)	A230 (Abs)	A260 (Abs)	A280 (Abs)	A260/A280	A260/A230
A1 BACILLUS	3169	28.550	63.381	31.846	1.99	2.22
A1 KLEB	4770.1	42.590	95.402	46.105	2.07	2.24
A1 STAPH	4225.3	38.238	84.505	40.846	2.07	2.21

Abs = Absorbent, A1 = eggs before the use of salt, A1 BACI = *Bacillus species* in eggs A1, A1 KLEB = *Klebsiella pneumonia* in eggs A1, A1 STAPH= *Staphylococcus succinus*in eggs A1.

products isolated and the type of amplified DNA bands generated by these primers. The molecular size of the PCR products generated by these primers ranged from 1500 - 30,000 bp. Fifty-one polymorphic bands were generated by two primers. The primer 1429R was found to be more potent, generating 07 unique bands while the latter primer produced 06 unique bands. The primer 27F revealed clear variation in RAPD products between the studied bacterial isolates.

Nucleotide found in egg samples of *Clarias gariepinus*

Table 3 showed that A1 KLEB has the highest nucleic acid (4770.1 ng/µl) followed by A1 STAPH (4225.3 ng/µl) while the least nucleic acid was recorded in A1 BACI (3169 ng/µl). Three kinds of absorbent (A), namely A230, A260, and A280, were recorded for all the sampled bacterial organisms, and their proportionality among the organisms follows the same trend as the proportion of the nucleic acids.

DISCUSSION

The mean total length and body weight of brood stocks of *Clarias gariepinus* used for this study indicated that the specimens had better growth where they were cultured. Similarly, the colours of the extracted eggs did not differ from conventional colours, as reported by Anetekhai (2017). However, the average weight of eggs extracted (208.33±5.83 g) could imply that the brood stocks were less fecund when compared with some smaller sizes of *Clarias gariepinus,* whose mean extracted eggs were about 600 g (Çoban *et al*., 2011). The molecular examination of the eggs of brood stocks of *C. gariepinus* examined in this study showed the presence of four different bacterial species: *Staphylococcus succinus*, *Staphylococcus epidermidis, Bacillus subtilis*, and *Klebsiella pneumonia.* Meanwhile, *Staphylococcus succinus* and *Staphylococcus epidermidis* observed in this study were also reported in the eggs of *C. gariepinus* isolated by Akinyemi (2009) using the biochemical method. However, *Salmonella sp, Proteus sp, Vibrio sp, Shigellasp, and Providencia rettgeri* found by Akinyemi were not present in this study. Also, micro-organisms (*Pseudomonas stutzeri, Acinetobacter generic, Enterobactercaviae, Acinetobacter haemolyticus,* and *Aeromonas caviae)* isolated by Awe *et al*. (2022) were not similar to those microbes found in this study. Therefore, the variation in the microbial load may be due to different sources of the broodstocks as well as the health status of their culturing media and the kind of feed that they were fed.

One of the several species discovered in this investigation was *Klebsiella spp*., which is widely distributed in the natural world. The mucosal surfaces of animals, such as humans, horses, or swine, are likely their second common home after the environment, where they can be found in surface water, saliva, soil, and plants. This is where the genus *Klebsiella* differs from *Shigella spp*. and *E. coli*, which are prevalent in humans but not in the environment. Instead, it is similar to *Enterobacter* and *Citrobacter* (Johnson and Russo, 2002; Touchon *et al.,*2009).

Furthermore, Awe *et al.* (2021) isolated the following bacterial organism from three different parts (skin, intestine, and gills) of African catfish (*Clarias gariepinus*) (Burchell, 1822) collected from private fish farmers along the Uren River in Odogbolu, Odogbolu Local Government of Ogun state, Nigeria. These include *Aeromonas veronii*, *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus agalactiae,* and *Enterococcus faecalis.*

In this study, before the use of salt and alcohol treatment, *Staphylococcus succinus* was the most prevalent bacterial species isolated from the eggs of the fish sample, with 84.84%. *Staphylococcus epidermidis* followed closely with 81.66%. After the use of common salt, only *Bacillus* species and *Klebsiella pneumonia* were detected, with a similarity of 98.08% and 82.67%, respectively. This observation indicated that *Bacillus species and Klebsiella pneumonia* are not susceptible to salt treatment. Therefore, the need to invent other methods of reducing their population in fish eggs cannot be over-emphasized. However, the treatment of salt effectively eliminated *Staphylococcus succinus* and *Staphylococcus epidermidis* from the egg samples. Furthermore, reports have demonstrated that salt kills some types of bacteria, effectively by sucking water out of them. In a process known as osmosis, water passes out of a bacterium so as to balance salt concentration on each side of its cell membrane. Meanwhile, some bacteria can tolerate salt; they are halotolerant. Certain strains of *Staphylococcus species*, *Bacillus species, and Klebsiella species* are salt-tolerant and halotolerant. Additionally, Sodium chloride (salt) has been proven as a safe, effective and economical disinfectant for controlling fungal and bacterial infection in freshwater fin and shellfish aquaculture, hence, increasing the hatching rate (Policar *et al.,* 2011; Bart *et al.,* 2013; Sarkheil, *et al.,* 2014).

Conclusion

The study has shown that the total length and body weight of broodstocks of the *Clarias gariepinus* eggs used did not differ from the conventional range in the reported literature. The molecular examination of the eggs of the brood stocks of *Clarias gariepinus* showed that common salt contributed immensely towards reducing the microbes and their nucleic acid contents in the fish eggs.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Recommendations

Salt should be used as a means of disinfecting bacterial organisms found in the eggs of catfish. Catfish culturing should be done in areas that are safe from human anthropogenic activities thereby reducing pollution from agricultural waste and run-off.

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