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# Isolation of *Pseudomonas* Species and Extended Spectrum beta-lactamase-producing *Escherichia coli* from Retail Imported Mackerel Frozen Fishes Sold in Abakaliki Metropolis

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## Abstract

**Purpose**: The cardinal objective of this study was to isolate, phenotypically characterize, and determine the antibiotic resistance patterns of extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Pseudomonas species* from retail imported mackerel frozen fishes sold in Abakaliki metropolis.

**Methods**: Exactly 100 mackerel frozen fish samples were collected from two selected markets within Abakaliki metropolis. They were analyzed for the presence of *Escherichia coli* and *Pseudomonas spp.* using standard microbiological techniques. Isolated *E. coli* and *Pseudomonas spp.* were screened for ESBL production using double disc synergy test and positive ESBL-producing *E. coli* were afterwards tested for their susceptibility to different classes of antibiotics using Kirby Bauer disc diffusion method.

**Results**: Results showed that out of 100 fish samples analyzed, 69 (69%) were positive for *Pseudomonas spp.* while 21 (21%) were positive for *E. coli.* Out of the 21 *E. coli* isolated, 7 (33.3%) were confirmed to be ESBL-producers while none (0) of the *Pseudomonas species* isolated produced ESBL. All the ESBL-positive *E. coli* were completely resistant (100%) to ceftriazone, amoxicillin, cefuroxime, ticarcillin/clavanic acid, cefepime, and piperacillin. They also exhibited resistance to chloramphenicol (83.5%), and tobramycin (58.5%). Interestingly, ciprofloxacin was the most active antibiotic against the ESBL-producing E. coli isolates as they were all completely susceptible (100%) to this fluoroquinolone antibiotic. The average multiple antibiotic resistance index (MARI) of the ESBL-producing *E. coli* isolates was 0.84 and this depicts their multi-drug resistance traits as they were resistant to at least two different classes of antibiotics.

**Conclusion**: This study has shown that mackerel fish might be a possible reservoir of ESBL-producing *E. coli* and may contribute to the spread of ESBL-producing bacterial strains to human through the food chain, thus resulting in food-borne illnesses and other public health problems. Therefore, it is imperative to holistically evaluate the drift of imported fish in Abakaliki and nationwide so as to curb possible public health consequences which could arise as a result of the consumption of imported fishes harbouring ESBL-producing bacteria.

**Keywords:** ESBL; *E. coli; Pseudomonas spp.*; Mackerel frozen fishes; Resistance

## Introduction

Over the past few decades, research has shown that the nutrients and minerals in seafood such as fish can bring about a possible change in the development and reproduction of brain and has emphasized the role of seafood in the function of the human body. Fish is also a source of vitamin A, and is needed for healthy skin and clear eyes, and vitamin D, which is needed to help the body absorb calcium for strong bones and teeth. Products from fishery, which are of enormous relevance for the nutrition of humans globally and contribute fairly to well-being can act as a source of food-borne pathogens and may be a possible source of infection [1]. The bacterial flora of marine fish, debris and sea water has been analyzed all over the globe and diseases caused by bacteria are mainly due to contaminated water and sea foods. The largest groups of pathogenic bacteria in fish are Gram-negative, aerobic and/or facultative anaerobic bacteria. The most common treatment for bacterial infections is the beta-lactam antimicrobial agents. The persistent exposure of bacterial strains to a multitude of beta-lactams has induced dynamic and continuous production and mutation of β-lactamases in these bacteria, expanding their activity even against the newly developed β-lactam antibiotics. These

enzymes are known as extended-spectrum beta-lactamases (ESBLs) [2]. Extended-spectrum beta-lactamases (ESBL) are enzymes that confer resistance to most beta-lactam antibiotics, including 2nd and 3rd generation cephalosporins, penicillins, and the monobactam (aztreonam). Infections with ESBL-producing organisms have been associated with poor outcomes. These enzymes (Extended Spectrum Beta-lactamases) open the beta-lactam ring, making the antibiotics to be inactive [3]. Treatment of infectious diseases has become a worldwide problem due to the resistance of pathogenic organisms to antibiotics. There is an increase use/misuse of antibiotics in human medicine, agriculture and veterinary which are contributing to it. There is heightened increase of antibiotic resistance in bacteria that cause either community infections or nosocomial infections; of particular interest are the multidrug resistant pathogens, e.g. *Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, methicillin-resistant Staphylococcus aureus, penicillin*-resistant *Streptococcus pneumoniae, vancomycin-resistance Enterococcus,* and *extensive drug-resistant Mycobacterium tuberculosis* [4]. This study is therefore designed to isolate, phenotypically characterize, and determine the antibiotic resistance patterns of extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Pseudomonas* species from retail imported mackerel frozen fishes sold in Abakaliki metropolis.

## Materials and Methods

### Study area

This study was conducted in Abakpa and Kpirikpiri market area in Abakaliki, the state capital of Ebonyi State. Ebonyi State is located in the South Eastern part of Nigeria. It is bounded by Enugu State in the West, Cross River in the East, Abia state in the South, and Benue state in the North. It is between longitude 7°30' and latitude 60°45' E. In the 2006 population and housing census, Ebonyi State had an estimated population of 2.3 million people.

### Sample collection

One hundred samples of mackerel frozen fishes were collected from various markets in Abakaliki, using sterile containers. The samples were labeled accordingly and transported within 2 hr in an ice-packed container to microbiology laboratory unit of Ebonyi State University, Abakaliki for bacteriological analysis.

### Sample preparation, culturing, and identification of isolates

Mackerel frozen fish preparations were made by cutting fish sample from the gill and intestine region with a sterile knife. The cut samples were crushed in a sterile mortar using pestle. From the crushed sample, one ml volume was measured out and homogenized in a clean, dry sterile test tube containing nine milliliters of peptone water. Preparation of serial dilutions was done and the crushed sample was diluted from 101 to 1010 for each fish sample [1]. The diluted crushed frozen fish samples were incubated at 37°C for 18-24 h. Turbid tubes with signify growth was cultured on eosin methylene blue (EMB) agar and MacConkey agar for the isolation of *E. coli*, and cetrimide agar for the isolation of *Pseudomonas spp*. The isolates were further identified and characterized using standard microbiological techniques [5].

### Preliminary determination of ESBL-producing *E. coli*

All the bacterial pathogens (*E. coli* and *Pseudomonas spp*) were screened for the production of ESBL. Single antibiotic discs containing cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), and aztreonam (30 µg) were placed aseptically at a distance of 30 mm apart on a Mueller-Hinton (MH) agar (Oxoid, UK) plates that was previously inoculated with standardized inoculums of the test bacteria. The plate was allowed for 30 minutes for pre-diffusion of antibiotics and was incubated for 18-24 h at 37°C. After the incubation, zones of inhibition were measured in millimeter using a metre rule and recorded. ESBL production was suspected if any of the test bacteria showed reduced susceptibility or is resistant to any of the antibiotics used for the screening studies according to the CLSI guidelines [6].

### Double disk synergy test (DDST)

ESBL production was confirmed on the bacterial isolates by double disk synergy test [6]. DDST was performed as a standard disk diffusion assay on Mueller-Hilton (MH) agar (Oxoid, UK) plates in line with CLSI criteria [7]. Sterile swab sticks were dipped into bacterial suspension (standardized to 0.5 McFarland turbidity equivalent) with reduced susceptibility to the 2nd and 3rd generation cephalosporins. Antibiotic discs of amoxicillin-clavulanic acid (30 µg) was placed at the center of the MH agar plate, and antibiotic discs containing cefotaxime (30 µg) and ceftazidime (30 µg) was each placed at a distance of 15 mm (centre to centre) from the central disk, amoxycillin/clavulanic acid (30 µg). The plate was incubated at 37°C for 18-24 h. A zone of inhibition ≥5 mm in diameter of either cefotaxime and ceftazidime tested in combination with amoxicillin/clavulanic acid against its zone when tested alone confirms ESBL production phenotypically [6,8].

### Antimicrobial susceptibility test

Antimicrobial susceptibility test of the ESBL-producing *E. coli* isolates was done using the standard Kirby-Bauer disk diffusion [9]. The ESBL-producing inoculum was prepared by suspending the freshly grown bacteria in 5 ml sterile nutrient broth and its turbidity adjusted to 0.5 McFarland standards. The antimicrobial susceptibility testing was performed on Mueller-Hinton agar using the following antibiotics; amoxicilin (AMC, 10µg), cefepime (FEP, 30 µg), cefuroxine sodium (CXM, 30 µg), ceftriazone (CRO, 30 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), ticarcilline/clavanic acid (TIM, 85 µg), piperacillin (PRL, 100 µg) and tobramycin (TOB, 10 µg).The plates were incubated aerobically at 37°C for 18-24 h. The zones of inhibition were measured with a metre rule and the results were recorded and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [7-12].


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### Determination of multiple-antibiotic resistance index (MARI)

This was calculated using the method described by Chitanand et al. [10]:

MARI=a/b

Where, a=Total number of resistance scored; b=Total number of antibiotics tested.

## Results and Discussion

Mackerel fishes can be exposed to pathogenic microorganisms during, before and after harvest; also during packaging, transportation, storage, and selling by handlers and consumers which in all is a health hazard to the consumer/public health. Infection of mackerel fishes by microbes may be as a result of human or animal wastes inundated with antibiotic resistant bacteria in the pond where these fishes are reared. This study reports the presence of *Pseudomonas spp.* and ESBL-producing *E. coli* from retail imported mackerel frozen fishes sold in Abakaliki metropolis, Ebonyi state Nigeria. A total of 100 mackerel frozen fish samples were collected from two selected markets (Abakpa and kpirikpiri) in Abakaliki and were analyzed for the isolation of extended spectrum beta-lactamase-producing *E. coli* and *Pseudomonas* spp. Out of the 100 mackerel frozen fishes sampled from the two markets; a total of 69 *Pseudomonas spp*. and 21 *E. coli* were isolated (**Tables 1 and 2**). The result of ESBL screening showed that all the *Pseudomonas spp.* isolated were ESBL-negative while 7 (33%) out of the 21 *E. coli* isolated were ESBL-positive (**Tables 3-6**). Four (57.1%) out of the 7 *E. coli* isolates from Abakpa market screened for confirmatory ESBL-production were positive while 3 (75%) were positive from the 4 *E. coli* isolates from kpirikpiri market (**Tables 7 and 8**).

Our result on the isolation of ESBL-producing *Escherichia coli* in this study is in agreement with the work carried out by Elhadi et al. [6] in Saudi Arabia who reported the presence of ESBL-producing *E. coli* in mackerel fishes. He further showed that mackerel fishes might be the possible reservoirs for ESBL-producing *E. coli* and may contribute to the dissemination and transfer of beta-lactamasegenes to humans through the food chain. ESBL-producing *E. coli* has also been reported in fishes by Sivaraman et al. [13] in India.

Our study is also in concord with the work of Eze et al. [7] in Nsukka, Nigeria who reported that frozen mackerel fish could be contaminated by a lot of microorganisms which include *Escherichia coli* and *Pseudomonas* species. Our result on antimicrobial susceptibility test showed that the isolated ESBL-producing *E. coli* from the two markets exhibited varying levels of susceptibilities and resistances to the test antibiotics. The ESBL-producing *E. coli* isolates from Abakpa market were completely resistant (100%) to ceftriaxone, chloramphenicol, amoxicillin, cefuroxine sodium, ticarcillin/clavanic acid, cefepime, and piperacillin (**Table 9**).

**Table 1.** Frequency of *Pseudomonas species* in mackerel frozen fish samples.

| No. of samples | No. of Isolate | Prevalence of isolate in samples (%) |
|---|---|---|
| 60 from Abakpa | 44 (63.8%) | 73 |
| 40 from Kpirikpiri | 25 (36.2%) | 63 |

**Table 2**. Frequency of *E. coli* in mackerel frozen fish samples.

| No. of samples | No. of Isolate | Prevalence of isolate in samples (%) |
|---|---|---|
| 60 from Abakpa | 13 (61.9%) | 22 |
| 40 from Kpirikpiri | 8 (38.1%) | 20 |

**Table 3.** Preliminary screening for ESBL production by *Pseudomonas spp*. isolated from mackerel frozen fishes sold in Abakpa market.

| Antibiotics used | Total number of isolate | Sensitivity (N (%)) | Resistance (N (%)) |
|---|---|---|---|
| Cefepime | 44 | 44 (100) | 0 (0) |
| Aztreonam | 44 | 44 (100) | 0 (0) |
| Cefotaxine | 44 | 44 (100) | 0 (0) |
| Ceftazidime | 44 | 44 (100) | 0 (0) |

**Table 4.** Preliminary screening for ESBL production by *Pseudomonas spp.* isolated from mackerel frozen fishes sold in Kpirikpiri market.

| Antibiotics used | Total number of isolate | Sensitivity (N (%)) | Resistance (N (%)) |
|---|---|---|---|
| Cefepime | 25 | 25 (100) | 0 (0) |
| Aztreonam | 25 | 25 (100) | 0 (0) |
| Cefotaxine | 25 | 25 (100) | 0 (0) |
| Ceftazidime | 25 | 25 (100) | 0 (0) |

**Table 5.** Preliminary screening for ESBL production by *E. coli* isolated from mackerel frozen fishes sold in Abakpa market.

| Antibiotics used | Total number of isolate | Sensitivity (N (%)) | Resistance (N (%)) |
|---|---|---|---|
| Cefepime | 13 | 7 (54) | 6 (46) |
| Aztreonam | 13 | 10 (77) | 3 (23) |
| Cefotaxime | 13 | 6 (46) | 7 (54) |

| | | | |
|---|---|---|---|
| Ceftazidime | 13 | 8 (62) | 5 (38) |

**Table 6.** Preliminary screening for ESBL production by *E. coli* isolated from mackerel frozen fishes sold in Kpirikpiri market.

| Antibiotics used | Total number of isolate | Sensitivity (N (%)) | Resistance (N (%)) |
|---|---|---|---|
| Cefepime | 8 | 4 (50.0) | 4 (50.0) |
| Aztreonam | 8 | 7 (87.5) | 1 (12.5) |
| Cefotaxine | 8 | 4 (50.0) | 4 (50.0) |
| Ceftazidime | 8 | 5 (62.5) | 3 (27.5 |

**Table 7.** Confirmatory screening for ESBL-producing *E. coli* isolated from mackerel frozen fishes sold in Abakpa market.

| No. of isolates screened | ESBL-ve (N) | ESBL+ve (N) |
|---|---|---|
| 7 | 3 (42.9%) | 4 (57.1%) |

**Table 8.** Confirmatory screening for ESBL-producing *E. coli* isolated from mackerel frozen fishes sold in Kpirikpiri market.

| No. of isolates screened | ESBL -ve (N) | ESBL +ve (N) |
|---|---|---|
| 4 | 1 (25%) | 3 (75%) |

**Table 9.** Antibiotic Susceptibility Patterns of ESBL-Producing *E. coli* isolated from mackerel frozen fishes sold in Abakpa market.

| S/N | Antibiotics | Total number of isolate | Sensitivity (N (%)) | Resistance (N (%)) |
|---|---|---|---|---|
| 1 | Ceftriaxone | 4 | 0 (0) | 4 (100) |
| 2 | Chloramphenicol | 4 | 0 (0) | 4 (100) |
| 3 | Tobramycin | 4 | 2 (50) | 2 (50) |
| 4 | Ciprofloxacin | 4 | 4 (100) | 0 (0) |
| 5 | Amoxicillin | 4 | 0 (0) | 4 (100) |
| 6 | Cefuroxin sodium | 4 | 0 (0) | 4 (100) |
| 7 | Ticarcillin/clavanic acid | 4 | 0 (0) | 4 (100) |
| 8 | Cefepime | 4 | 0 (0) | 4 (100) |
| 9 | Piperacine | 4 | 0 (0) | 4 (100) |

They also showed resistance to tobramycin (50%) but were completely susceptibility (100%) to ciprofloxacin. This is in agreement with the report of Elhadi et al. [6] who in their work reported that the least frequency of resistance was observed for ciprofloxacin while the highest frequency of resistance was observed for piperacillin and ceftriazone. The ESBL-producing *E. coli* isolates from kpirikpiri market were completely resistant (100%) to ceftriaxone, amoxicillin, cefuroxime sodium, ticarcillin/clavanic acid, cefepime, and piperacillin and strangely to ciprofloxacin (**Tables 10-12**). Some level of susceptibility (33%) was observed for chloramphenicol and tobramycin. The result of the multiple antibioticresistance index (MARI) showed that the isolated ESBL-producing *E. coli* in our study had an average MARI of 0.84. This depicts the multi-drug resistance traits of the ESBL-producing *E. coli* isolates as they were resistant to at least two different classes of antibiotics such as the carbapenems, penicillins, aminoglycosides, and fluoroquinolones.

**Table 10.** Antibiotic Susceptibility patterns of ESBL-producing *E. coli* isolated from mackerel frozen fishes sold in Kpirikpiri market.

| S/N | Antibiotics | Total number of isolate | Sensitivity (N (%)) | Resistance (N (%)) |
|---|---|---|---|---|
| 1 | Ceftriaxone | 3 | 0 (0) | 3 (100) |
| 2 | Chloramphenicol | 3 | 1 (33) | 2 (67) |
| 3 | Tobramycin | 3 | 1 (33) | 2 (67) |
| 4 | Ciprofloxacin | 3 | 0 (0) | 3 (100) |
| 5 | Amoxicillin | 3 | 0 (0) | 3 (100) |
| 6 | Cefuroxin sodium | 3 | 0 (0) | 3 (100) |
| 7 | Ticarcillin/clavanic acid | 3 | 0 (0) | 3 (100) |
| 8 | Cefepime | 3 | 0 (0) | 3 (100) |
| 9 | Piperacine | 3 | 0 (0) | 3 (100) |

**Table 11.** MARI values for the ESBL-producing *E. coli* strains isolated from mackerel frozen fishes sold in Abakpa market.

| S/N | Organism | MARI Value |
|---|---|---|
| 1 | *E. coli* | 0.89 |
| 2 | *E. coli* | 0.89 |


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| | | |
|---|---|---|
| 3 | *E. coli* | 0.78 |
| 4 | *E. coli* | 0.89 |
| Total | | 3.45; Average=0.86 |

**Table 12.** MARI values for the ESBL-producing *E. coli* strains isolated from mackerel frozen fishes sold in Kpirikpiri market

| S/N | Organism | MARI Value |
|---|---|---|
| 1 | *E. coli* | 0.89 |
| 2 | *E. coli* | 0.67 |
| 3 | *E. coli* | 0.89 |
| Total | | 2.45; Average=0.82 |

## Conclusion

The results of our study have shown that some frozen mackerel fishes are heavily contaminated with *Pseudomonas* spp. and ESBL-producing *E. coli* pathogens that can cause infections in humans via the consumption of undercooked or ill-processed fishes mostly those who consume the gills and intestine of these fishes. Those who cut fishes at home for preparation and fish sellers in the market, who often cut these fishes for their buyers without washing their hands properly, can serve as potential reservoirs for the transmission of these pathogens which eventually leads to serious public health consequences. It is therefore imperative that strict hygienic practices be maintained when handling fishes for consumption. Also, proper monitoring and evaluation of environment, especially where fishes are sold, is very important to ensure that their wastes are properly disposed. Concerted effort to monitor the storage conditions of these imported fishes is also vital to curb or reduce the spread of food-borne illnesses/diseases.

## Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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