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Original Article **Open Access**

First report of enteropathogenic and enteroinvasive *Escherichia coli* with multiple antibiotic resistance indices from African catfish (*Clarias glariepinus*) in Nigeria

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Abstract

Background: There are increasing reports of food safety issues associated with intensive production of fish which increase the chances of disease outbreaks from stressful growth conditions accompanying mass production and presence of bacterial pathogens.
Methodology: Two hundred gastrointestinal tract (GIT) samples from two hundred African Cat Fish (*Clarias glariepinus*) were assessed for the presence of enteric *Escherichia coli* species including *E. coli* 0157, Enteropathogenic *E. coli* (EPEC) and Enteroinvasive *E. coli* (EIEC) which are traditionally associated with infantile gastroenteritis. The antibiotic resistance profile and Multiple Antibiotic Resistance Index (MARI) for these isolates were determined. The serogrouping of the *E. coli* isolates was done using *E. coli* agglutinating sera (Oxoid) and *E. coli* 0157 latex reagent (Oxoid). Antibiotic susceptibility was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.
Results: A total of 35 (17.5%) *E. coli* isolates were recovered from the fish intestines among which 9 (25.7%) were EPEC and 2 (5.7%) were EIEC. No *E. coli* 0157 strain was recovered. Thirty-three (94.0%) isolates had a MARI greater than 0.2. Antibiotic resistance to cefoxitin and amoxicillin-clavulanic acid were 77.1% and 74.3% respectively. All isolates were susceptible to meropenem and amikacin but all EPEC and EIEC isolates were AmpC (resistance to all penicillins, cephalosporins and beta lactamase inhibitors) positive.
Conclusion: The isolation of EPEC and EIEC which can cause fatal gastroenteritis coupled with high MARI among isolates in this study represents a public health concern. Strict monitoring of administration of antibiotics in aquaculture is recommended.

Keywords: EPEC; EIEC; Multiple antibiotic resistance**;** Aquaculture

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Premier signalement d'*Escherichia coli* entéropathogène et entéro-invasif avec plusieurs indices de résistance aux antibiotiques chez le poisson-chat africain (*Clarias glariepinus*) au Nigéria

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Abstrait

Contexte: On signale de plus en plus de problèmes de sécurité sanitaire des aliments associés à une production intensive de poisson, qui augmentent les risques d'épidémies dues à des conditions de croissance stressantes accompagnant une production de masse et la présence d'agents pathogènes bactériens
Méthodologie: Deux cents échantillons du tractus gastro-intestinal (GIT) de deux cents poissons chats africains (*Clarias glariepinus*) ont été évalués pour la présence d'espèces entériques d'*Escherichia coli* comprenant *E. coli* 0157, *E. coli* entéropathogène (EPEC) et *E. coli* Enteroinvasive (EIEC). qui sont traditionnellement associées à la gastro-entérite infantile. Le profil de résistance aux antibiotiques et l'indice de résistance multiple aux antibiotiques (MARI) de ces isolats ont été déterminés. La sérogroupe des isolats de *E. coli* a été réalisée à l'aide de sérums agglutinants de *E. coli* (Oxoid) et du réactif latex *E. coli* 0157 (Oxoid). La sensibilité aux antibiotiques a été déterminée conformément aux directives du Clinical and Laboratory Standard Institute (CLSI).
Résultats: Au total, 35 isolats d'*E. coli* (17,5%) ont été retrouvés dans l'intestin des poissons, dont 9 (25,7%) étaient des EPEC et 2 (5,7%) des EIEC. *E. coli* 0157 n'a pas été retrouvé. Trente-trois (94,0%) des isolats avaient un IRS supérieur à 0,2. La résistance aux antibiotiques de la céfoxitine et de l'amoxicilline-acide clavulanique était respectivement de 77,1% et 74,3%. Tous les isolats étaient sensibles au méropénème et à l'amikacine, mais tous les isolats d'EPEC et EIEC étaient positifs pour AmpC (résistance à toutes les pénicillines, céphalosporines et inhibiteurs de la bêta-lactamase).
Conclusion: l'isolement des EPEC et des EIEC pouvant provoquer une gastro-entérite fatale, associé à un IAR élevé parmi les isolats de cette étude, constitue un problème de santé publique. Une surveillance stricte de l'administration d'antibiotiques en aquaculture est recommandée.

Mots-clés: EPEC; EIEC; Résistance multiple aux antibiotiques; Aquaculture

Introduction

Aquaculture is currently one of the fastest growing food production sectors with fish contributing about 60% of the world supply of protein (1). Fish and fish products are usually highly nutritious and safe, however there are increasing reports of food safety and environmental issues associated with intensive production of fish which increases the chances of disease outbreaks (2, 3). The common causes of such disease outbreaks in aquaculture include stressful growth conditions associated with mass production and presence of bacterial pathogens (4, 5). This has led to huge dependence on antibiotics in the management of bacterial infection in aquaculture which has in turn resulted in emergence of antibiotic resistance among micro-organisms isolated from fish. *Escherichia coli* is regarded as a commensal organism found in the GIT of humans and warm-blooded animals where they usually co-exist in a mutually beneficial relationship with the host organism, contributing to metabolic processes (6). In some instances, *E. coli* may cause opportunistic infections and other strains are considered to be truly pathogenic (7). *E. coli* serves as the most preferred indicator organism to test food and environmental samples for faecal contamination (8).

In developing countries like Nigeria, the artificial aquaculture of fish (especially the African Catfish) is popular, and no regulatory body exists to monitor and regulate the practice. In such settings, aquaculture practices are an issue of public health concern, being an important source of environmental pollution and possible contributor to the problem of antibiotic resistance. The aim of this study was to determine the prevalence of *E. coli* strains (EPEC, EIEC and *E. coli* O157) in the GIT of African Catfish (ACF) and the antimicrobial resistance profile of these strains.

Materials and methods

Sample collection:

Between October and December of 2016, intestinal contents of 200 ACF (one per ACF) were collected into sterile polythene bags during fish evisceration from the Jos Main Fish Market on alternate days between 7: 00a.m. to 12 noon. The GIT contents were placed on ice and transported to the diagnostic laboratory within sixty minutes of collection (9).

Isolation and identification:

In the diagnostic laboratory, the intestinal contents of each ACF were obtained aseptically using a sterile scalpel blade to dissect the intestine after which sterile cotton swabs were used to collect the intestinal contents; these were placed in peptone water and incubated overnight in aerobic conditions. The pre-enriched samples were then inoculated on Eosin Methylene Blue (EMB) Agar and MacConkey Agar, and incubated for 18- 24 hours at 37^{o}C (10).

Biochemical identification:

Single colonies from each sample were identified biochemically as *E. coli* using standard procedures including the Gram staining, morphology observation under microscope, Indole-Methyl Red-Voges Proskauer-Citrate (IMViC), lysine decarboxylase tests and triple sugar ion reactions. The *E. coli* isolates were seeded on nutrient agar for further processing.

Sero-grouping of isolates:

Further characterization of *E. coli* isolates was done using the *E. coli* agglutinating sera and *E. coli* 0157 latex agglutination assay according to the manufacturer's instructions (Oxoid, Basingstoke, UK).

Antimicrobial susceptibility testing:

The antimicrobial susceptibility test for each identified *E. coli* isolate was performed using the modified Kirby-Bauer disk diffusion method (11). Isolates were inoculated into peptone broth and incubated at 35-37^{o}C for 16 -18 hours in ambient air. The isolated *E. coli* were seeded onto the surface of freshly prepared, dry surfaced Mueller Hinton agar using sterile swabs after standardization of the inoculum. Using sterile forceps, the antimicrobial discs were placed on the agar plates and incubated at 35 -37^{o}C for 16 -18 hours in ambient air. The zone of inhibition was measured using a standard meter rule and results interpreted using the CLSI breakpoints (11).

All isolates were tested for sensitivity to the following antibiotics: amoxycillin (30µg), gentamicin (10µg), amikacin (30µg), sulfamethoxazole trimethoprim (30µg), ciprofloxacin (5µg), cefuroxime (30µg), cefoxitin (30µg), ceftriaxone (30µg), ceftazidime (30µg), amoxicillin-clavulanic acid (30µg), piperacillin tazobactam (30µg), and meropenem (10µg).

Screening for AmpC production:

The isolates were screened for AmpC beta lactamase production by testing their susceptibility to cefoxitin (30µg) using Kirby Bauer disk diffusion method as described by Tanushree and colleagues (12). The inhibition zone sizes were interpreted according to the CLSI guidelines (11). All the isolates with an inhibition zone diameter of less than 18 mm were presumed positive for AmpC β-lactamases production.

Extended spectrum β-lactamase detection:

This was carried out by the double disk synergy test (DDST). All isolates with reduced susceptibilities or resistance to an extended-spectrum cephalosporin namely ceftriaxone or ceftazidime were subjected to DDST to detect the presence of ESBL enzyme as described by CLSI (11). Mueller Hinton agar plates were inoculated with a 0.5 McFarland standard inoculum of *E. coli*. Control strains: *E. coli* ATCC 35218 served as positive control while *E. coli* ATCC 25922 served as negative control.

Multiple Antibiotic Resistance Index (MARI) determination:

The MARI of each isolate was determined using the formula first described by Krupperman (13). The MARI when applied to a single isolate is defined as a/b where; 'a' represents the number of antibacterial agents to which the isolate was resistant to, and 'b' represents the number of antibacterial agents to which the isolate was exposed to.

Results:

A total of 35 out of 200 ACF (*C. glariepinus*) GIT samples were positive for *E. coli*, giving a prevalence rate of 17.5 %. Serogrouping of the isolates revealed a total of 31.4% (11/35) of specimens were either Enteropathogenic *E. coli* (EPEC) or Enteroinvasive *E. coli* (EIEC). Among these, 14.3% were identified as EPEC with agglutinating sera for serotypes 026, 055, 0111, 0119, 0126; 11.4% for serotypes 086, 0114, 0125, 0127, 0128 and another 5.7% were identified as EIEC with agglutinating sera for serotypes 044, 0112, 0124 and 0142. None of the *E. coli* isolates was identified as *E. coli* 0157 using the Oxoid *E. coli* 0157 latex agglutination assay (Table 1).

The antimicrobial susceptibility testing of all isolates revealed high level resistance to cefoxitin (77.1%) and amoxicillin-clavulanic acid (74.3%), with other susceptibility patterns as shown in Table 2. A combined resistance to cefoxitin and amoxicillin-clavulanic acid is a phenotypic marker for *ampC* genes which usually confer resistance on the organism to all penicillins and cephalosporins including the extended spectrum beta lactamases (ESBLs) and beta lactamase inhibitors**.**

None of the isolates showed the characteristic dumbbell shape description for a positive ESBL phenotype on DDST**.**

Table 1: Serogroups of *E. coli* isolated from ACF sold within Jos, Nigeria

N = 35 Serogroups (Serotypes)	Frequency (%)
E. coli 0157	0 (0.0)
EPEC I (026, 055, 0111, 0119, 0126)	5 (14.3)
EPEC II (086, 0114, 0125, 0127, 0128)	4 (11.4)
EIEC (044, 0112, 0124, 0142)	2 (5.7)
Total	11 (31.4)

Key: EPEC = Enteropathogenic *E. coli*, EIEC = Enteroinvasive *E. coli*

All the 11 (100%) EPEC and EIEC isolates presumptively carried the *ampC* gene. The multiple antibiotic resistance indices showed that more than 90% of the isolates had a MARI greater than 0.2 (Table 3).

Table 3: Multiple Antibiotic Resistance Indices of *E. coli* Isolates from ACF sold within Jos, Nigeria

MARI	Frequency (%)
0.00 - 0.10	0 (0.0)
0.10 - 0.20	2 (5.7)
0.21 - 0.30	8 (22.9)
0.31 - 0.40	10 (28.6)
0.41 - 0.50	8 (22.9)
0.51 - 0.60	5 (14.3)
0.61 - 0.70	2 (5.7)
Total	35 (100.0)

Table 2: Antimicrobial susceptibility profile of *E. coli* isolated from ACF in Jos, Nigeria

Antibiotic	Susceptible (%)	Intermediate (%)	Resistant (%)
amoxycillin (30µg)	13 (37.1)	4 (11.4)	18 (51.4)
gentamicin (10µg)	31 (88.6)	0 (0.0)	4 (11.4)
amikacin (30µg)	35(100.0)	0 (0.0)	0 (0.0)
Sulfamethoxazole/trimethoprim (30µg)	19 (54.2)	1 (2.9)	15 (42.9)
ciprofloxacin (5µg)	5 (14.3)	23(65.7)	7 (20.0)
cefuroxime (30µg)	33 (94.2)	1 (2.9)	1 (2.9)
cefoxitin (30µg)	1 (2.9)	7(20.0)	27 (77.1)
ceftriaxone (30µg)	32 (91.4)	2 (5.7)	1 (2.9)
ceftazidime (30µg)	32 (91.4)	2 (5.7)	1 (2.9)
Amoxicillin/clavulanic acid (30µg)	0 (0.0)	9 (25.7)	26 (74.3)
Piperacillin/tazobactam (30µg)	28 (80.0)	6 (17.1)	1 (2.9)
meropenem (10µg)	35(100.0)	0 (0.0)	0 (0.0)

Discussion

Escherichia coli are regarded as commensal microflora in several living organisms including humans, animals and the African Catfish (14, 15). The presence of *E. coli* is also utilised as an indicator organism to monitor for faecal contamination of foods. In this study of ACF (*C. glariepinus*) sold at fish markets in Jos, Nigeria, the overall prevalence of *E. coli* was 17.5%. This prevalence is lower than what has been observed from studies on *E. coli* in ACF and pond water from various regions within and outside Nigeria (16-19).

Amande and Nwaka observed a 42% prevalence of *E. coli* in ACF harvested from ponds in Uyo, South-South Nigeria (16). Danba and co-workers obtained a prevalence of 54.27% in Kano, North-West, Nigeria (14). Studies in Ekiti, South-West Nigeria recorded 25.8% (17). Egbebi and colleagues recorded 24% prevalence also in Ondo, South-West Nigeria (18). However, a higher prevalence of 72.7 % from freshwater fish was observed in China (19). Nonetheless, Grema and colleagues in an analysis of bacterial flora of catfish obtained from different fish markets in Maiduguri, North-East Nigeria recorded a lower prevalence of 9% *E. coli* (20). In the West African country of Ghana, Takyi and colleagues documented a prevalence of 0% and 14.3% of *E. coli* in catfish obtained from two different fish farms (21).

The lower prevalence of *E. coli* in African catfish in this study compared to others, could have been influenced by the quality of the water source for aquaculture which would vary in the different studies, although, it can be argued that since the samples in this study came from the fish market, it would also serve as representative of various water sources. However, it may also signify that fish farmers in our study area pay closer attention to hygiene and their source of water for aquaculture. Fish obtained directly from ponds are also likely to have a higher load of microorganisms resulting from poor management, poor sanitary conditions in the farms and substandard hygiene practices associated with many artificial ponds especially in developing

countries (14). These practices provide favourable conditions for bacteria reproduction and development (14).

However, observations in this study revealed that fish marketers kept the fish for sale in large basins containing clean water without adding fish feeds. They also changed the water regularly perhaps to reduce bacteria growth and enhance sales. This practice could have lowered the chance of isolating *E. coli* from the fish. The average temperature of Jos is between 13^{o}C and 22^{o}C and can drop as low as 5^{o}C in the months of December and January (22). High temperature affects the population dynamics of *E. coli* and favours bacterial growth with peaks observed in the summer months (23). Hence, the lower temperatures that prevails in Jos could have contributed to the lower prevalence of *E. coli* obtained in this study. On the other hand, lower prevalence of *E. coli* from other studies in relation to this study (20, 21) could be because the fish samples were obtained from regulated markets and probably an indication of better management practices that prevail in the farms.

The occurrence of EPEC in fresh fish as revealed by this study emphasises that fresh fish could be a potential source of human infection, thus making this an issue of public health concern. The spread of such infectious agent to humans could occur not only by consumption of raw or undercooked fish, but also by environmental spread during handling or contact with contaminated surfaces, disposal of waste water from ponds, local transportation of the fish from farms to retail market in addition to gross mishandling and other human activities.

It is important to state that the *E. coli* serogroups found in this study have been identified in humans to cause severe infections including fatal cases of infantile gastroenteritis (24). They have also been isolated from animals (dogs, rabbits, monkeys, sheep, birds) and food items such as vegetables and other food products from animal source such as raw milk or cheese (24, 25). Barbosa and others recorded an overall prevalence of 43% EPEC serogroup from water and fresh fish in Brazil (9). The disparity in their observation in comparison with this study could be attributed to differences in the prevailing strains of *E. coli* colonising the humans and those found in the different environments. Similarities of many human and animal EPEC based on clonal relationship and virulent properties in other studies suggest interspecies transmission (9). The incidence of EIEC in this study was 5.7%. Reports of this pathotype in the environment or in food are rare. However, Barbosa et al., reported a similar EIEC incidence of 5% from fresh fish and water in Brazil (9).

Furthermore, ACF serves as a major source of protein in most developing countries like Nigeria. The identification of EPEC and EIEC in ACF could have major consequences. Particularly, EPEC and EIEC transmission via the food chain would affect nutrition, increase infection rates; increase hospital visits, stretch medical care resources thereby increasing poverty and might create a vicious circle of malnutrition, disease and poverty. With lack of safe food practices available, fish handlers in the markets were seen handling fish and equipment without proper hygienic practices. The lack of biosecurity and tight hygienic controls or policies within the fish market could have also contributed to the introduction of some of the pathogenic *E. coli* that were isolated.

It has been observed that some pathogenic and potentially pathogenic microorganism including *E. coli*, *Staphylococcus* and some anaerobes survive when uncooked and precooked fish foods were stored at freezing point (26). With the advent of grilled fish at bars, restaurants and eateries and the demand for fresh catfish in many places within Jos and other parts of the world, there is the danger of the transfer of these pathogens to both human and animals through anthropogenic activities. To the best of our knowledge this is the first

report of EIEC and EPEC from ACF in Nigeria.

Isolates in this study were tested against several classes of drugs including penicillin, cephalosporins, aminoglycoside, fluoroquinolones and carbapenem. Similar to our findings, Hleba et al., did not detect any *E. coli* resistant to meropenem and ceftriaxone from fresh water fish but found *E. coli* resistant to ampicillin and chloramphenicol (27). Also, Ryu et al., isolated 179 *E. coli* from commercial fish and sea food which were resistant to ampicillin (12 isolates) and to chloramphenicol (21 isolates). However, these authors found resistant strains to ceftriaxone in 3 isolates of *E. coli* (28). Lower sensitivity rates were observed with trimethoprim sulfamethoxazole and ciprofloxacin. Although, in an analysis of a large number of *E. coli* strains isolated from seawater samples collected from three beaches in Brazil, there were no strains resistant to ampicillin, cephalothin, gentamicin, tetracycline, sulfamethoxazole trimethoprim, chloramphenicol or ciprofloxacin (29).

The *E. coli* isolates in our study showed very little or no susceptibility to cefoxitin (2.9%) and amoxicillin-clavulanic acid (0%). This is an interesting finding, especially in the absence of ESBL production in the identified isolates. If indeed all the EPEC and EIEC *E. coli* isolates were harbouring Amp C type β-lactamase resistance, then cross transmission of these strains to humans could have catastrophic outcomes especially as there is little or no therapeutic options available. Isolates carrying *ampC* gene are usually resistant to all penicillins and cephalosporins including the extended spectrum and beta lactamase inhibitors. We were unable to confirm presence of *ampC* gene in the isolates due to lack of facility for genetic study. Further antibiotic resistance genomic studies are required to correctly identify what resistance genes were present in the isolates. However, the resistance to amoxicillin-clavulanic acid in this study is in agreement with the investigation of Adedeji et al., who reported 100% resistance to amoxicillin-clavulanic acid among bacterial isolates including *E. coli* from ACF (17).

The MARI of the isolates in this study ranged from 0.17 - 0.66. When the use of antibacterial agents in an aquaculture is seldom or low (low risk exposure), the MARI value is usually below or equal to 0.2. MARI value greater than 0.2 implies high level exposure to antibiotics (30). In this study, 94.3% of isolates had MARI value greater than 0.2, indicating high level exposure of fish in Jos to antibiotics. Varying MARI values have also been reported for different bacterial isolates from ACF (31). This corroborates other findings that there is high level exposure to antibacterial agents in ACF sold within Jos metropolis and other parts of Nigeria.

The high incidence of resistance among the isolates implies that practices such as use of sub-therapeutic doses of antibacterial agents and drug administration through feed medication which exposes both infected and uninfected fish population to antibacterial agents, are high in aquaculture in this environment. These practices enhance selection pressure and transfer of resistant genes among the fish population. This is a major risk to public health due to the resulting development of acquired antimicrobial resistance in fish pathogens and other aquatic bacteria. Bacteria in fish can act as reservoirs of resistance genes, from which such genes can disseminate to even commensal human pathogens (28, 29).

Conclusion

The occurrence of *E. coli* in ACF sold within the Jos metropolis is of public health and infection control significance. The pathotypes (EIEC and EPEC) identified are traditionally associated with gastroenteritis. The high rates of MARI in *E. coli* isolates from ACF also suggest overuse of antimicrobials in aquaculture practice in Jos. The aquaculture industry is

experiencing massive growth in many regions of the world and is of great importance for food and health. However, efforts are needed to prevent the widespread, intensive and unregulated use of antimicrobial agents in this area of animal food production, especially in developing countries such as Nigeria. International cooperation from organizations such as WHO and FAO is needed to support and assist developing countries in educating farmers, capacity building and implementation of preventive measures in animal husbandry and aquaculture.

The use of contaminated water sources for aquaculture should be prevented through adequate treatment. Hygienic practices should also be encouraged among fish farm workers and fish handlers in markets to reduce the risk of contamination during handling. It is important that governmental agencies set up hazard analysis and critical control point systems to monitor quality of foods available to the community at all times.

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