# ISOLATION AND IDENTIFICATION OF MULTI-DRUG-RESISTANT *Pseudomonas* ISOLATES FROM SHELLFISHES FROM A FISH MARKET IN LAGOS, NIGERIA

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## ABSTRACT

Shellfishes are a delicacy worldwide and they are usually found at the bottom of water bodies. These aquatic resources can harbour pathogenic organisms, which cause food-borne infections when the shellfish is not adequately processed. The microbial quality of shellfish is therefore a major public health concern for marketers, processors and consumers. This study investigated the microbial quality of shrimp and crab samples from a fish market located in Lagos, Nigeria. Bacteria were isolated from shellfishes and characterized using standard microbial analysis techniques. The susceptibility of selected isolates to 15 antibiotics was determined using the modified disc diffusion method. Total heterotrophic count ranged from $1.20 \times 10^7$ to $>1.0 \times 10^8$ $cfu^{-1}g$, while the coliform count was between $4.08 \times 10^4$ and $>1.0 \times 10^8$ $cfu^{-1}g$. The isolates identified belonged to the seven genera: *Pseudomonas, Proteus, Alcaligenes, Vibrio, Salmonella, Staphylococcus* and *Bacillus*. Antibiotic susceptibility test showed that the three *Pseudomonas* spp. isolated were resistant to 3-6 different antibiotics, with multiple antibiotic resistance values ranging from 0.2 to 0.4. The presence of these organisms poses a potential health risk, as they can cause spoilage of shellfish, result in food-borne infections and confer antibiotic resistance to normal gut floral. There is therefore a need for regular monitoring of the safety of shellfishes sold in the market and creation of awareness on the dangers of improper handling and inadequate processing of shellfishes.

**Keywords: Shellfish, Bacteria, Antibiotic-resistance, Makoko, *Pseudomonas***

## INTRODUCTION

Fish constitutes approximately 16% and 17% of animal protein consumed globally and in Africa, respectively (FAO, 2007; Allison *et al*., 2009). Fish can harbour pathogenic organisms on their skin and in their tissue, due to exposure to contaminated/polluted water. These makes fish and fisheries products potential health risks (Gufe *et al*., 2019). Bacteria thrive well on substrates with high moisture content, and harvested fish are mostly prone to microbial deterioration because of their high moisture content and low pH (Morshdy *et al*., 2019).

Bacterial infections from fish usually result from improper handling and inadequate processing of fish for consumption. Most of the food borne diseases associated with the consumption of seafood are caused by *Escherichia, Salmonella, Vibrio, Staphylococcus, Pseudomonas* and other enteric organisms (Yagoub, 2009, Hossain *et al*., 2010). In addition, bacteria genera such as *Aeromonas, Serratia, Flavobacterium, Pseudomonas, Listeria, Klebsiella, Salmonella* have been isolated from shell fishes (Jeyasekaran *et al*., 2006, Sichewo *et al*., 2014).

Shellfishes such as shrimps, crabs and oysters are naturally associated with bacteria, parasites and viruses; because of anthropogenic activities, environmental conditions of the water bodies and post-harvest operations (Flick and Granata, 2010). *Pseudomonas* are gram negative, rod-shaped bacteria. They are free living and present aquatic organisms, water and sediments. *Pseudomonas* have been reported as potential pathogens in fish (Koutsoumanis and Nychas, 2000; Jeyasekaran *et al*., 2006; Yagoub, 2009; Ali *et al*., 2021). They are common spoilage organisms of fish and fishery products, with the consumption of contaminated products resulting in gastrointestinal and skin infections (Benie *et al*., 2017; Losito *et al.,* 2022).

Antibiotic-resistant *Pseudomonas* from fish have been reported by a number of authors (Akond *et al.,* 2009, Kathleen *et al*., 2016, Gufe *et al*., 2019). The emergence of antimicrobial resistance in bacteria found in animals is a serious public health concern, as this can lead to the transfer of resistant genes to humans (Cabello, 2006) and the abuse of antibiotics is one of the factors that has contributed to the development of antimicrobial resistance (Kathleen, 2016).

Makoko Fish market is a popular fish market that provides fresh fish, mostly obtained from the Lagos Lagoon in Nigeria. The lagoon is prone to contamination from domestic waste, untreated sewage and industrial effluents (Okoye *et al.* 2010). It is therefore important to examine the safety (in terms of microbial and antimicrobial resistance) of fish obtained from the market. In addition, the health implications fish products from the market on fish mongers, buyers and consumers require assessment. This study was therefore carried out to isolate and identify the bacteria associated with shellfishes (shrimp and crab) from Makoko market. The study also evaluated the antibiotic susceptibility pattern of *Pseudomonas* spp. isolated from the shellfishes.

## MATERIALS AND METHODS

### *Sample Collection*

Shellfish samples (shrimps - *Penaeus monodon*, *Penaeus notialis*, *Macrobrachium vollenhovenii* and crab - *Callinectes amnicola*) were randomly purchased from vendors at Better Life Fish Market, Makoko, Lagos Mainland Area, Nigeria. The samples were collected between June 2019 and June 2020. Five samples each were purchased and transported in ice boxes to the Microbiology Laboratory of the Fish Technology and Product Development Department, Nigerian Institute for Oceanography and Marine Research, Victoria Island, Lagos. The samples were identified at the Marine Biology Section of the Institute. All samples were analyzed within 3 hours, after collection.

### *Isolation of Bacteria*

The shells of the samples were aseptically removed and 10 g of the de-shelled sample was weighed into a universal bottle containing 0.1% sterile peptone water and homogenized. The mixture was serially diluted and plated out on different media, such as *Pseudomonas* isolation Agar (PIA), Thiosulphate Citrate Bile Salt (TCBS) Agar,

*Salmonella Shigella* Agar (SSA), Mannitol salt Agar (MSA), Nutrient Agar (NA) and Lactose Broth (LB). The media were prepared according to the manufacturers' instructions. Total heterotrophic bacteria count was done by plating out aliquots of the serially-diluted samples on NA, coliforms were enumerated using LB. The presence of other bacteria of public health importance was determined by plating out aliquots of the serially-diluted samples on SSA and TCBS. These helped to detect *Salmonella* and *Shigella;* and *Vibrio* species, respectively. The isolates were purified severally, until pure cultures were obtained and then used for morphological characterization.

### *Identification of Isolates*

The cultural characteristics of the colonies such as colour, shape, edge and orientation were observed and further biochemical tests such as oxidase, citrate, Urease, sugar fermentation, and Triple Sugar Iron (TSI) were carried out on the pure cultures to tentatively characterize the bacterial isolates, following the methods described by Cheesbrough (2006).

The DNA of selected isolates were extracted and subjected to Polymerase Chain Reaction (PCR) (Omoya and Ajayi, 2020). The PCR product obtained was sequenced and the sequences were compared with those available in the GenBank using the NCBI Basic Local Alignment Search Tool (BLAST) for the molecular identification of the isolates.

### *Antibiotic Susceptibility Testing*

The susceptibility of the isolates to fifteen selected antibiotics was done by the modified disc diffusion method (Bauer *et al.*, 1966) as described by Olajuyigbe *et al.* (2019).

The antibiotics used were: Amoxicillin Clavulanate (AUG) - 30 µg, Cefotaxime (CTX) - 25 µg Imipenem/ Cilastatin (IMP) - 10/10 µg, Cefuroxime (CXM) - 30 µg, Ofloxacin (OFX) - 5 µg, Erythromycin (ERY) - 15 µg, Gentamicin (GN) - 10 µg, Azthromycin (AZN) - 15 µg, Ceftriaxone/ sulbactam (CRO) - 45 µg, Levofloxacin (LBC) - 5 µg, Ciprofloxacin (CIP) - 5 µg, Nalidixic acid (NA) - 30 µg, Nitrofurantoin (NF) - 300 µg, Ampiclox (ACX) - 10 µg, Cefexime (ZEM) - 5 µg. The isolates were defined as either resistant (R), Intermediate (I) or Susceptible (S), according to the standards of CLS1 (2018).

### *Multiple Antibiotic Resistance Indexing*

Isolates that were resistant to more than three antibiotics were recorded as having multiple antibiotic resistance. The multiple antibiotic resistance (MAR) value for each isolate was obtained according to the method of Odeyemi *et al.* (2012). This was done by dividing the total number of antibiotics the isolate was resistant to with the total number of antibiotics tested.

## RESULTS

The total heterotrophic count ranged from $1.20\times10^7$ to $>1.0\times10^8$ $cfu^{-1}g$, while coliform count was between $4.08\times10^4$ and $>1.0\times10^8$ $cfu^{-1}g$. A total of eighty-six bacteria isolates were identified and belonged to

*Pseudomonas, Proteus, Alcaligenes, Vibrio, Salmonella, Staphylococcus* and *Bacillus* genera. *Pseudomonas* which are common spoilage organisms with high percentage of antimicrobial resistance was selected for the antimicrobial resistance testing. The *Pseudomonas* isolates were susceptible to between nine and twelve antibiotics and exhibited different antibiotic susceptibility patterns (Table 3).

The isolates were resistant to between three and six antibiotics (Table 4), thus implying multidrug resistance isolates. The Multiple Antibiotic Resistance index ranged from 0.2 to 0.4.

**Table 1. Morphological Characteristics of *Pseudomonas* Isolates from Shellfishes in Makoko Lagos, Nigeria**

| S/N | Isolate code | Suspected organism | Form | Margin | Elevation | Optical Density | Colour on Nutrient Agar | Colour on MacConkey Agar |
|---|---|---|---|---|---|---|---|---|
| 1 | C2BVY1 | *Pseudomonas* sp. | Circular | Irregular | Raised | Opaque | Greenish | Colourless |
| 2 | S3AT2 | *Pseudomonas aeruginosa* | Circular | Irregular | Raised | Opaque | Greenish | Colourless |
| 3 | C2BVB | *Pseudomonas aeruginosa* | Circular | Entire | Raised | Opaque | Greenish | Colourless |

The sequences of the *Pseudomonas aeruginosa* strain S3AT2, C2BVB, have been deposited in the Gen bank with accession number MT785873 and MT785904, respectively.

**Table 2. Biochemical Characteristics of Isolates from Shellfishes in Makoko Lagos, Nigeria**

| Characteristics | Suspected Organisms | | |
|---|---|---|---|
| | ***Pseudomonas* sp. (C2BVY1)** | ***Pseudomonas aeruginosa* (S3AT2)** | ***Pseudomonas aeruginosa* (C2BVB)** |
| Gram Reaction | Negative | Negative | Negative |
| Shape | Rod | Rod | Rod |
| Catalase | Positive | Positive | Positive |
| Oxidase | Positive | Positive | Positive |
| Methyl Red | Negative | Negative | Negative |
| Voges Proskauer | Negative | Negative | Negative |
| Indole | Negative | Negative | Negative |
| Citrate utilization | Positive | Positive | Positive |
| Urease | Negative | Negative | Negative |
| Nitrate | Positive | Positive | Positive |
| Hydrogen sulphide | Negative | Negative | Positive |

**Table 3. Antibiotic Susceptibility Profile of Isolates from Shellfishes in Makoko Lagos, Nigeria**

| Antibiotics | Isolates | | |
|---|---|---|---|
| | ***Pseudomonas* sp. (C2BVY1)** | ***Pseudomonas aeruginosa* (S3AT2)** | ***Pseudomonas aeruginosa* (C2BVB)** |
| Amoxicillin Clavulanate (AUG) | R | R | S |
| Cefotaxime (CTX) | S | R | R |
| Imipenem/ Cilastatin (IMP) | R | S | R |
| Ofloxacin (OFX) | S | S | S |
| Gentamicin (GN) | S | S | S |
| Nalidixic acid (NA) | R | S | S |
| Nitrofurantoin (NF | R | S | S |
| Cefuroxime (CXM) | R | S | R |
| Ceftriaxone sulbactam (CRO) | S | R | S |
| Ampiclox (ACX) | R | R | S |
| Cefixime (ZEM) | S | R | S |
| Levofloxacin (LBC) | S | S | S |
| Erythromycin (ERY) | S | S | S |
| Azthromycin (AZN) | S | S | S |
| Ciprofloxacin (CIP) | S | S | S |

KEY: S = Susceptible R= Resistant

**Table 4. Pattern of Antibiotic Resistance of Isolates from Shellfishes in Makoko Lagos, Nigeria**

| Isolate code | No. of Sensitive Antibiotics | No. of Resisted Antibiotics | List of Resisted Antibiotics | MAR Index |
|---|---|---|---|---|
| ***Pseudomonas* sp. (C2BVY1)** | 9 | 6 | AUG, IMP, NA, NF, CXM, ACX | 0.4 |
| ***Pseudomonas aeruginosa* (S3AT2** | 10 | 5 | AUG, CTX, CRO, ACX, ZEM | 0.3 |
| ***Pseudomonas aeruginosa* (C2BVB)** | 12 | 3 | CTX, IMP, CXM | 0.2 |

## DISCUSSION

The total heterotrophic bacteria count ($1.20 \times 10^7$ to $>1.0 \times 10^8$ $cfu^{-1}g$) and coliform count ($4.08 \times 10^4$ to $>1.0 \times 10^8$ $cfu^{-1}g$) exceeded the acceptable limit recommended by the International Commission on Microbiological Specification for Foods (ICMF, 1986). Edun *et al.* (2016) and Omoya and Ajayi (2020) also observed high microbial load in shrimps from Rivers and Ondo States, Nigeria. This high microbial presence was attributed to water pollution as a result of anthropogenic activities. In this study, the shellfishes could potentially cause food borne infection and therefore, require adequate processing to prevent food intoxication. Since, the shellfishes were generally from the Lagos lagoon, this implies that the level of environmental pollution was high in the lagoon., which is susceptible to indiscriminate discharge of untreated sewage and industrial wastes. Most of the isolates obtained (*Pseudomonas, Alcaligenes, Proteus, Staphylococcus* and *Bacillus*), have been associated with crabs in Nigeria, Benin and India (Mahalaxmi *et al.,* 2013; Edun *et al.*, 2016; Omoya and Ajayi, 2020).

Benthic organisms such as shrimps and crabs have the tendency to harbour more pathogenic organisms as a result of the accumulation of pollutants in sediment. *Pseudomonas* has been identified as a pathogen with antimicrobial resistance in seafoods (Koutsoumanis and Nychas, 2000; Jeyasekaran *et al*., 2006; Yagoub, 2009; Benie *et al*., 2017; Ivanisova *et al.,* 2019).

*Pseudomonas aeruginosa* strains have been shown to possess natural resistance to many antibiotics. This has been attributed to hypermutator phenotypic qualities of some strains. This allows them to develop resistance to drugs; they were initially sensitive to. The presence of plasmids has also been reported to be responsible for the development of resistance to various antibiotics (Peng *et al.*, 2014; Pereira *et al*., 2014).

The isolation of *Pseudomonas* strains with multi-drug resistance from fish is a serious public health concern; as this could lead to the transfer of Antimicrobial Resistance (AMR) genes in the aquatic ecosystem (Banquero *et al.,* 2008). The consumption of inadequately processed fish contaminated with *Pseudomonas aeruginosa* could lead to the transmission of Multi-Drug-Resistant (MDR) plasmids (Shahrokhi *et al*., 2022). The emergence of MDR bacteria can lead to a reduction in the efficacy of antibiotics used in the treatment of infections (Delannoy *et al*., 2022). The isolates restricted different groups of antibiotics, such as the Beta lactamase group. The isolation of bacteria with MAR index > 0.2 showed some level of antibiotic abuse. This probably has resulted in the development of antibiotic resistance among the isolates.

## CONCLUSION

The shellfishes obtained from Makoko fish market were contaminated with potential pathogenic organisms that could cause food-borne infection and has the potentials of conferring antibiotic resistance on normal gut floral. With proper handling and adequate processing, these negative health implications can be averted. There is

therefore a need for proper handling and adequate processing of fish in order to reduce the potential risks associated with shellfish consumption. There is also a need for regular monitoring of the safety of fishes sold in the market. The various stakeholders should be sensitised on the health implications of handling and consuming bacteria-contaminated fish.

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