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MOLECULAR CHARACTERIZATION OF SOME MICROBES ISOLATED FROM SMOKE-DRIED FISH SOLD IN PORT HARCOURT AND THEIR PUBLIC HEALTH SIGNIFICANCE

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ABSTRACT

The study investigated molecular characterization of smoke-dried fish *Clarias gariepinus* and Oreochromis niluticus (Catfish and Tilapia) sold in some markets in Port Harcourt. Eighty (80) fish samples were collected from Oil Mill, Mile 3, Rumuokoro and Borokiri markets and their antibiotics susceptibility pattern determined. The fish samples were swabbed and analyzed microbiologically using standard procedure. The bacteria isolates were then characterized molecularly using 16S rRNA gene sequencing. The antibiotics susceptibility patterns were determined using Kirby-Bauer disc diffusion method. The bacteria isolated from the smoke-dried fish were *Proteus* 25% followed by *Bacillus* sp 15%, *E. coli, Providencial* sp and *Klebseilla sp* had 10% each while other bacteria strains had total of 5% each. The mean value of the smoke-dried fish measured were Borokiri 3.6 x 10^5, Mile/3 4.7 x 10^5, Oil Mill 3.8 x 10^5 and Rumokoro market 3.7 x 10^5 respectively. The study shows divers bacteria species exist in smoke-dried fish. Catfish had a mean count of 4.3 x 10^5 ± 2.4 x 10^5 and tilapia had 3.7 x 10^5± 2.7 x10^5CFU/mL which was not significant, the mile/3 market had the highest microbial load among all the markets in the study. The present study took cognizance of the educational level of the fish sellers, fish storage, and age of fish sellers as well as contamination awareness of fish sellers through questionnaire which could lead to unhygienic practices thereby resulting to more microbial load of the smoke-dried fish through faecal contamination. 63.6% of isolates from the result is among the smoke-dried fish stored on the floor. In the study there was no resistance on any of the antibiotics, the antibiotics used were Gentamycin 10mg, Ampiclox 10mg, Rifampicin 10 mg, Amoxil 20 mg, Streptomycin 30 mg, Norfloxacin 30 mg, Chloramphenicol 30 mg, Ciprofloxacin 30 mg, Erythromycin 20mg and Levofloxacin 20 mg. Rifampicin, Streptomycin and Levofloxacin were the most sensitive antibiotics among others. The study isolated some opportunistic pathogens but they were all sensitive to the antibiotics used.

KEYWORDS: *Bacillus* sp 15%, *E. coli, Providencial* sp and *Klebseilla sp.*

1 INTRODUCTION

Preservation of fish by smoking is carried out after it has been caught and eaten without further cooking. From the processing centres to the market centres smoked fishes are often contaminated with bacteria among other microorganisms, also during handling of fish the natural flora of the environment will be contaminated with organisms associated with man, such as members of Enterobacteriaceae and *Staphylococcus* which can grow well at 30–37^{0}C (Adams and Moses, 2010).

The rapid increase in the quantity of fish caught was caused by the increasing world demand for protein, as human population expanded rapidly in the 1950s and 1960s there was an urgent need for more dietary protein at low cost. Research has shown that fish smoking is the most widely practiced and recommended method of preservation where sophisticated equipment for more improved methods is lacking. Smoking of food is achieved by lowering of the water activity via application of gentle heat. The surface of food which will normally support most commensal organisms is dried while the heat and chemicals inherent in the smoke deprives microbes of necessary growth factors (Brown, 2016). Smoking is one of the oldest methods of preserving fish or any other meats for that matter. Long before there were refrigerators and freezers our fishing ancestors learned to use a combination of salt and smoke to keep fish from spoiling. Today smoking is no longer "necessary", but it remains popular for the flavor it gives to such fish as salmon, tuna, trout etc.

Bacterial contamination in food often results in food spoilage as well as life-threatening health hazards like food poisoning (Prescott *et al.*, 1999). Preservation thus helps in the maintaining food quality and public health enhancement. These facts greatly influenced the interest

of this study which one of the aims is assessing the bacterial load of retailed smoke-dried fish.

There are four main factors responsible for fish spoilage once it is out of its natural environment (water) and these include: Autolysis which usually proceeds bacterial spoilage and involves the breakdown of protein and lipids to amino acids and fats by muscle enzymes. The activity of microorganism is another factor which uses the amino acid produced by autolysis for spread rapidly. Others are chemical deterioration and insect attack which cause abnormal consideration, according to Emeikpe, (2011) it has been reported that there was a serious disease outbreak in both man and animals after eating of some dried fish feed and food. It could be as a result of food borne pathogen. Other microorganisms of primary concern are *Listeria monocytogenes* and *Clostridium botulinium*. Too much handling provides opportunities for other food borne pathogens to contaminate products if sufficient attention is not giving during smoking process (Akinwumi, 2014).

Fish is among the healthiest food on the planet, it is loaded with important nutrients such as Protein and vitamin D. It is also the best source of omega–3fatty acids. It is important for the body and brain, Iodine, vitamins and minerals. People who eat more fish have a lower risk of heart attacks, slow rates of cognitive decline. Studies have shown that those who eat more fish have more grey matter in the centers of the brain that regulate emotion memory. Fish consumers are less likely to become depressed. Fish can improve your quality of life (Felix *et al.*, 2015).

Fish intake has been linked to reduced risk of type I diabetes and several other auto immune diseases. Fish may protect your vision in old age. A disease called macular degeneration is a leading cause of vision impairment and blindness, mostly affects older individuals. Fish with omega -3 fatty acids can protect against the above mentioned disease (Felix *et al.*, 2015).

There is preliminary evidence that eating fish with omega-3 fatty acids like salmon may lead to improved sleep. Due to some health challenges most persons go for fish because of its low fat content or cholesterol level. Fish also contain most of the important essential amino acids like Lysine, Methionine & Tryptophan that are lacking in plant proteins. Vitamins and minerals are also contained in the fish that promote healthy living. Fish is a low fat food. Over the years agriculture has increase its interest due to the importance of fish.

According to Felix *et al.* (2015) fish possess medicinal value outside its food value such as healing of Goitre, Cancer, Arthritis, Coronary Heart Diseases and Asthma. The microorganisms associated with smoke-dried fish pose a great threat to the populace as the transfer of the microorganisms attack the immune system of the consumer, usually man, thereby giving man room for invasion of disease**.**

2 MATERIALS AND METHODS

2.1 Sample Source and Preparation

The study was conducted in four different locations of Port Harcourt and Obio/Akpor local government areas of Rivers state (Oil mill, Rumuokoro, Mile 3 and Borokiri these are four large markets found in these metropolis Obio-Akpor is bounded by Port Harcourt local government area to the south, Oyigbo to the east, lkwerre to the north, and Emohua to the west. Obio-Akpor is generally a lowland area with average elevation below 30 m above sea level. The thick mangrove forest, raffia palms and light rainforest are the major types of vegetation. Smoke-dried fish is one of the lucrative businesses of dwellers of the study areas. According to Chinedu, (2011) Port Harcourt's growth is further due to its position as the commercial center and foremost industrial city of the former Eastern Region; its position in the Niger Delta; and its importance as the center of social and economic life in Rivers State. In Port Harcourt where the study took place has its total population estimated as 2,000,000 in 2009, making it one of the largest metropolitan areas in Nigeria (Njoku, 2008). The smoke-dried *Clarias gariepenus and Oreochromis niluticus* were randomly purchased in above mentioned markets. All samples were transported to the laboratory within one hour of purchase using a sterile polythene bag.

2.2 Microbiological Analysis

Each sample was plated on MacConkey agar, Nutrient agar and Sabouraud agar. Replicate plates for each preparation were incubated aerobically at 37°C for 24 hours. The samples were plated using pour plating method (Chessbrough, 2000). The bacterial isolates were identified and classified using a combination of the methods as recommended by Chessbrough, (2000). Distinct colonies developing on the culture plates were observed for their pigmentation, margin, elevation and opacity. Gram's staining was done using 24 hours pure culture. The stained slides were then examined under the microscope with oil immersion objective lens (x100). From the Gram's reaction, shape and size of the cells were examined microscopically.

2.3 Molecular Identification

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000rpm for 3 minutes. The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer.

2.4 16S rRNA Amplification

The 16s rRNA region of the genes of the isolates were amplified using the 27F: 5' AGAGTTTGATCTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on ABI 9700 Applied Biosystems thermal cycler at a final

volume of 25 microlitres for 35 cycles. Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10µl.

2.5 Statistical Analysis

All data obtained were analyzed with statistical package of social science (SPSS) version 21 and express as mean ± standard deviation (SD) and P-value less than 0.05 were considered significant.

3 RESULTS

Out of the eighty samples both gram positive and gram negative bacteria were isolated in 65 plates which is 81.2% of the samples. However, 20 isolates were sequenced for their DNA. The colony counts were 8.5 x 10^6cfu/ml. This is higher than the maximum recommended bacteria count by International commission on microbiological specification for food (ICMSF) (5.0 x 10^5) cfu/ml. In this study the mean value of the catfish was 4.3 x 10^5 while that of the tilapia was 3.7 x $10^{5.}$ There is no significant value between the catfish and the tilapia which is found in Table 1.0. In Table 2.0 the bacteria count from different market locations from Borokiri catfish had the mean count of 5.0 x 10^5 while the tilapia had mean value of 2.3 x 10^5 in Borokiri market there was a significant value between the catfish. The Tilapia while in Mile 3 market there was no significant value between the catfish and the tilapia fish which had 5.0 x 10^5 and 4.5 x 10^5 respectively, no significant value in Oil mill market the tilapia had a mean value of 3.0 x 10^5 and catfish 4.7 x 10^5 while Rumuokoro had a mean value of 2.5 x10^5 for catfish and 5.0 x 10^5 for tilapia which was significant.

Table 3.0 shows the percentage of various bacterial species isolated from the smoke-dried fish using molecular technique with *Proteus* having the highest of 25% followed by *Bacillus sp* 15% *E. coli, Providencia sp* and *Klebsiella* sp had 10% each while other bacteria strains recorded a total of 5% each. *Proteus* sp *was* the most prevalent in this study.

The susceptibility of different strains varied with the type of antibiotic used as shown in Figure 1.0 without any being resistance to the antibiotic. Levofloxacin, streptomycin and Rifampicin being the most sensitive antibiotic, Amoxil and Erythromycin were 25% intermediate followed by Gentamycin and Ampiclox that were 10% each and others 20% which means an increase dose of the antibiotics can be effective if taking accordingly. Following these antibiotics pattern if these drugs are taking accordingly and when due they will be able to take care of any infection that is caused by these organisms, misused and inadequate use of antibiotics cause resistance. Contamination awareness is very necessary in this study 55% of the fish sellers are less aware of smoke-dried fish contamination which can increase microbial load of the fish while 45% of the fish sellers are much aware of fish contamination which is indicated in Table 4.0.

Most of the fish sellers store their smoke-dried fish on the floor causing a high microbial load due to the unhygienic environment of the floor which is shown in Table 5.0**.** Plate 1.0 is the PCR products obtained following amplification with the 16srRNA sequences were estimated to be 1,600bp in size the results of the sequence similarity [%] by the BLASTIN in gene bank of the National center for biotechnology information [NCBI] library.

Table 1.0: The Comparison of Mean Count of Bacteria Load of the CatFish and Tilapia for the Study

	Mean		t-test value	Df	P-value	Remark
Cat Fish	4.3 x 10^5	±2.4x10^5				
Tilapia	3.7 x 10^5	± 2.7x10^5	1.089	1	0.279	N/S
Total	40	40				

Key: N/S - Not significant

Table 2.0: Bacteria count of fishes from different market locations in the study area.

Market Location	Fish Type	Mean	t-test value	df	P-value	Remark
Borokiri 10	Catfish	5.0 x10^5± 2.0x10^5	3.006	1	0.01	Sig
10	Tilapia	2.3 x10^5 ± 2.1 x 10^5				
Mile 3 10	Catfish	5.0 x10^5 ± 2.0 x 10^5	0.495	1	0.63	N/S
10	Tilapia	4.5 x 10^5 ± 2.8 x 10^5				
Oil Mill 10	Catfish	4.7 x 10^5 ± 2.9 x 10^5	1.296	1	0.21	N/S
10	Tilapia	3.0 x 10^5 ±3.1 x 10^5				
Rumuokoro 10	Catfish	2.5 x 10^5 ±2.0 x 10^5	-2.883	1	0.01	Sig
10	Tilapia	5.0 x 10^5 ± 2.0 x 10^5				
Total 80						

Key*: Sig = Significant < 0.05 N/S= Non Significant >0.05 df= Degree of freedom.*

Table 3.0: Prevalence of bacteria obtained using molecular technique.

Isolates	Bacterial Occurrence	Prevalence (%)
Proteus sp	5	25
Bacillus sp	3	15
E. coli	2	10
Klebsiella sp	2	10
Providencia sp	2	10
Enterobacter sp	1	5
Staphylococcus aureus	1	5
Enterococcus feacalis	1	5
Shewanlla chilikensis	1	5
Chryseobacterium sp	1	5
Pseudomonas xiamenenisis	1	5
Total isolates	20	100

Key: Most prevalent

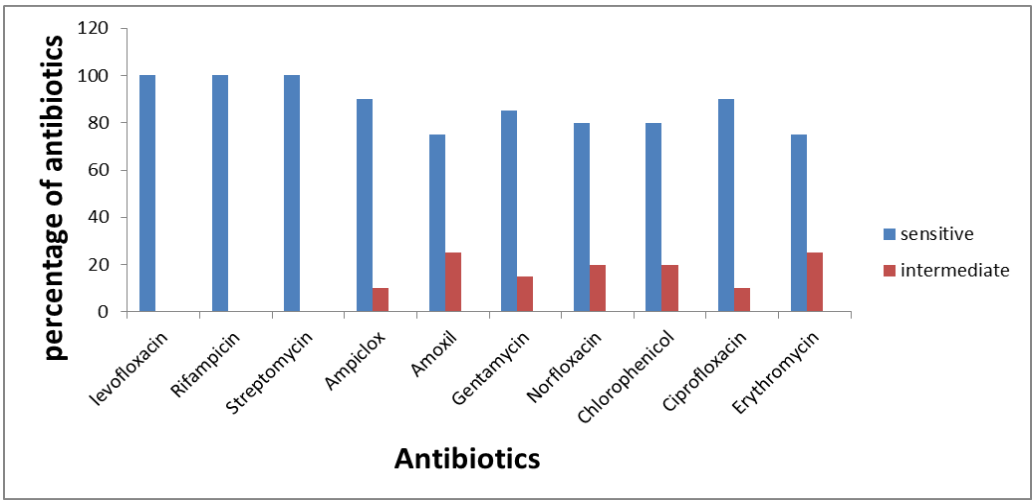

Figure 1.0: Susceptibility Pattern of the Isolates.

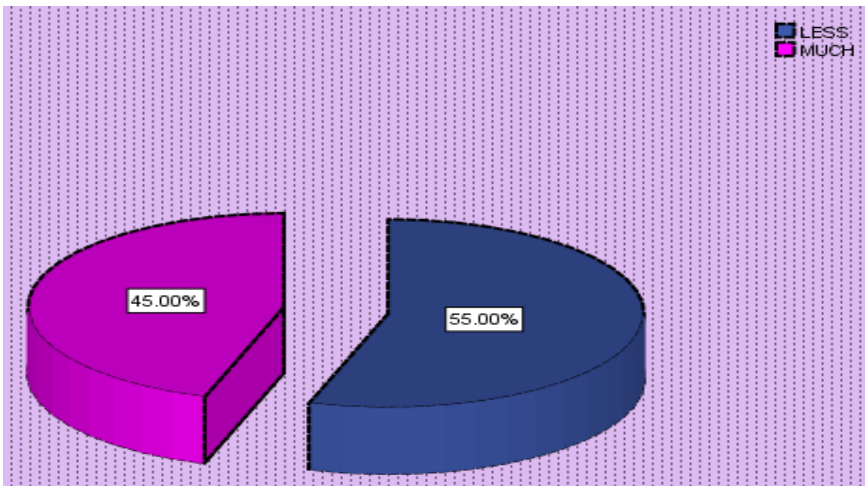

Figure 2.0: Pie Chart Showing Level of Contamination Awareness.

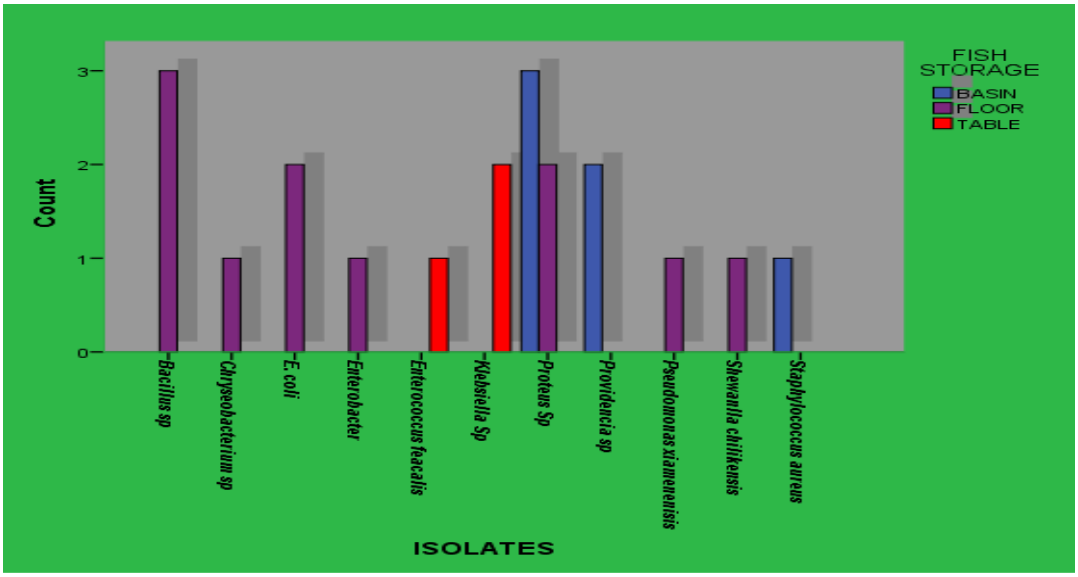

Fig. 3.0: Storage of Smoke-dried Fish.

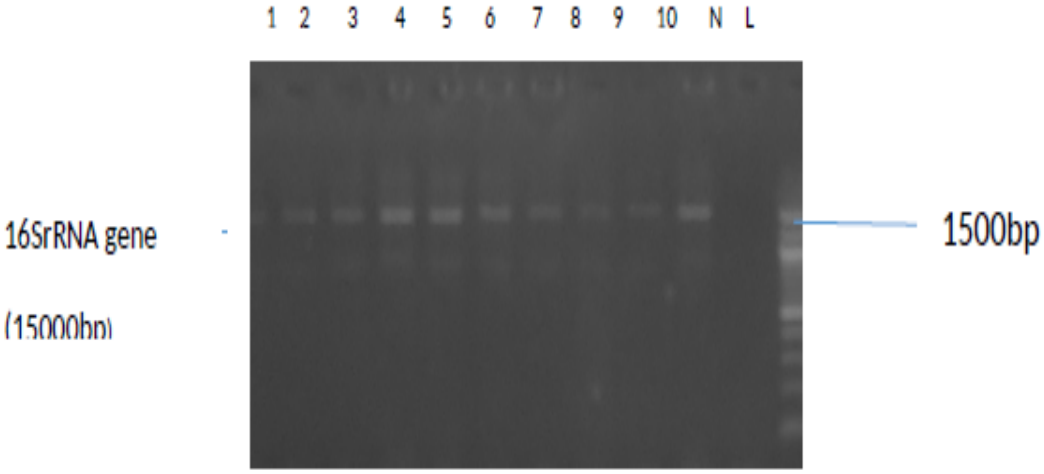

Plate 1: Agrose gel electrophoresis of the 16SrRNA gene of some selected bacterial isolates. Lane 1, 3-12 represent the 16S-rRNA gene bands (1500bp), lane 2, failed amplification, lane N represents the negative control, lane L represents the 100bp molecular ladder.

5 DISCUSSION

The mean value of the catfish is 4.3 x10^5 CFU/ml and Tilapia 3.7 x 10^5 CFU/ml in Table 1.0 indicating that there is no significant difference between the Catfish and the Tilapia. In Table 2.0 it shows the bacteria count of the smoke-dried Catfish and Tilapia from various market locations. Table 3.0 of this present study revealed that various bacteria are present in smoke-dried Catfish and Tilapia, the bacteria isolated from the fish includes *Proteus* sp 25%, *Escherichia coli, Pseudomonas* sp *and Klebsiella* sp 10% *Bacillus* sp 15% while other bacteria had 5% each. This result is not in line with Akinyemi and Oyelakin, (2014) who conducted similar research on the isolated bacteria from farm-raised Catfish and recorded *Comamonas* strains to be the highest frequency found in the smoke-dried fish 25% followed by *Proteus* strain which was 15% while other strains of bacteria occurred uniformly with a total of 5% for each strain. The result of this study shows that Mile 3 market had the highest level of microbial contamination of mean value of 5.0 x 10^5CFU/ml for catfish and 3.0 x 10^5CFU/ml for Tilapia. This could be due to the unhygienic environment of the market, as the market was observed to be highly congested and surrounded by some dumping sides and dirty gutters.

Atmospheric exposure and unhygienic handling of the smoke-dried fish could probably be the reasons for the high bacteria contamination. This is because the smoke-dried fishes were displayed on open trays and tables thereby exposing them to insects, dust particles and even consumers who were observed to be making direct contact with the smoke-dried fish with bare hands while bargaining. Adebayo-Toyo *et al.* (2012b) noted a similar scenario in Uyo, Nigeria, where retailers displayed

smoked fish samples in open trays and tables beside gutters and refuse heaps.

Mile 3 market was closely followed by Rumuokoro (Slaughter market) in terms of high microbial contamination with mean value of 5.0 x 10^5 for tilapia and 2.5 x 10^5 for catfish. This could be associated with the proximity of the slaughter market bridge which was observed to be highly polluted due to anthropogenic activities, such as defecating and refuse dumping. The present study took cognizance of the educational level of the fish sellers and contamination awareness which could lead to unhygienic practices thereby resulting to more microbial load of the smoke-dried fish through fecal contamination therefore fish sellers should be health educated on hygienic practices to avoid cross contamination of the fish.

Bascillus sp, *Chryseobacterium* sp, *Escherichia coli, Enterobacter* sp, *Proteus* sp, *Pseudomonas* sp and *Shewanella chilikensis* were found among the smoke-dried fish store on the floor as in Figure 4.7. On the other hand, *Staphylococcus aureus, Providencia* sp and *Proteus* sp were found among the smoke-dried fish stored in the basin and other isolates were among those stored on tables.

In this study Levofloxacin, Streptomycin and Rifampicin were 100% sensitive while Ampiclox and Ciprofloxacin were 90% sensitive in Figure 4.3 Amoxil and Erythromycin are 25% intermediate while Norfloxacin and Chloramphenicol are 20% intermediate and others 10% and 15% respectively. The level of antimicrobial sensitivity of these bacteria is clinically relevant, all bacteria strains were sensitive to the antibiotics which means they were found to be more effective as all the bacterial strains were susceptible to these antibiotics. This result is not in agreement with the findings reported by Akinyemi, (2011) who reported that all the bacteria strains isolated were resistance to Levofloxacin. Ciprofloxacin was effective on the following isolates *Klebsiella sp, Proteus sp Escherichia coli, Enterococcus feacalis, Shewanella chilikensis* while chloramphenicol was effective on *Providencia* sp, *Chryseobacterium* sp *and Klebsiella* sp. This implies that some bacteria showed variation in sensitivity to these antibiotics. In 2018 and 2019, Monsi and co-researchers showed that some bacteria develop resistance to antibiotics after prior sensitization to herbal substances. Some of the fish preservatives could be a potential source of antimicrobial resistance seen in some isolates. One of the mechanisms responsible for resistance in the study conducted by Monsi *et al.* (2018) suggested that opportunistic pathogens get sensitized by exposure to herbal extracts hence inducing biofilm formation.

The antimicrobial resistance in bacterial pathogens is a major impediment to successful therapy, and in several instances, bacterial strains have high resistance capacity to most available antimicrobial treatments. The public health consequences of antimicrobial resistance to many antibiotics have been debated. Recently clear evidence of health risk was evaluated to the multiple drug resistance of bacteria these drugs sent an extremely public health problem and it has always been associated with the outbreak of major epidemic throughout the world (Prescott *et al.*, 1999) but in this study there was no case of resistance, however if anyone is infected with any of the bacteria strain in the study there will be a complete cure unless there is a bridge of therapy intake which is one of the reasons for drug resistance. Those antibiotics that fall under intermediate were counted among the sensitive because prolong intake of antibiotics will take care of the ill health.

6 CONCLUSION

This study isolated some potentially pathogenic bacteria which none were resistant to antibiotics used in the study. Fish processors and retailers should take extra care in ensuring proper processing and handling of fish and fish products by observing sanitary and hygienic rules.

Owing to the potential hazard of some pathogens, isolated in this study it is clearly necessary to put more emphasis on food hygiene. Therefore surveillance of potential contaminant bacteria in smoke-dried fish is crucial for sustenance of public health. It is therefore recommended that people should be educated on the unhealthy implication of water pollution as this goes a long way in contaminating the aquatic animals proper processing, storage, and handling procedures should be cultivated. Adequate processing methods before consumption should be employed.

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