Original Research Article

Pisciculture and Fish parts as non-clinical Sources of CTX-M and TEM Extended Spectrum Beta-lactamases producing *Escherichia coli* in Southeastern Nigeria

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 ~~(60)~~ 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 combined 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 pisculuture and fish parts and it is important in understanding the mechanism of resistance operating in these common pathogens, which are also endemic in most pisiciculture 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*

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* is 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). ESBLs, generally found in Enterobacteriaceae such as *E. coli*, are a class of enzymes conferring resistance to penicillins, 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].

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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.

Materials and Methods

Sample collection and Bacteria Isolation

A total of sixty ~~(60)~~ fish parts were collected from pisciculture farms A, C, F and G within Abakaliki metropolis, 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 ™ *Escherichia coli*/Coliform Selective agar (Thermo Fisher Scientific, U.S.A) plate. The plates were incubated aerobically for 18-24 hours at 37 °C. Bacterial colonies with purple colonies were inferred as the presence *Escherichia coli*. All discrete colonies were purified by plating onto nutrient agar (Thermo Fisher Scientific, U.S.A). The sub-cultured plates were incubated at 37 °C for 24 hrs. Discrete colonies were purified by plating onto nutrient agar (Thermo Fisher Scientific, U.S.A) for further studies [9, 10].

Screening of *Escherichia coli* for ESBL enzymes

ESBL enzymes were phenotypically detected in *E. coli*~~,~~ isolates using the combined 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 and cefotaxime) tested in combination with AMC (20/10µg) compared to its inhibition zone diameter when tested alone was inferred as ESBL positive phenotypically.

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]. This was carried out using a PCR master mix (50 µl) comprising 5x GoTaq (10µl), 25 mM MgCl2 (3µl), dNTPs (10 mM) 1µl, forward primers (1µl), reverse primers (1µl) [14], 10 pmol 1 µl, DNA Taq (1000 U) 25µl, Ultrapure Water 8µl. The PCR conditions used are an amplification cycle at 95 °C for 5 minutes; 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)
β-lactamase	TEM-F	TTGGGTGCACGAGTGGGTTA	506
	TEM-R	TAATTGTTGCCGGGAAGCTA	

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CTX-M F	ATGTGCAGYACCAGTAARGTKATGGC	592
CTX-M R	TGGGTRAARTARGTSACCAGAAYSAGCGG	

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Statistical analysis

Basic descriptive statistics such as frequency distribution was calculated. Statistical analysis was performed using the statistical package for social sciences (SPSS) computer software (Version 25), IBM software, USA. ANOVA for statistically significant difference in the occurrence of *E. coli* and ESBL-*E. coli* among the different samples while Pearson chi-square test or Fisher's exact test after checking the applicability conditions. A statistically significant difference was considered at p value ≤ 0.05 [15, 16]

RESULT AND DISCUSSION

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 $P>0.05$.

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 $P>0.05$.

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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 figure 1.

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

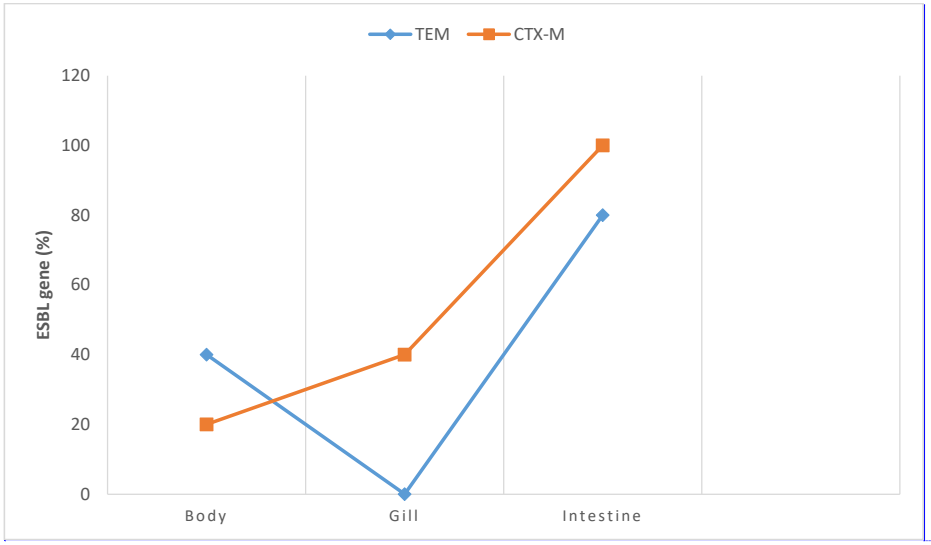

Figure 1 shows the rate of TEM and CTX-M ESBL gene in Fish part

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 haves reported their occurrence; Similar to this study were CTX-M and *TEM* were the most prevalence ESBL gene. Elsewhere blaCTX-M genes have been identified in three *E. coli* respectively [17]; in Thailand, the most prevalent ESBL gene found in the samples was *bla*CTX-M, which was detected in tilapia (100%, n = 30) imported from Thailand 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 is identified as the most prevalent type of ESBLs worldwide [17, 21] and as their name suggest, they displayed the most powerful 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. Barlow *et al.* [24] reported that the *bla*CTX-M genes have been mobilized to plasmids almost 10 times more frequently than other class A β-lactamases. CTX-M also impart resistance against other advanced generation cephalosporins (e.g., cefepime, ceftazidime) and have become the most prevalent ESBLs worldwide with many variants described [21, 25]. Interestingly, in this study, some of the strains of *E. coli* that produced *bla*CTX-M ESBL can be used to explain persistent use of 3 and 4GCs. The implication of this is the potential for spread of emerging *bla*CTX-M producing *E. coli* in pisciculture in Abakaliki metropolis, which will add to the prevailing public health burdens in the state.
TEM-gene was identified in fish body and gills. In pisciculture farms this gene has been reported [26, 27]. A possible reason for most Aztreonam and Cephalosporin resistancet was as a result of TEM β-lactamase which has the ability to hydrolyze only monobactam, first generation cephalosporin to extended spectrum cephalosporins. This observation substantiates the current literature [22, 28]. The observed high occurrence of blaCTX-M and blaTEM genes in the study indicate that these genes were located in the same plasmid, which carried the *ISEcp1* element upstream of the blaCTX-M gene to facilitate mobilization and expression [29]. The present study is important in understanding the mechanism of resistance operating in these common pathogens, which are also endemic in most pisiciculture area in Nigeria.

Conclusion
Our findings reports the presence of ESBL gene in pisculuture 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

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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.

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