

Full Length Research Paper

Phenotypic screening of multidrug-resistant *E. coli* from water and fish collected from different fish farms within Abakaliki metropolis, Nigeria

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The aim of this study was to screen fish farm water and fishes reared in fish farms within Abakaliki metropolis for multi-drug resistant *Escherichia coli*. Exactly 30 fish samples and 30 water samples were obtained from 10 different fish farms using sterile bottles from January to June, 2018. Samples were analyzed by standard microbiology methods. Susceptibility test to antibiotics was done by Kirby-Bauer disc diffusion technique. Double-disc synergy test was used to screen isolates for Extended Spectrum Beta-Lactamase (ESBL)-production. Exactly 54 (60%) *E. coli* and 12 (40%) *E. coli* were recovered from 90 different fish parts and 30 farm water samples, respectively. Isolates exhibited resistance (54% - 100%) to ceftazidime, aztreonam, cefotetan, cefuroxime, cefoxitin, piperacillin/tazobactam, ofloxacin, and ceftriaxone, but were susceptible to imipenem (80%), cefotaxime (60%), and gentamicin (57%). All the *E. coli* isolates from water and fish samples were negative for ESBL production. An average multiple antibiotic resistance index (MARI) value of 0.71 was recorded for the isolates. High prevalence of *E. coli* with multidrug-resistant traits in fish and water samples in our study area is a serious public health concern as this will make the treatment of infections, especially *E. coli*-associated foodborne diseases very difficult, thus leading to increase in health care cost, morbidity, and mortality.

Key words: *Escherichia coli*, fish farm, multidrug-resistance, farm water, fish.

INTRODUCTION

The widespread use of antimicrobial agents in humans, aquaculture, and veterinary medicine has led to the continued emergence of multidrug-resistant microbes which is now a worldwide public health problem (Ahmad,

2008). Beta-lactam antibiotics are usually used in the treatment of various bacterial infections in humans, farm animals, and aquaculture (Ahmad, 2008). Beta-lactamase is an enzyme produced by bacteria to counter the action

of β -lactam antibiotics (Cheng et al., 2014). The term "Extended Spectrum Beta-Lactamase (ESBL)" refers to a special type of β -lactamase which can hydrolyse the entire group of β -lactam antibiotics, starting from narrow spectrum to broad spectrum, especially the 2nd and 3rd generation cephalosporins. The widespread and indiscriminate use of antibiotics coupled with transmissibility of antibiotic-resistant genes leads to the increasing emergence of multidrug-resistant *Escherichia coli* (Cantos et al., 2016). Infections caused by ESBL-producing *E. coli* are always difficult to treat (Bradford, 2001). Since *E. coli* is the most common commensal bacteria existing in human and animal intestinal tract; it can become an opportunistic and an obligate pathogen when it co-exists with pathogenic strains (Bush and Jacoby, 2010). *E. coli* strains that produce ESBL are also becoming Multi Drug-Resistant (MDR), and are considered to be one of the emerging pathogens worldwide (Cheng et al., 2014). Multiple antibiotic-resistant *E. coli* have been reported in most food products such as raw milk, meat, chicken, and fish (Elhadi and Alsamman, 2015). Beta-lactam antibiotics constitute the main therapeutic choice for treating human infections caused by Enterobacteriaceae. In recent years, ESBL-producing *E. coli* has gained recognition as a major clinical problem worldwide due to their increased antibiotic-resistant traits to most beta-lactam antibiotics, including carbapenems, penicillin, and third generation cephalosporins. The increased resistant traits to beta-lactams are due to the ability of bacteria to produce β -lactamase enzymes capable of hydrolysing β -lactams, rendering the antibiotic inactive (Nordmann et al., 2012). Fishes may be contaminated with faecal material before, during or after harvest. This may bring about incidence of infectious diseases or even food poisoning. Also, the leading cause of most microorganism acquiring resistance to both first and second-line antibiotics is due to frequent use of antibiotics employed in fish farming for the treatment of contaminated ponds; thereby causing serious failure in the treatment of infectious disease. There is still paucity of information on the occurrence and prevalence of multidrug-resistant bacterial pathogens in fish products sold in Nigeria. Hence, this study was designed to screen fish farm water and fishes reared in different fish farms for multi-drug resistant *E. coli* in Abakaliki, Nigeria.

METHODOLOGY

Area of study

This research was done in different fish farms within Abakaliki

metropolis, Ebonyi State. Abakaliki is the capital city of Ebonyi State, Eastern Nigeria. The 2006 population census conducted in Nigeria pegged the population of Ebonyi State at an estimated population of 4.3 million. The land mass of Ebonyi State is 5,935 km². Ebonyi State is known for its large deposit of lead, zinc, salt, limestone, and its farming activities.

Collection of fish samples

A total of thirty (30) fish samples were harvested (3 from each of the 10 fish farms, alphabetically named A - J) by casting net in the fish pond, and then put in different sterile polythene bags (1 fish per bag). Fish samples were transported within 1 h to the Department of Applied Microbiology, Faculty of Science, Ebonyi State University, Abakaliki for bacteriological analysis.

Collection of water samples

A total of 1000 ml of 30 different well-mixed homogeneous water samples were collected (3 samples from each of the 10 fish ponds) from different locations of the fish ponds by inverting sterile polystyrene bottle to about 30 m below the surface. Exactly 250 ml each of the collected water samples were immediately transported within 1 h in icebox to the Department of Applied Microbiology, Faculty of Science, Ebonyi State University, Abakaliki for bacteriological analysis.

Bacteriological analysis of fish and water samples

Fish samples were collected for bacteriological analysis by swabbing the fish skin surface, the intestine (viscera), and gills during evisceration using sterile moist cotton swab. Fish swab samples were then incubated at 37°C for 24 h in nutrient broth medium. Loopfuls of fish swab samples in nutrient broth and fish farm water samples were then aseptically streaked on MacConkey agar medium and incubated for 24 h at 37°C. Plates were then observed for typical *E. coli* growth (red or pink colonies) on MacConkey agar after completion of incubation, sub-cultured on Eosin Methylene Blue (EMB) agar medium, and further incubated for 24 h at 37°C. EMB agar plates were then observed for *E. coli* growth (green metallic sheen appearance). These suspected bacterial isolates were characterized by standard microbiology methods such as Gram-stain, motility test, sugar fermentation test, and some biochemical tests such as methyl red, Voges-Proskauer, indole, citrate, motility and urease test (Osek, 1999; Cheesebrough, 2006; Moses et al., 2018).

Antibiotics susceptibility test

Antibiotic susceptibility of the isolates was done by Kirby-Bauer disc diffusion technique in line with the Clinical and Laboratory Standard Institute recommendations (CLSI, 2015; Moses et al., 2018). Isolates were sub-cultured on nutrient agar and incubated at 37°C for 18 to 24 h to obtain fresh colonies. Fresh bacterial colonies were then subsequently adjusted to 0.5 McFarland turbidity standard (equivalent to 1.5×10^8 cfu/mL) and incubated for 10 min. Standardized broth culture of isolates was thereafter inoculated

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onto Mueller-Hinton (MH) agar plates using sterile swab sticks. To ensure even distribution of standardized inoculum, the surface of the MH agar medium was streaked in four directions while rotating at approximately 60°. Inoculated MH agar plates were allowed to dry for a few minutes. The antibiotics tested against the bacterial isolates were cefuroxime (30 µg), cefotetan (30 µg), imipenem (10 µg), amoxicillin/clavulanic acid (20/10 µg), aztreonam (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), gentamicin (10 µg), ceftazidime/clavulanate (30/10 µg), and ofloxacin (5 µg) (Oxoid, UK). Antibiotic discs were evenly placed on inoculated MH agar using sterilized forceps in such a way that the discs were about 15 mm from the edge of the plate and not closer than 25 mm from one disc to another. After 30 min, the seeded plates were inverted and incubated for 24 h at 37°C. A metre rule was then used to measure the inhibition zone diameter (IZD) in mm on the underside of the plate. The IZD was interpreted as susceptible or resistant according to the criteria of CLSI (2015).

Detection of ESBL by double-disc synergy test (DDST)

Bacterial isolates that exhibited low susceptibility to 2nd and 3rd generation cephalosporins were phenotypically confirmed for ESBL production (Iroha et al., 2013). DDST was performed on Mueller-Hinton (MH) agar (Oxoid, UK) plates according to CLSI criteria (CLSI, 2015). Standardized inoculums (0.5 McFarland turbidity standards) were inoculated onto Mueller-Hinton (MH) agar plates using sterile swab sticks. Amoxicillin/Clavulanic acid (20/10 µg) disc was placed at the center of MH agar plate and cefotaxime (30 µg) and ceftazidime (30 µg) antibiotics were each placed at a distance of 15 mm (center to center) from the central disc (amoxicillin/clavulanic acid). Plates were then incubated for 18 to 24 h at 37°C. ESBL production was suspected when the IZD of the cephalosporins (cefotaxime and ceftazidime) increased in the presence of amoxicillin/clavulanic acid disk. A ≥ 5 mm increase in the IZD for either of the cephalosporins (cefotaxime and ceftazidime) tested in combination with amoxicillin-clavulanic acid versus its zone when tested alone confirmed ESBL production (Iroha et al., 2010).

Multiple antibiotic resistance index (MARI) determination

Determination of isolates' MARI was calculated as the ratio of the number of antibiotics to which isolates exhibited resistance (a) divided by the total number of antibiotics tested against the isolates (b) as described previously by Moses et al. (2020).

RESULTS

All 30 (100%) fish intestine swab samples tested positive for *E. coli* while 21 (70%) fish gill swabs and 3 (10%) fish skin swab samples also tested positive for *E. coli* as shown in Table 1.

In total, 54 (60%) and 12 (40%) *E. coli* were isolated from 30 fish samples (90 different parts) and 30 water samples collected from ten different fish farms, respectively (Table 2). All the *E. coli* isolated from fish and water samples were non ESBL producers (Table 2).

Antibiotic susceptibility results showed that *E. coli* isolates were absolutely resistant (100%) to ceftazidime, aztreonam, cefotetan, and cefuroxime. Isolates also

exhibited resistance to ceftazidime (71%), piperacillin/tazobactam (64%), ofloxacin (57%), and ceftazidime (54%) (Table 3). However, isolates were very susceptible to imipenem (80%), cefotaxime (60%), and gentamicin (57%) (Table 3).

The MARI values of *E. coli* isolates ranged from 0.54 to 1.0 (Table 4).

DISCUSSION

This study focused on the phenotypic screening of water and fishes collected from different fish farms within Abakaliki metropolis for the presence of *E. coli* with multiple antibiotic-resistant traits. Results from our study have shown that fishes are heavily contaminated with multidrug-resistant non-ESBL-producing *E. coli* that can cause infections in humans, especially through the consumption of undercooked or ill-processed fishes. The present study is in line with the report of Dang and Dalsgaard (2012) who reported that *E. coli* was the predominant bacterial species in the intestinal tract of fishes. The presence of *E. coli* in food indicates the possible cause of many gastro-intestinal diseases, and may constitute potential danger of antibiotic resistance transfer through the food chain, from bacteria in aquatic systems to humans (Nataro and Kaper, 1998). All isolates in this study were non-ESBL producers. This current study is not in agreement with the work of Elhadi and Alsamman (2015) in Saudi Arabia who reported that fish might be the possible ESBL-producing *E. coli* reservoir, and could contribute to the transfer and dissemination of β -lactamase genes to humans through the food chain. Isolates in this study exhibited resistance to different antibiotic classes, such as the cephalosporins, carbapenems, penicillins, monobactam, aminoglycosides, and fluoroquinolones tested. *E. coli* isolates in our study were completely resistant (100%) to ceftazidime, aztreonam, cefotetan, and cefuroxime. Isolates also exhibited resistance to ceftazidime (71%), piperacillin/tazobactam (64%), ofloxacin (57%), and ceftazidime (54%). This is in agreement with the research of Ajah et al. (2016) who also reported the resistance of *E. coli* to third generation cephalosporin such as ceftazidime (83.7%), and cefotaxime (97.2%) and 80% resistance frequency to ofloxacin. The average MARI value of isolates in this study was 0.71. This further implies misuse of antibiotics in our study area. However, *E. coli* isolates exhibited susceptibility to imipenem (80%), cefotaxime (60%), and gentamicin (57%). This study agrees with the work of Adedeji and Abdulkadir (2009) who reported that *E. coli* exhibited susceptibility to gentamicin. Iroha et al. (2010) also reported *E. coli* susceptibility frequency of 76.5% to gentamicin. Studies carried out by Overvest et al. (2011) and Albuquerque et al. (2007) indicated that antibiotic resistance in Gram-negative bacteria, especially in the Enterobacteriaceae family, has dramatically increased over the decade. This

Table 1. Isolation frequency of *E. coli* from gill, intestines, and skins of fish samples collected from fish farms in Abakaliki.

Fish body parts	No. of fish body parts	No. of <i>E. coli</i> isolated (%)
Gills	30	21 (70)
Intestines	30	30 (100)
Skin	30	3 (10)
Total	90	54

Table 2. Percentage occurrence of *E. coli* from fish and water samples collected from fish farms.

Sample type	No. of samples	<i>E. coli</i> isolated [n (%)]	No. of ESBL producers
Fish (90 different parts)	30	54 (60)	0
Fish pond water	30	12 (40)	0
Total	-	66	0

Table 3. Antibiotic susceptibility profile of *E. coli* isolated from fish and water samples in different fish farms in Abakaliki.

S/N	Antibiotics used	Resistance (%)	Susceptibility (%)
1	Ceftazidime	100	0
2	Cefotaxime	0	60
3	Cefotetan	100	0
4	Aztreonam	100	0
5	Ceftriaxone	54	46
6	Cefoxitin	71	29
7	Cefuroxime	100	0
8	Imipenem	0	80
9	Piperacillin/tazobactam	64	36
10	Ofloxacin	57	43
11	Gentamicin	43	57

Table 4. MARI values of *E. coli* isolates from fish and water samples from fish farms.

Farm (A-J)	MARI value
A	0.90
B	0.90
C	0.54
D	0.81
E	0.64
F	0.72
G	0.81
H	0.81
I	0.72
J	1.0
Average	0.71

has further provided evidence that there is an increase in the emergence of antimicrobial resistance in isolates originating from fish and fish handlers. The use of

antibiotics in human and veterinary medicine has influenced the emergence of antibiotic-resistant bacteria (ARB), contributed to the problem of resistance, and

complicates the choice of treatment in infectious diseases management (Novotny et al., 2004). The transmission of ARB between aquatic animals and humans through the food production line has been documented, and can also pose serious threats to public health (Grema et al., 2015). The world organization for animal health, aquatic animal health code recommends the continuous monitoring and surveillance of antimicrobial resistance in microorganisms associated with aquatic animals (Smith et al., 2013; Geidam et al., 2012). Therefore, the presence of ARB in fish and fish handlers poses risk of disease to fishes and also public health hazard to fish handlers and consumers in general. The results of our study have shown that some fishes in fish farms in our study area are heavily contaminated with multidrug resistant non ESBL producing *E. coli* that are potent and can cause infections in humans. Data generated from this study will help/assist in monitoring ponds where fishes are being grown and the application of antimicrobial stewardship program in all sectors to increase the level of knowledge of physician and farmers with regards to judicious use of antibiotics.

Conclusion

This study has shown that fishes and fish water from fish farms in Abakaliki metropolis are heavily contaminated with multidrug-resistant non-ESBL-producing *E. coli*. Isolates in this study exhibited resistance to different antibiotic classes, such as the cephalosporins, penicillins, monobactams, aminoglycosides, and fluoroquinolones. Interestingly, imipenem, a carbapenem, was very effective against the *E. coli* isolates. Fishes, contaminated with these multidrug-resistant *E. coli* isolates could serve as a route through which human infections can occur especially through the consumption of undercooked or ill-processed fishes. It is therefore imperative to establish strong regulation and strict measures of control to reduce the usage of antimicrobials in fish husbandry to curtail the increasing menace of antimicrobial resistance (AMR) in aquaculture. This calls for concerted efforts to implement the usage of alternatives like immunostimulants and probiotics which will ensure healthy fishes, and in turn further curtail the development of superbugs in future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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