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Distribution and Antibiotic Resistance Profile of Extended Spectrum Beta-Lactamase Producing *Escherichia coli* from Fish Farms within Abakaliki Metropolis

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Authors' contributions

This work was carried out in collaboration among all authors. Authors JOA, NDU helped to conceptualized the data. Author CSI, FAI did Data curation and Formal analysis. Authors IUP, IRI performed Methodology. Author JOA did Project administration. Authors ALO and IUP Supervised the data and wrote the original draft. Authors IRI, IUP and KAN wrote, reviewed and edited the manuscript. All authors investigated the study, did literature searches and did data Validation and Visualization. All the authors reviewed and approved the final draft, and are responsible for all aspects of the work

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ABSTRACT

Background and Objectives: The emergence of antibiotic resistant determinant in fish farms and its spread is on the increase and has evolved into strains that are resistant to many classes of antibiotics. Thus, it is critical to identify the distribution and antibiotic resistance profile of Extended Spectrum Beta-lactamase (ESBL) producing *Escherichia coli* from fish farms within Abakaliki Metropolis.
Methodology: Aseptically, fifty (50) milliliters of fishpond water was collected from twenty locations in fifteen (15) fish farms and were analyze using standard microbiological culture and identification of *Escherichia coli.* Detection of phenotypic extended spectrum β-lactamases production was performed using Double-Disk Synergy Test (DDST). Antibiotic susceptibility studies of extended spectrum β-lactamases producing *Escherichia coli* was determined using the Kirby–Bauer disk diffusion method and the results were construed using the Clinical Laboratory Standard Institute (CLSI) zone diameter breakpoints.
Results: Extended spectrum beta-lactamase producing *Escherichia coli* distribution from fishpond water revealed overall occurrence rate of 34(11.3 %). The proportion of ESBL producing *Escherichia coli* was 5(25.0 %) from fish farm L followed by Farm A, Farm E, Farm G, which both accounted for 20.0 % respectively while the least occurrence of 1(5.0 %) was recorded against Farm I. ESBL-producing *E. coli* were resistant to cephalosporin particularly Ceftriaxone (88.2%), Ceftazidime (91.2 %), Cefotaxime (94.1) and Cefepime (85.3 %). This was followed by Amoxicillin-Clavulanate (91. 2 %), Azetronam (97.1 %). In all, Ciprofloxacin (82.4 %), Imipenem (97.1 %), and Meropenem (100 %) were the most effective antibiotic against ESBL-producing *E. coli* isolate.
Conclusion: This study reveals the prevalence of the ESBL phenotype in fish farms. The increasing prevalence of resistance to routinely used antibiotics in medical and veterinary therapies among the study isolates from aquaculture products poses a significant challenge to the treatment of human and animal diseases. As a result, adequate antibiotic intervention is essential to ensure the continued efficacy of antibiotics for aquaculture and human health, as well as the industry's viability.

Keywords: Escherichia coli; extended spectrum beta-lactamase; antibiotic resistance; fish farm.

1. INTRODUCTION

Fish farm (aquaculture) is a rapidly growing field of food production since the demand for fish is increasing worldwide, including Nigeria being the largest fish consumers in Africa and among the largest fish consumers in the world [1] with about 3.2 million metric tons of fish consumed annually [1,2]. It has been projected that Nigeria needs an average annual increase of 3.8% in fish production to keep up with the demands of an ever-increasing population [3]. According to Gazal et al. [4], population growth, rising incomes and urbanization are factors that contribute to the increase in production. However, the possible emergence of bacterial diseases and the need to treat sick fish also increase [4]. Gram negative bacteria especially members of the Enterobacteriaceace family are the main pathogens that cause diseases in fish [5,6,7,8]. These strains 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 [4]. Worldwide, there is a massive increase in fish farming, which is associated with intensive use of antibiotics to combat bacterial infections [9].

Many factors are known to favor the emergence of antibiotic resistant determinant in aquaculture and its spread to other sectors. This includes high stocking densities leading to elevated stress and infections in shrimp, widespread use of various chemicals (such as spawning aids, disinfectant and herbicide use in pond maintenance), nutrient rich environment in the ponds, occupational human exposure to Antimicrobial resistant bacteria, release of untreated water/waste to local environment [10].

Antibiotic-resistant bacteria, including human and zoonotic pathogens have been reported from various aquaculture settings [11,12]. Among these, members of Enterobacteriaceae are of particular concern owing to their considerable 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, 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 [12, 13].

There is strong evidence that the other Antibiotic Resistant (AR) determinants and ESBL encoding genes are found on integron that has the ability to integrate, express and facilitate their transfer among bacteria of different genera and kingdoms [14,15,16]. Although Nigeria is the world's largest consumers of farmed fish, no studies on the distribution of Enterobacteriaceae with resistance determinant to critical antibiotics (extended-spectrum beta-lactam) in fish farm environment exist to the best of our knowledge within our region. Identification of ESBL phenotype in the bacterial isolates could help to trace the prevalence among bacteria presence within fish farming environment.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out using fifteen (15) fish farms denoted by A, B, C, D, E, F, G, H, I, J, K, L M, N and O in Abakaliki, Ebonyi State, Nigeria. Abakaliki town is the capital city of Ebonyi State. It is located in 6.32^{o}N latitude and 8.12°E and longitude and is situated at an elevation of 117 meters above sea level. Abakaliki is populated and inhibited by indigenes and people from other parts of Nigeria. Ebonyi State shares border with Benue State to the north, Enugu State to the west, Imo and Abia to the south and Cross River to the east. The climate is characterized by a hot dry period which stretches from November-April, while the rainy season is from May - October. The maximum temperature during dry season is 37.6°C while the minimum temperature is 27.1°C [17]. The major occupations of people in Abakaliki are farming and trading, there are also civil servants and students and all these people engage in a busy activity of life.

2.2 Sample Collection and Processing

Aseptically, fifty (50) milliliters of fishpond water was collected from twenty locations in fifteen fish farms and each pond was subjected to ten folds serial dilution. Exactly 0.5ml of dilution factor three for each randomly serially diluted water samples were spread plated on plate count agar and incubated at 37°C for 24 hrs. After 24 hrs of incubation, colonies were counted using Colony counter (Techmel and Techmel, USA) and a loopful of each colony were aseptically streaked on solidified eosin methylene blue agar plate. The plates were incubated aerobically for 18-24 hours at 37°C. Bacterial colonies with greenish-metallic sheen on eosin methylene blue agar plate for were infer as the presence of *Escherichia coli*. All discrete colonies were purified by plating onto nutrient agar (Hi-Media, India). The sub-cultured plates were incubated at 37°C for 24 hrs. Discrete colonies were purified by plating onto nutrient agar (Hi-Media, India). Isolates was characterized based on their colonial morphology (color, consistency, texture), microscopic techniques (Gram staining and motility test) and biochemical characteristics, including oxidase, indole, citrate utilization, triple sugar iron test, methyl red, Voges-Proskaeur test, coagulase test, catalase and carbohydrate fermentation tests such as mannitol, sucrose, glucose and lactose [18,19].

2.3 Detection of Extended Spectrum Beta‑Lactamase Producing *E. coli*

ESBL production was phenotypically confirmed in only the bacteria isolates that showed reduced susceptibility to the 3rd-generation cephalosporins (such as cefotaxime and ceftriaxone) using the double disk synergy test (DDST) technique. Standardized inoculum of the isolate (adjusted to 0.5 McFarland turbidity standards) were aseptically swabbed on MH agar plates; and amoxicillin/clavulanic acid disc (20/10 µg) was placed at the center of the plate while cefotaxime (30 µg) and ceftazidime (30 µg) discs each was placed at adjacently distance of 15 mm away from the amoxycillin-clavulanic acid disc. The plates were incubated at 37^{o}C for 18 - 24 hrs; and ESBL production was phenotypically inferred by expansion of the zone of inhibition of either cephalosporin in the presence of amoxicillin-clavulanic acid than in its absence giving a dumbbell shape [20].

2.4 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was carried out using the Kirby-Bauer disc diffusion method as

outlined in the current Clinical and Laboratory Standards Institute (CLSI) guidelines. In brief, overnight culture of the test bacterial suspension ($1x10^6$ colony forming unit per milliliter (cfu/ml) was adjusted to 0.5 MacFarland turbidity standard and was spread over the entire surface of solidified Mueller-Hinton agar using a sterile cotton-tipped swab stick. This was allowed to stand for 15 mins to enable the inoculated organisms to pre-diffuse. The following antibiotics: ceftazidime (30 µg), ceftriaxone (30 µg), cefotaxime (30 µg), , cefepime (30µg), imipenem (10µg), amoxicillin-clavulanic acid (20/10µg), ciprofloxacin, (5µg), aztreonam (10µg), gentamicin (10µg), imipenem (10µg), meropenem (10µg), ofloxacin (5µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg) was aseptically placed onto the surface of solidified Mueller-Hinton agar plates with a sterile forceps and gently pressed to ensure even contact. The plates were incubated at 37°C for 18-24 hrs and zones of inhibition after 24 hrs of incubation was taken. The inhibition zone diameters (IZD) around each antibiotic disk were measured using a calibrated transparent ruler and recorded in millimeters. A standardized Table was used to determine if each bacterium was 'resistant', 'intermediate,' or 'sensitive.' For analysis, isolates with intermediate or resistant results was merged as resistant [21,22].

3. RESULT S

3.1 *E. Coli* Distribution from fishpond water

E. coli distribution from fishpond water revealed an overall occurrence rate of 97(32.3 %) consisting of a high occurrence rate of 9(45.0 %) in both fish farms H and L followed by Farm A, Farm D, Farm E, Farm N, Farm M, which both accounted for 40.0 % respectively while the least occurrence of 20.0 % was recorded against Farm B and Farm G respectively as presented in Table 1.

3.2 Distribution of Extended Spectrum Beta-Lactamase Producing *Escherichia coli* from Different Fishpond water from fishpond within Abakaliki Metropolis

Extended spectrum beta-lactamase producing *Escherichia coli* distribution from fishpond water revealed overall occurrence rate of 34 (11.3 %) consisting of high occurrence rate of 5(25.0 %) from fish farm L followed by Farm A, Farm E, Farm G, which both accounted for 20.0 % respectively while the least occurrence of 1 (5.0 %) was recorded against Farm I. Non- Extended spectrum beta-lactamase producing *Escherichia coli* accounted for 63 (21.0 %) as presented in Table 2.

The ESBL-producing *E. coli* from fish farm showed varying percentage rate of resistance and susceptibility to the test antibiotic. From the chart section (Fig. 1), a high rate of resistance by ESBL-producing *E. coli* was shown against cephalosporin particularly Ceftriaxone (88.2%), Ceftazidime (91.2 %), Cefotaxime (94.1 %) and Cefepime (85.3 %). This was followed by Amoxicillin-Clavulanate (91. 2 %), Azetronam (97.1 %). In all, Ciprofloxacin (82.4 %), Imipenem (97.1 %) and meropenem (100 %) were the most effective antibiotic against ESBL-producing *E. coli* isolate in this study (Fig. 2).

Table 1. Distribution *E. coli* from fishpond water

Aquaculture	No. Sampled	*E. coli* (%)
Farm A	20	8(40.0)
Farm B	20	4(20.0)
Farm C	20	6(30.0)
Farm D	20	8(40.0)
Farm E	20	8(40.0)
Farm F	20	5(25.0)
Farm G	20	4(20.0)
Farm H	20	9(45.0)
Farm I	20	7(35.0)
Farm J	20	7(35.0)
Farm K	20	4(20.0)
Farm L	20	9(45.0)
Farm M	20	5(25.0)
Farm N	20	8(40.0)
Farm O	20	5(25.0)
Total	300	97(32.3)

Table 2. Distribution of extended spectrum beta-lactamase producing *Escherichia coli* from different fish water from fishpond within Abakaliki Metropolis

Aquaculture	No. sampled	*E. coli* (%)	ESBL (%)	Non-ESBL (%)
Farm A	20	8(40.0)	4(20.0)	4(20.0)
Farm B	20	4(20.0)	3(15.0)	1(5.0)
Farm C	20	6(30.0)	2(10.0)	4(20.0)
Farm D	20	8(40.0)	2(10.0)	6(30.0)
Farm E	20	8(40.0)	4(20.0)	4(20.0)
Farm F	20	5(25.0)	3(15.0)	2(10.0)
Farm G	20	4(20.0)	4(20.0)	0(0.0)
Farm H	20	9(45.0)	2(10.0)	7(35.0)
Farm I	20	7(35.0)	1(5.0)	6(30.0)
Farm J	20	7(35.0)	0(0.0)	7(35.0)
Farm K	20	4(20.0)	0(0.0)	4(20.0)
Farm L	20	9(45.0)	5(25.0)	4(20.0)
Farm M	20	5(25.0)	1(5.0)	4(20.0)
Farm N	20	8(40.0)	3(15.0)	5(25.0)
Farm O	20	5(25.0)	0(0.0)	5(25.0)
Total	300	97(32.3)	34(11.3)	63(21.0)

Key: ESBL extended spectrum beta-lactamase

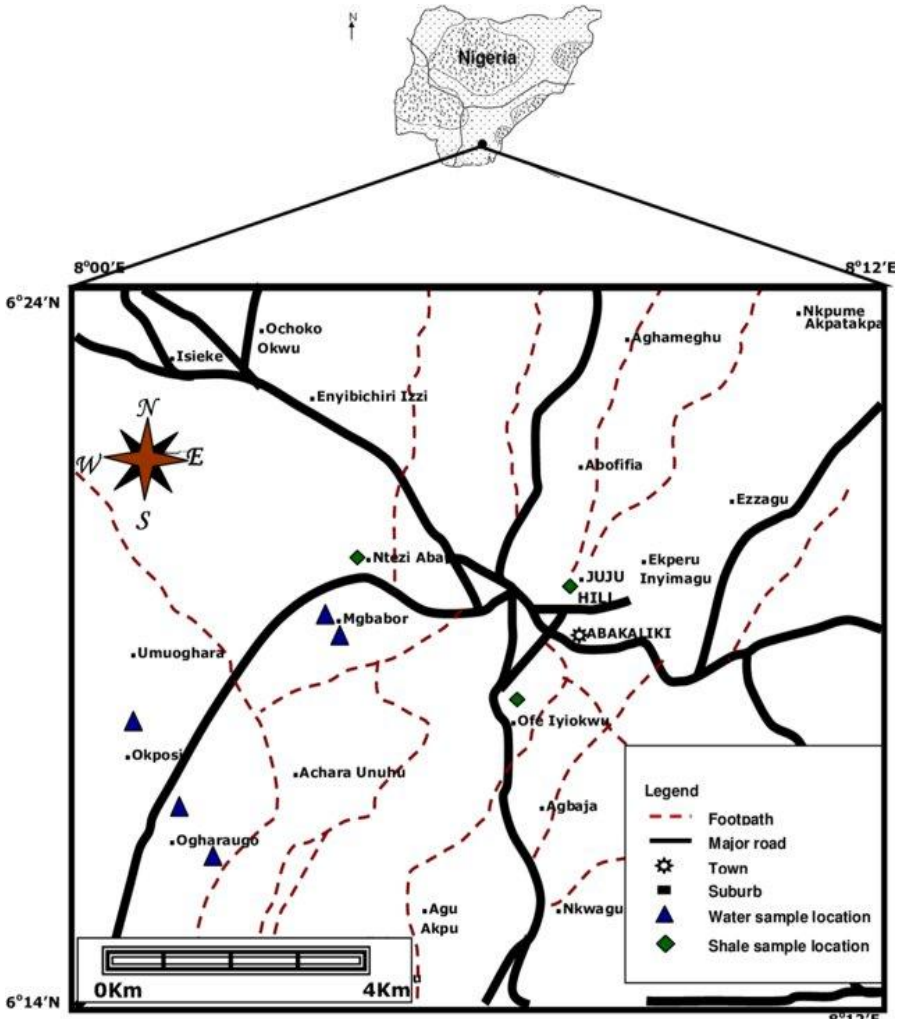

Fig. 1. Map showing Abakaliki in Ebonyi State the study area [17]

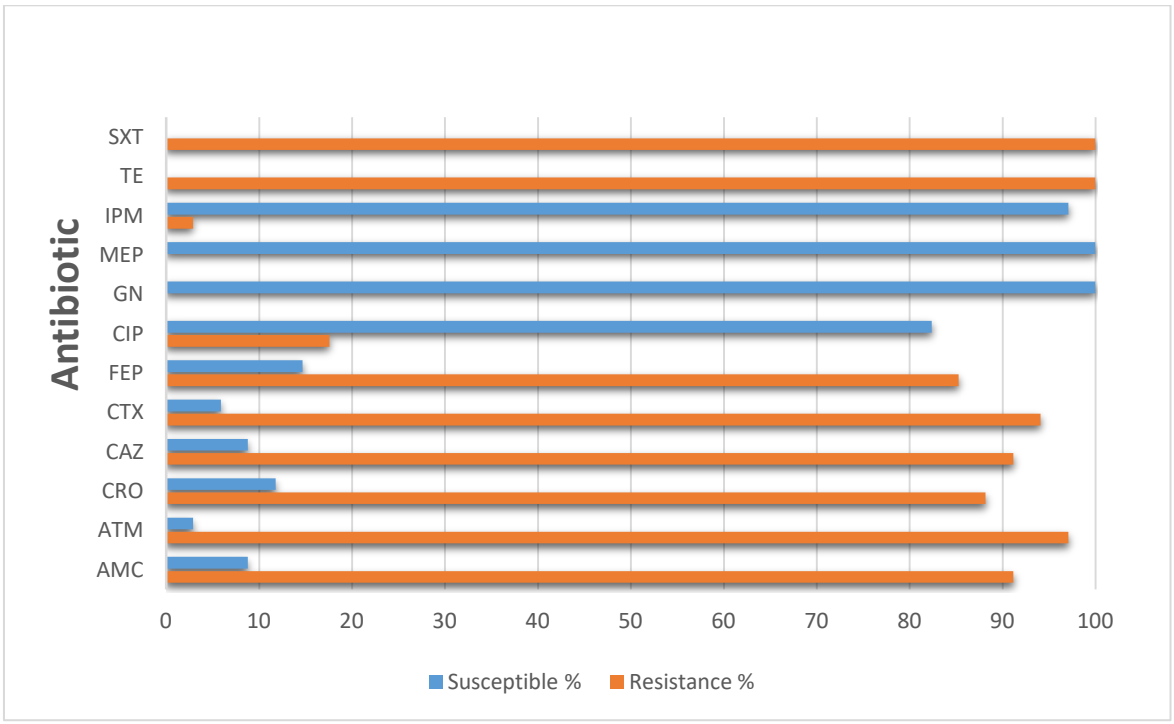

Fig. 2. Chart showing Antibiogram of ESBL-producing *E. coli*

Key: Amoxicillin-Clavulanate =AMC, Azetronam = ATM, Ceftriaxone = CRO, Ceftazidime = CAZ, Cefotaxime = CTX, Cefepime = FEP, Ciprofloxacin = CIP, Gentamicin = CN, Meropenem = MEP, Imipenem = IPM, Tetracycline = TE, Trimethoprim-Sulfamethoxazole = SXT

4. DISCUSSION

E. coli distribution from fishpond water revealed an overall occurrence rate of 97 (32.3 %). The major findings of this study reiterate with report in Northern Ethiopia [23], Nigeria [24], Bangladesh [25], Northwest of Borneo [26] and Malaysia [27] where these bacteria were isolated in fishpond. *Escherichia coli* has been traditionally recognized as an indicator organism of fecal contamination of water, and fish [23,28]. The source for the occurrence of the organism might have come from the point of distribution, poor water treatment, flies (*Musca domestica*) that wander around the pond, and poor handling practices by farm keepers and visitors operating in the study sites through the introduction of contaminated materials.

Prevalence of ESBL strain accounted for 34 (11.3 %) among samples from fishpond water. Although there is a paucity of information on phenotypic ESBL occurrence in aquaculture but few studies have reported the numeric presence of ESBL-producing *E. coli* in fish guts [25,29] and fish ponds [30]. Unfortunately, as with other livestock, antimicrobials usage in the aquaculture industry is not monitored, and therefore, accurate data are not available. The occurrence of ESBL phenotype could stern from the frequent incorporation of antimicrobial agents into the fish pellet as well as being used as a prophylactic agent in healthy fish which may facilitate prolonged selective pressure on bacteria in the aquatic environment. Actually, water ESBL strain could be indigenous to aquatic environments, or exogenous, transiently, and occasionally present in the water as a result of shedding from animal, vegetal, or soil surfaces [31, 32, 33, 34]. The occurrence of the ESBL strains could also be explained by the possibility of the heavy metal use of these compounds in aquaculture, several of which are non-biodegradable, thus increasing antibiotic selective pressure in water, facilitating the transfer of antibiotic-resistant determinants between aquatic bacteria, including fish and human pathogens, and allowing the presence of residual antibiotics in commercialized fish and products.

The findings highlighted the high resistance level of *E. coli* isolated towards antibiotics categorized as a priority and critically important for human use and as veterinary critically important drugs for food-producing animals, indicating important risk to public and animal health. Indeed,

tetracycline resistant accounted for 100 % in all isolates. Few studies have reported a similar pattern of resistant from aquaculture; 26 ESBL-producing isolates from fish demonstrated 61.5 % resistant to tetracycline [35]; Gufe et al. [36] reported 63.0 %, Dewl et al. [27] reported tetracycline resistance in fish: 31.2 % and water 53.3 %, in Tanzania, Extended-Spectrum β-Lactamase-Producing *Escherichia coli* in the Aquatic Environment and Nile Perch (*Lates niloticus*) of Lake Victoria showed resistance to tetracycline 90.9 % (10/11) [37] while tetracycline 100 % susceptibility has been reported [26]. Clearly, the findings of Lihan et al. [26] have shown that the force driving tetracycline resistance in aquaculture may differ between the two settings. However, oxytetracycline is frequently incorporated into the fish pellet for Streptococcosis treatment in the fishery as well as being used as a prophylactic agent in healthy fish [38]. Most of the isolates found were conferring resistance to tetracyclines, which could indicate the history of oxytetracycline use in the aquaculture. However, the presence of tetracycline resistance genes has been previously observed also in fish farms and pond sediment environments [39,40, 41].

ESBL-producing *E. coli* from aquaculture 66.7-100 % resistant to beta-lactam antibiotic such as aztreonam, ceftriaxone, cefotaxime and cefepime has not change from report from existing literature; Sapugahawatte et al. [29] reported cefepime (35.6 %), ceftriaxone (100 %), and cefotaxime (100 %); also in Tanzania, extended spectrum beta lactamase producing *Escherichia coli* in integrated agro-aquaculture in Morogoro, showed 70.0 % resistivity to Cefotaxime (70 %) [41]; also in Ghana, antibiotic sensitivity patterns of microbial isolates from fish ponds showed that 15 isolates of *Escherichia coli* showed resistance to Cefuroxime 70% [42] and in Abakaliki, phenotypic screening of multidrug-resistant *E. coli* from water and fish collected from different fish farms revealed that the Isolates exhibited resistance (54%-100%) to ceftazidime, aztreonam, cefuroxime, and ceftriaxone [43] while aquaculture and fishery in Asian revealed the mean resistance to third-generation and fourth-generation cephalosporins 69.6 % (95 % CI 65 to 75 %) [44]. A recent study has reported the presence of CTX-M and TEM ESBL genotype in pisciculture and fish parts [45]. The high prevalence of ESBL-producing *E. coli* with 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 an increase in health care cost, morbidity, and mortality. Notably, antibiotics resistance arises quickly and spreads rapidly, especially when resistance genes are horizontally transferred via plasmids and integrons among individuals, among species, and even among bacterial kingdoms [46, 47]. Much of the problem of antimicrobial resistance is due to the presence of transferable plasmids encoding Multidrug Resistant (MDR) and their dissemination among different enterobacterial species and it is common for a single plasmid to simultaneously mediate resistance to multiple antimicrobials and to be shared among different bacterial genera [47,48,49,50]. Also, an important consideration should be paid to the spread of resistance in aquaculture through potential sources of product contamination in the supply chain at the nursery periods of fingerlings and fry linked to humans, where poor or inappropriate personal hygienic practices during transport, methods of breeding and the potential implications of treated wastewater used for growth/nursery phase in the dissemination of antibiotic-resistant also should not be neglected.

5. CONCLUSION

This study highlights the occurrence rate of ESBL phenotype in fish farms. The presence of ESBL contribute to phenotypic antibiotic resistance in bacteria. The increasing level of resistance among the study isolate from aquaculture product to commonly used antibiotics in medical and veterinary therapies poses a great challenge to the treatment of human and animal diseases. Hence, appropriate intervention of antibiotic use is required to ensure the continuous efficacy of antibiotics for aquaculture and human health and the sustainability of the industry. The isolated ESBL producing bacteria pose high risk to the environment, human and animal health. Strict guidelines and supervision of aquaculture activities, as well as food safety training for farm owners/breeders on many elements of excellent hygiene standards, are strongly advised. Further study on the use of heavy metals in aquaculture, several of which are non-biodegradable, should be correlated with the transfer of antibiotic-resistant determinants between aquatic bacteria, including fish and human pathogens.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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