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# Pisciculture and Fish Parts as Non-clinical Source of CTX-M and TEM Extended Spectrum Beta-lactamases Producing *Escherichia coli* in Southeastern Nigeria

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Authors JOAN and CSI wrote the protocol. Author KAN was the English text/draft editor. Authors IUP and IRI wrote the first draft of the manuscript. Authors CIE, ALO, FAI and IRI managed the molecular analyses of the study. All authors read and approved the final manuscript.*

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## ABSTRACT

**Background and Objectives:** Antimicrobial agent use in pisciculture exerts a selective pressure, resulting in a reservoirs of extended spectrum beta-lactamase producing *Escherichia coli* in the aquatic environment. Here we screened pisciculture and fish parts as non-clinical source of CTX-M and TEM Extended Spectrum Beta-lactamases (ESBL) producing *Escherichia coli* in Southeastern Nigeria

**Methodology:** A total of sixty fish parts [fish gills, body part and intestinal swab samples] were collected from pisciculture farm A, C, F and G within Abakaliki metropolis. The samples were bacteriologically analyzed using standard microbiological techniques for isolation and identification. ESBL enzymes were phenotypically detected in *E. coli,* isolates using the double disk diffusion technique. The presence of TEM and CTX-M genes was determined by polymerase chain reaction using specific primers.

**Results**: *Escherichia coli* and Extended spectrum beta-lactamase producing *Escherichia coli* accounted for overall occurrence rate of 27(45.0 %) and 17(28.3 %) respectively. There was no statistically significant difference in the occurrence of *Escherichia coli* and ESBL-*E. coli* among the different samples $P>0.05$. The presence of TEM and CTX-M gene 80 % and 100 % respectively were confirmed among the isolates.

**Conclusion:** Our findings reports the presence of ESBL gene in pisciculture and fish parts and it is important in understanding the mechanism of resistance operating in these common pathogens, which are also endemic in most pisciculture area. Strict rules and monitoring/surveillance of antimicrobial agent used in pisciculture activities combined with food safety training of farmer owners/breeders on various aspects of good hygiene practices are strongly recommended.

*Keywords: Pisciculture; CTX-M; TEM; Escherichia coli.*

## 1. INTRODUCTION

Pisciculture is the process of growing or breeding fish most often for food, in fish tanks or artificial enclosures such as fish ponds. In recent time, the rapidly demand for fish is increasing worldwide, including Nigeria being the largest fish consumers in Africa [1]. In south eastern, Nigeria, rising incomes, urbanization and population growth are mainstay that contribute to the increase in production of pisciculture. Worldwide, there is a massive increase in pisciculture, which is associated with intensive use of antibiotics to combat bacterial infections [2]. Most bacteria strains such as *E. coli* are responsible for different infectious diseases, such as skin lesions, abscesses, bleeding, and sepsis; these pathogens increase morbidity and mortality in fish and cause significant economic loss [3]. As a result of ever-increasing use of antimicrobial agent such as disinfectant, spawning aids, antibiotic and herbicide in pisciculture, *E. coli* has the ability to acquire resistance determinant to various antimicrobials and to disseminate widely. This in large part is due to the highly diverse and rapidly evolving group of beta-lactamase determinant such as extended-spectrum beta-lactamases (ESBLs) [3]. ESBLs, generally found in Enterobacteriaceae such as E. coli, are a class of enzymes conferring resistance to penicillin, first-, second- and third-generation cephalosporins, and aztreonam, and are usually inhibited by beta-lactamase inhibitors such as clavulanic acid [4,5]. Enzyme families with ESBL phenotype are mainly described in class A (TEM, SHV, CTX-M, GES, and VEB families) and class D (OXA family) beta-lactamases [4,6]. Most of the ESBLs prevalent initially were TEM or SHV variants possessing amino acid substitutions which changed their substrate profile to include extended-spectrum cephalosporins [4,6]. In contrast, the CTX-M type ESBLs originated by the mobilization of chromosomal bla genes of Kluyvera species, an innocuous rhizosphere bacterium [7]. Since 2000, CTX-M type enzymes gained prominence over other ESBLs and disseminated widely around the world resulting in a “CTX-M pandemic” with Escherichia coli being

the predominant pathogen producing these enzymes [4,8]. Both CTX-M and TEM are ESBLs gene widely reported among clinical source where they are capable of truncating the effectiveness of numerous antibiotic resulting in significant morbidity and mortality among patients. Due to the rapid dissemination of CTX-M and TEM ESBLs gene, fish bacteria particularly Escherichia coli can exchange these resistance genes with human and animal bacteria through integrons and (or) plasmids [2,4]. This encourages the development of antibiotic resistance in bacteria present in fish and the surrounding environment. On the contrary, the presence of CTX-M and TEM gene among E. coli in pisciculture products has been much less described in region, as this studies in this field show the interest of an increased surveillance to molecularly characterize the collected isolates for the presence of ESBL genes.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection and Bacteria Isolation

A total of sixty fish parts were collected from pisciculture farm A, C, F and G within Abakaliki metropolis south eastern Nigeria, the fish gills, body part and intestinal swab samples were aseptically and separately inoculated and suspended in a sterile nutrient broth (Thermo Fisher Scientific, U.S.A) and incubated aerobically at 37°C for 24 hrs. After overnight incubation, turbid broth culture of fish part swab samples were streaked on solidified Brilliance TM Escherichia coli/Coliform Selective agar (Thermo Fisher Scientific, U.S.A) plate. The plates were incubated aerobically for 18-24 hours at 37 $^{o}$C. Bacterial colonies with purple colonies were inferred as the presence *Escherichia coli.* The bacteria were confirmed using VITEK® 2 Compact system (BioMerieux, France). All discrete colonies were purified by plating onto Nutrient agar (Thermo Fisher Scientific, U.S.A) for further studies [9,10].

### 2.2 Screening of *Escherichia coli* for ESBL Enzymes

ESBL enzymes were phenotypically detected in E. coli, isolates using the double disk diffusion technique as per our previous method [11]. Based on this protocol, all test isolates were swabbed on Mueller Hinton (MH) agar plates and subjected to the requisite antibiotic disks, particularly the third-generation cephalosporin (for ESBL detection). A ≥5mm increase in the inhibition zone diameter for any of the third-generation cephalosporin (ceftazidime 30 µg and cefotaxime 30 µg) tested in combination with Amoxicillin-Clavulanic acid (20/10µg) compared to its inhibition zone diameter when tested alone was inferred as ESBL positive phenotypically [11].

### 2.3 Molecular Typing of the ESBL Genes

The *E. coli* plasmid DNA was extracted from the test bacterial isolates using the Zymo Plasmid miniprep kit™ (Epigenetics Company, USA) [12,13]. The primer sequence that were used for the PCR amplification of ESBL genes are shown in Table 1. The purified DNA fragment was quantified using a NanoDrop (Thermo Scientific, USA). PCR experiment was conducted according to the procedures described previously [11,14]. A PCR master mix (50µl) of 5x GoTaq (10µl), 25 mM MgCl2 (3µl), dNTPs (10 mM) 1µl, forward primers (1µl), reverse primers (1µl) [14], 10 pmol 1µl, DNA Taq (1000 U) 25µl, Ultrapure Water 8µl was used. An amplification cycle at 95 °C for 5 minutes was employed, followed by 35 cycles at 96 °C for 30 seconds, 58 °C for 90 seconds, and 72 °C for 60 seconds, and a final extension step at 72 °C for 10 minutes.

**Table 1. Primer sequences and their amplicon sizes**

| Criteria | Primers | Nucleotide sequence | Base pair (bp) | References |
|---|---|---|---|---|
| **β-lactamase** | TEM-F | TTGGGTGCACGAGTGGGTTA | 506 | [14] |
| | TEM-R | TAATTGTTGCCGGGAAGCTA | | |
| | CTX-M-F | ATGTGCAGYACCAGTAARGTKATGGC | 592 | [14] |
| | CTX-M-R | TGGGTRAARTARGTSACCAGAAYSAGCGG | | |

*Key: R=Reverse, F=Forward*

## 2.4 Statistical Analysis

The frequency distribution and other basic descriptive statistics were computed. The statistical package for social sciences (SPSS) computer software (Version 25), IBM software, USA, was used for the analysis. After validating the applicable conditions, ANOVA was used to determine whether there was a statistically significant difference in the occurrence of E. coli and ESBL-*E. coli* among the different samples, followed by the Pearson chi-square test or Fisher's exact test. At $p \leq$ value 0.05, a statistically significant difference was evaluated [15,16].

## 3. RESULT AND DISCUSSION

### 3.1 Distribution of *Escherichia Coli* From Different Fish Body Parts From Pisciculture A, C, F and G

*Escherichia coli* accounted for overall occurrence rate of 27(45.0 %) consisting of high proportion in Farm A 9(60.0%), and Farm G 7(46.7 %) over Farm C and F recording 26.7 % and 46.7 % respectively. From Farm A, the Intestine accounted for 5(100 %) over Gills 3(60.0 %) and body 1(20.0 %) while in Farm C, the gills accounted for 1(20.0 %) and intestine 3(60.0 %). From Farm G, the Intestine accounted for 4(80.0 %) over Gills 1(20.0 %) and body 2(40.0 %) as shown in Table 2. There was no statistical significant difference in the occurrence of E. coli among the different samples P>0.05.

### 3.2 Distribution of Extended Spectrum Beta-lactamase Producing *Escherichia coli* from Different Fish Body Parts from Pisciculture A, C, F and G

Extended spectrum beta-lactamase producing Escherichia coli accounted for overall occurrence rate of 17(28.3 %) consisting of proportion in Farm G 7(46.7 %) over Farm A 4(26.7 %), Farm C and F both recording 3(20.0 %) respectively. From Farm A, the Intestine accounted for 60.0 % over Gills 20.0 % and body 0.0 % while in Farm C, the body and accounted for 0.0 %and intestine 60.0 %. From Farm G, the Intestine accounted for 4(80.0 %) over Gills 20.0 % and body 40.0 % as shown in Table 3. There was no statistically significant difference in the occurrence of ESBL-*E. coli* among the different samples P>0.05.

TEM gene accounted for 0.0 %, 40.0 % and 80.0 % in fish gill, body and intestine respectively. The CTX-M ESBL gene were detected in fish body 20.0 %, gill 40.0% and intestine 100 % as shown in Fig. 1.

The highest occurrence rate of TEM gene and CTX-M accounted for 80 % and 100 %. Although there is paucity of information on the epidemiology of ESBL-producing E. coli in fish pisciculture but few studies have reported their occurrence; Similar to this study were CTX-M and TEM were the most prevalence ESBL gene. Elsewhere blaCTX-M genes have also been discovered in three different E. coli strains [17]; The most common ESBL gene found in Thailand samples was blaCTX-M, which was found in tilapia (100%, n = 30) and carfoo (100%, n = 5), milkfish (60%, n = 24), catfish (52.3%, n = 34), and tilapia imported from India (34.8%, n = 24) [18] and other studies [19,20,21]. The CTX-M group has been identified as the most common type of ESBL gene worldwide [17,21], and as their name suggests, they demonstrated the strongest hydrolytic activity against Cefotaxime [22].

CTX-M was identified in *E. coli* from both gill, intestine and body. It widespread among fish milieus is due to the wide use of third generation cephalosporins, especially ceftriaxone and cefotaxime or may be associated with high mobilization of the encoding genes [23] within the pisciculture. According to Barlow *et al*. [24], the $_{bla}$CTX-M genes have been mobilized to plasmids about ten times more frequently than other class A beta-lactamases. CTX-M also confers resistance to other advanced generation cephalosporins (e.g., cefepime, ceftazidime) and has become the most common ESBL globally, with numerous variations identified [21,25]. Interestingly, several of the E. coli strains that produced $_{bla}$CTX-M ESBL in our work can be utilized to explain the continued use of 3 and 4GCs. This has the potential to disseminate new $_{bla}$CTX-M generating E. coli in pisciculture in Abakaliki metropolis, adding to the state's existing public health challenges.

The TEM gene was identified in fish body and gills. This gene has been found in pisciculture farms [26,27]. Most Aztreonam and Cephalosporin resistance could be attributed to TEM-lactamase, which has the potential to hydrolyze monobactam, first generation cephalosporins, and extended spectrum cephalosporins. This observation supports the

existing research [22,28]. The high frequency of $_{bla}$CTX-M and $_{bla}$TEM genes found in the study suggests that these genes were positioned in the same plasmid, which carried the ISEcp1 element upstream of the $_{bla}$CTX-M gene to enable mobilization and expression [29]. The current study is significant in understanding the mechanisms of resistance functioning in this common pathogen, which are also endemic in most of Nigeria's farms.

**Table 2. Distribution of *Escherichia coli* from different fish body parts from pisciculture A, C, F and G**

| Aquaculture | Fish part | No. sampled | *E. coli* | *P-value* |
|---|---|---|---|---|
| **Farm A** | | | | |
| | Body | 5 | 1(20.0) | .8766 |
| | Gills | 5 | 3(60.0) | |
| | Intestine | 5 | 5(100) | |
| **Sub-total** | | **15** | **9(60.0)** | |
| **Farm C** | | | | |
| | Body | 5 | 0(0.0) | |
| | Gills | 5 | 1(20.0) | |
| | Intestine | 5 | 3(60.0) | |
| **Sub-total** | | **15** | **4(26.7)** | |
| **Farm F** | Body | 5 | 2(40.0) | |
| | Gills | 5 | 1(20.0) | |
| | Intestine | 5 | 4(80.0) | |
| **Sub-total** | | **15** | **7(46.7)** | |
| **Farm G** | Body | 5 | 2(40.0) | |
| | Gills | 5 | 1(20.0) | |
| | Intestine | 5 | 4(80.0) | |
| | | **15** | **7(46.7)** | |
| **Overall Total** | | **60** | **27(45.0)** | |

**Table 3. Distribution of extended spectrum beta-lactamase producing *Escherichia coli* from different fish body parts from fishpond, A, C, F and G within Abakaliki Metropolis**

| Aquaculture | Fish part | No. sampled | *E. coli* | ESBL (%) | Non-ESBL (%) | P-value |
|---|---|---|---|---|---|---|
| **Farm A** | | | | | | |
| | Body | 5 | 1(20.0) | 0(0.0) | 1(20.0) | .7522 |
| | Gills | 5 | 3(60.0) | 1(20.0) | 2(40.0) | |
| | Intestine | 5 | 5(100) | 3(60.0) | 1(20.0) | |
| **Sub-total** | | **15** | **9(60.0)** | **4(26.7)** | **5(33.3)** | |
| **Farm C** | | | | | | |
| | Body | 5 | 0(0.0) | 0(0.0) | 0(0.0) | |
| | Gills | 5 | 1(20.0) | 0(0.0) | 1(20.0) | |
| | Intestine | 5 | 3(60.0) | 3(60.0) | 0(0.0) | |
| **Sub-total** | | **15** | **4(26.7)** | **3(20.0)** | **1(6.7)** | |
| **Farm F** | Body | 5 | 1(20.0) | 1(20.0) | 0(0.0) | |
| | Gills | 5 | 1(20.0) | 0(0.0) | 1(20.0) | |
| | Intestine | 5 | 4(80.0) | 2(40.0) | 2(40.0) | |
| **Sub-total** | | **15** | **6(40.0)** | **3(20.0)** | **3(20.0)** | |
| **Farm G** | Body | 5 | 2(40.0) | 2(40.0) | 0(0.0) | |
| | Gills | 5 | 1(20.0) | 1(20.0) | 0(0.0) | |
| | Intestine | 5 | 4(80.0) | 4(80.0) | 0(0.0) | |
| | | **15** | **7(46.7)** | **7(46.7)** | **0(0.0)** | |
| **Overall Total** | | **60** | **26(43.3)** | **17(28.3)** | **9(15.0)** | |

*Key: ESBL extended spectrum beta-lactamase*


**Fig. 1. Shows the rate of TEM and CTX-M ESBL gene in fish part**

## 4. CONCLUSION

The findings report the presence of ESBL gene in pisciculture and fish milieus. The risks to public health is related to the spread of resistant bacteria or resistance ESBL genes and the presence of residues of these agents in fish and the environment, which can be transferred to humans in the food chain. Further studies should showcase the mechanisms underlying the propagation and persistence of ARGs in different pisciculture environments and whole genomic sequence of different ARG, mobile genetic element (MGE) in numerous bacteria genera. Strict rules and monitoring/surveillance in pisciculture activities combined with food safety training of farmer owners/breeders on various aspects of good hygiene practices are strongly recommended.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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