Original Article

Prevalence of Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* in Imported Frozen Freshwater Fish in Eastern Province of Saudi Arabia

Nasreldin Elhadi

Department of Clinical Laboratory Science, College of Applied Medical Sciences, University of Dammam, Dammam 31441, Saudi Arabia

Correspondence: Dr. Nasreldin Elhadi, Department of Clinical Laboratory Science, College of Applied Medical Sciences, University of Dammam, P. O. Box. 2435, Dammam 31441, Saudi Arabia. E-mail: nmohammed@uod.edu.sa

ABSTRACT

Background: The prevalence of extended-spectrum β-lactamase (ESBL) production and antimicrobial susceptibility testing in the *Escherichia coli* in frozen freshwater fish imported into Saudi Arabia have not been investigated.

Objective: The aim of this study was to investigate the prevalence of ESBL-producing *E. coli* in frozen freshwater fish imported into Saudi Arabia and retailed in various supermarkets and food stores in the Eastern Province of Saudi Arabia.

Materials and Methods: A total of 405 imported freshwater fish samples: Catfish (n = 65); mrigal (n = 45); tilapia (n = 135); carfoo (n = 50); rohu (n = 75); and milkfish (n = 35) were purchased from supermarkets and screened for ESBL-producing *E. coli* using ESBL chromogenic selective agar. The phenotypically confirmed ESBL isolates were further tested for antimicrobial susceptibility testing against 21 antimicrobial agents and amplification of bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes using polymerase chain reaction.

Results: A total of 110 out of the 405 (27.2%) freshwater fish samples were found to be positive for ESBL producing *E. coli* and yielded 224 confirmed isolates. The highest rates of multi-drug resistant patterns to antimicrobial agents were observed in *E. coli* isolated from catfish, mrigal, and tilapia imported from Thailand and milkfish imported from Vietnam. The most prevalent ESBL gene found in the samples was $bla_{CTX\text{-}M}$, 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).

Conclusion: The results confirmed the imported frozen freshwater fish is pool reservoir of antibiotic resistance and ESBL producing *E. coli*.

Key words: Antimicrobial resistance, *Escherichia coli*, extended-spectrum β-lactamases, freshwater fish

ملخص البحث :

تهدف هذه الدراسة إلى تحديد معدل انتشار إنزيم (β-lactamase) واختبار حساسية مضادات الميكروبات لبكتيريا (*E.coli*) في الأسماك المجمدة المعروضة للبيع بأسواق المملكة العربية السعودية. شملت الدراسة 405 من هذه الأسماك المستوردة. وخلصت إلى أن أعلى معدلات أنماط المقاومة لمضادات الميكروبات لوحظت في (*E.coli*) التي عزلت من الأسماك المستوردة من تايلند واسماك الحليب المستوردة من فيتنام، وأن أعلى انتشار لجينات (FSBL) كان ($bla_{CTX\text{-}M}$) وتم رصده في أنواع الأسماك بنسب متفاوتة.

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INTRODUCTION

Extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* have become an emerging pathogen worldwide with a wide range of antibiotic resistance.[1] Recently, several studies from different parts of the world have documented the occurrence and the prevalence of ESBL-producing *Escherichia coli* in cattle, meat, fish, and raw milk.[2-4] A recent study from the United Kingdom has shown that wastewater effluent contributes to the highly resistant enteric bacteria and dissemination of CTX-M-type in the natural environment, which poses a threat to human and animal health.[5] This high rate of food contamination with ESBL producing *E. coli* represents a substantial source of transmission and dissemination of ESBL genes to the human intestinal bacterial flora.

According to the Food and Agriculture Organization, aquaculture is growing more rapidly than all other animal food production sectors and its contribution to global supplies of several species of fish, crustaceans, and mollusks increased from 3.9% of total production by weight in 1970 to 33%, in 2005.[6] It has been estimated that fisheries and aquaculture supplied the world with about 110 million metric tons of food fish per year.[6] The consequences of the extensive use of antibiotics in aquaculture have resulted in multi-drug resistant bacteria that are passed to the human gut commensal flora when the fish are eaten.[7] The intensive use of antimicrobial agents such as tetracycline, florfenicol, and nitrofurantoin has resulted in an increase in antibiotic-resistant pathogens; consequently quinolones are increasingly the preferred agents.[7] The most common route for the delivery of antibiotics to fish occurs by mixing the antibiotic with specially formulated feed. It is well-known that fish do not effectively metabolize antibiotics and will pass them largely unused back into the environment in their feces.[8] It has been estimated that 75% of the antibiotics fed to fish are excreted into the water.[8] The public health hazards related to antimicrobial use in aquaculture include the development and spread of antimicrobial-resistant bacteria and resistance genes and the presence of antimicrobial residues in aquaculture products and the environment.[9] A prevalence study from China reported the presence of high levels of mobile genes conferring a reduced susceptibility to fluoroquinolones, as well as the presence of ESBL genes in fish produced in fish farms in China.[3]

To the best of the author knowledge, this is the first report on the ESBL producers in frozen fishery products imported into Saudi Arabia. This study investigated the prevalence of ESBL-producing *E. coli* in frozen imported freshwater fish retailed in different supermarkets and food stores in the Eastern Province of Saudi Arabia.

MATERIALS AND METHODS

Between December 2012 and February 2013, a total of 405 imported freshwater fish samples (65 catfish, 45 mrigal, 135 tilapia, 50 carfoo, 75 Rohu, and 35 milkfish) were randomly purchased from different supermarkets in the Eastern Province of Saudi Arabia and examined for the presence of ESBL-producing *E. coli*. The samples originated from four countries, namely Thailand (n = 260), India (n = 75), Vietnam (n = 35), and Myanmar (n = 35). The milkfish purchased originated from India, Vietnam, and Myanmar, while the other species namely catfish, mrigal, tilapia, carfoo, and rohu, originated from Thailand. All the purchased frozen freshwater fish were labeled with information indicating the country of origin, labeled as frozen belly gutted or nongutted fish, date of production, date of expiry, weight, and storage temperature (−18°C). The label did not indicate whether the fish were farmed-raised or wild-caught fish. Furthermore, some labels did not indicate the name of manufacturing company.

After purchase, the samples were transported on ice in coolers to the Microbiology Research Laboratory, University of Dammam. Upon arrival, the samples were kept intact on the ice and within 3-5 h they were processed and analyzed in aseptic conditions.

Examination of the freshwater fish samples was carried out, according to the Methods of Bacteriological Analytical Manual and other published protocols.[10,11] Briefly, 25 g of each fish sample (fish gills and intestinal and skin samples) was placed into a stomacher bag containing 225 ml of EC broth (Oxoid, UK) and homogenized using a stomacher (Seward Stomacher 400 Circulator, UK) for 2 min and incubated for 18-24 h at 35°C. After enrichment incubation, 0.1 ml was streaked on ESBL chromogenic agar (CHROMagar, France). After overnight incubation of CHROMagar, three to five pink to reddish isolated colonies with distinct morphological appearance were picked and subcultured on Trypticase Soy Agar to carry out biochemical tests. The positive indole and oxidase-negative isolates were further confirmed by using API 20E (bioMerieux, France) and for phenotypic confirmation of ESBL production, the ESBL *E*-test strips combining ceftazidime, cefotaxime, and cefepime (Oxoid, UK) were used.

Antimicrobial susceptibility was determined by the disk diffusion method on Muller-Hinton agar, as described by Kirby-Bauer, in accordance with the guidelines of the Clinical and Laboratory Standards Institute.[12] All the isolates were tested against 21 antibiotics, which included: Amikacin, ampicillin, augmentin, aztreonam, cefepime, cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, ertapenem, gentamicin, kanamycin, nalidixic acid, nitrofurantoin, noroxin, piperacillin, tetracycline, tobramycin, trimethoprim, and trimethoprim/sulfamethoxazole. The antibiotic disks were obtained from Oxoid (Basingstoke, United Kingdom).

All bacterial isolates from the samples confirmed as ESBL producers were further screened for the presence of the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes by polymerase chain reaction (PCR) amplification.[13,14] A DNA template was extracted by using a standard heat boiling lysis method.

RESULTS

The freshwater fish samples originated from four countries of the Asian subcontinent, namely Thailand, India, Myanmar, and Vietnam [Table 1]. Of the 405 samples, 110 tested positive for putative ESBL producing *E. coli* with prevalence rate of 27.2%. From the 110 positive samples, 224 putative ESBL producing *E. coli* isolates were identified [Table 1]. The prevalence of positive samples with putative ESBL producing *E. coli* on ESBL chromogenic agar are being reported with catfish representing 49.2% (n = 65), mrigal 6.7% (n = 5), tilapia 30% (n = 30), carfoo 4% (n = 5), Rohu 12.5% (n = 10), tilapia 37.3% (n = 69), and milkfish 54.3% (n = 40)] [Table 1]. The CHROMagar ESBL used in this study showed good sensitivity and specificity to investigate the prevalence of ESBL producing *E. coli* in imported freshwater fish, thereby reducing the number of man hours required.

A total of 224 ESBL producing *E. coli* isolates were recovered from the 405 freshwater fish samples. The antimicrobial resistant patterns of 224 isolates of *E. coli* against 21 antimicrobial agents exhibited high rates of resistance to ampicillin, cefotaxime, ceftriaxone, chloramphenicol, ciprofloxacin, nalidixic acid, tetracycline, and trimethoprim/sulfamethoxazole [Table 2]. The highest rates of multiresistant patterns to antimicrobial agents were observed in *E. coli* isolates isolated from catfish, mrigal, and tilapia, imported from Thailand and milk fish imported from Vietnam. The lowest multi-resistant patterns to antimicrobial agents were observed in *E. coli* isolates isolated from tilapia imported from India [Table 2].

All the 224 *E. coli* isolates obtained from the samples were screened for ESBL genes (bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$). Among the 224 *E. coli* isolates, PCR of β-lactamase genes revealed 122 ESBL-producing *E. coli* isolates. The most prevalent ESBL genes in imported freshwater fish were $bla_{CTX\text{-}M}$ (52.2%, n = 117). The highest rates of $bla_{CTX\text{-}M}$ were detected in tilapia (100%) [Figure 1], carfoo [Figure 2] imported from Thailand, and milkfish (60%) [Figure 3] imported from Vietnam [Table 3]. The bla_{SHV} gene were detected with low prevalence rate (2.2%, n = 5). The bla_{SHV} was detected only in 5 strains isolated from catfish imported from Thailand [Table 3 and Figure 4]. Figures 5 and 6 show the positive $bla_{CTX\text{-}M}$ samples of catfish and tilapia fish imported from Thailand and India, respectively. In this study, two strains isolated from catfish imported from Thailand were identified with ESBL gene combinations $bla_{CTX\text{-}M}/bla_{SHV}$. The isolates isolated from mrigal and rohu imported from Thailand were negative for β-lactamase genes.

Table 1: Prevalence of ESBL-producing *Escherichia coli* in imported frozen freshwater fish imported to Eastern Province of Saudi Arabia

Country of origin	Fish type	Number of Samples	Number of positive samples on ESBL chromogenic agar	Prevalence (%)	Number of isolates
Thailand	Catfish	65	32	49.2	65
	Mrigal	45	3	6.7	5
	Tilapia	60	18	30.0	30
	Carfoo	50	2	4.0	5
	Rohu	40	5	12.5	10
India	Tilapia	75	28	37.3	69
Vietnam	Milkfish	35	19	54.3	40
Myanmar	Rohu	35	0	0	0
Total		405	110	27.2	224

ESBL: Extended-spectrum β-lactamase

Table 2: Antibiotic resistant patterns of 224 *Escherichia coli* isolates against 21 antimicrobial agents isolates according to the country of origin and fish type

Country of origin	Fish type	Antibiotic resistant patterns	Number of isolates
Thailand	Catfish	AP-T-PRL-NA	2
		AP-T-CRO-PRL-NA-K	1
		AP-T-C-CRO-CTX-PRL	5
		AP-T-CRO-CTX-PRL-NA	8
		AP-T-CRO-CTX-PRL-NA-AM	1
		AP-T-CRO-CTX-PRL-TS-K-TM	1
		AP-T-CRO-CTX-PRL-NA-TS-TM	15
		AP-T-GM-C-CRO-CTX-PRL-NA-TS-TN	1
		AP-T-C-CRO-CTX-PRL-NA-TS-CIP-NOR-TM	24
		AP-T-GM-C-CRO-CTX-PRL-NA-TS-CIP-TN-NOR-TM	4
		AP-T-C-CRO-CTX-PRL-NA-AK-TS-CIP-ATM-NOR-TM	1
		AP-T-C-CRO-CTX-PRL-NA-AUG-TS-CIP-NOR-TM-FEP	1
		AP-T-GM-C-CRO-CTX-PRL-NA-TS-K-CIP-CAZ-TN-NOR-TM	1
		Sub-total	65
	Mirgal	AP-T-C-CRO-CTX-PRL-NA-TS-K-TN-TM	3
		AP-T-GM-C-CRO-CTX-PRL-NA-TS-ATM-TM	2
		Sub-total	5
	Tilapia	AP-CRO-CTX-PRL	1
		AP-T-PRL-TS-TM	1
		AP-T-GM-CRO-PRL-TS	1
		AP-T-CRO-CTX-PRL-ATM	1
		AP-CRO-CTX-PRL-CAZ-ATM-FEP	4
		AP-T-GM-PRL-NA-CIP-NOR-TM	1
		AP-T-CRO-CTX-PRL-NA-TS-CIP-NOR-TM	3
		AP-T-C-CRO-CTX-PRL-NA-TS-CIP-NOR-TM	3
		AP-T-C-CRO-CTX-PRL-NA-TS-CIP-ATM-NOR-TM	14
		AP-T-C-CRO-CTX-PRL-NA-TS-CIP-ATM-NOR-TM-FEP	1
		Sub-total	30
	Carfoo	AP-CRO-CTX-PRL	2
		AP-CRO-CTX-PRL-ATM-FEP	2
		AP-CRO-CTX-PRL-CAZ-ATM	1
		Sub-total	5
	Rohu	AP-T-GM-C-PRL-TN	2
		AP-T-GM-C-CRO-PRL-NA	3
		AP-T-GM-C-CRO-CTX-PRL-NA-TS-ATM-TN-TM	5
		Sub-total	10
India	Tilapia	AP	2
		AP-PRL	1
		AP-AUG	19
		AP-CRO-PRL	2
		AP-PRL-AUG	14
		AP-PRL-NA-TM	5
		AP-AUG-CIP-TM	1
		AP-CRO-CTX-PRL	9
		AP-CRO-PRL-NA-AP-TM	6
		AP-CRO-CTX-PRL-NA-TS-CIP-NOR-TM	9
		AP-T-CRO-CTX-PRL-NA-AK-AUG-TS-CIP-NOR-TM	1
		Sub-total	69
Vietnam	Milkfish	AP-PRL	5
		AP-CRO-PRL-NA-CIP	5
		AP-CRO-PRL-NA-TS-TM	5
		AP-CRO-CTX-PRL-NA-CIP-NOR	9
		AP-T-CRO-PRL-NA-CIP-TN-NOR-TM	1
		AP-T-C-CRO-CTX-PRL-NA-TS-CIP-NOR-TM	6
		AP-T-CRO-CTX-PRL-NA-TS-CIP-ATM-NOR-TN	3
		AP-T-GM-C-CRO-CTX-PRL-NA-TS-CIP-TN-NOR-TM	6
		Sub-total	40
		Total	224

AK: Amikacin (30 µg), AP: Ampicillin (10 µg), AUG: Augmentin (30 µg), ATM: Aztreonam (30 µg), FEP: Cefepime (30 µg), CTX: Cefotaxime (30 µg), CAZ: Ceftazidime (30 µg), CRO: Ceftraxone (30 µg), C: Chloramphenicol (30 µg), CIP: Ciprofloxacin (5 µg), GM: Gentamicin (10 µg), K: Kanamycin (30 µg), NA: Nalidixic acid (30 µg), NOR: Noroxin (10 µg), PRL: Piperacillin (100 µg), T: Tetracycline (30 µg), TN: Tobramycin (10 µg), TM: Trimethoprim (5 µg) and TS: Trimethoprim/sulfamethoxazole (25 µg)

DISCUSSION

This study investigated the prevalence of ESBL producing *E. coli* in imported frozen freshwater fish retailed in markets due to the importance of the ESBLs, global reports on the dissemination of the CTX-M-type ESBLs and limited published data describing the prevalence of ESBL-producing *E. coli* in fishery or aquaculture products, including those available for purchase in the Middle East and more specifically, Saudi Arabia.

The result of the current study has shown an alarming prevalence (27.2%) of ESBL producing *E. coli* in freshwater fish imported to the Eastern Province of Saudi Arabia. This study found that 52.2% of the ESBL isolates identified harbored the $bla_{\text{CTX-M}}$ gene, which has also been found in *E. coli* isolated from farmed fish in China, although at a lower prevalence rate [Table 3].[3] Several studies have been reported that the ESBL blaCTX-M gene producing E. coli strains has been isolated from cattle in France, chicken meat in Germany and wastewater effluent in the United Kingdom.[5,15,16]

Table 3: Distribution of ESBLs genes in *Escherichia coli* isolated from imported frozen freshwater fish imported to Eastern Province of Saudi Arabia

Country of origin	Fish type	Number of isolates	Number of isolates positive for ESBL genes		
			bla_{TEM} (%)	bla_{SHV} (%)	$bla_{\text{CTX-M}}$ (%)
Thailand	Catfish	65	0	5 (7.7)	34 (52.3)
	Mirgal	5	0	0	0
	Tilapia	30	0	0	30 (100)
	Carfoo	5	0	0	5 (100)
	Rohu	10	0	0	0
India	Tilapia	69	0	0	24 (34.8)
Vietnam	Milkfish	40	0	0	24 (60)
Myanmar	Rohu	0	0	0	0
Total		224	0	5 (2.2)	117 (52.2)

ESBL: Extended-spectrum β-lactamase

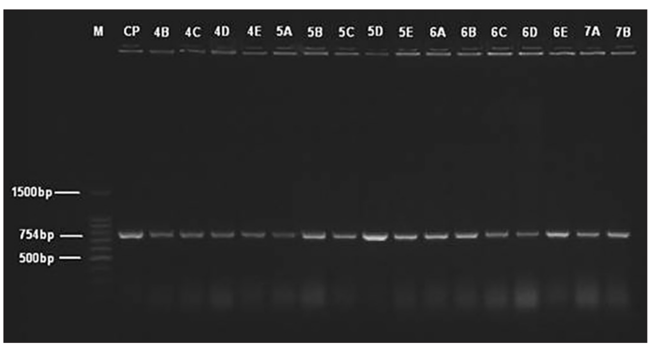

Figure 1: Representative polymerase chain reaction amplified fragments of the blaCTX-M gene (amplicon size 754 bp) on 1.5% Agarose gel electrophoresis of *Escherichia coli* isolates isolated from tilapia fish imported from Thailand. Lane M: Bench Top 100 bp DNA ladder (Promega, USA), lane coat protein: In-house positive control, Lane 4B to 7B: Positive isolates

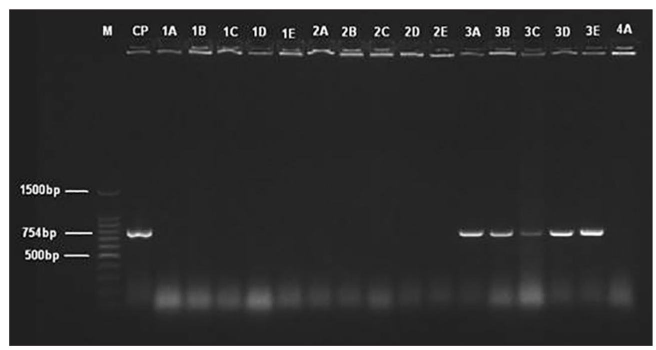

Figure 2: Polymerase chain reaction amplified fragments of the blaCTX-M gene (amplicon size 754 bp) on 1.5% Agarose gel electrophoresis of *Escherichia coli* isolates isolated from carfoo and rohu fish imported from Thailand. Lane M: Bench Top 100 bp DNA ladder (Promega, USA), Lane coat protein: In-house positive control, Lane 1A to 2E: Negative isolates of rohu fish, Lane 3A to 3E: Positive isolates of carfoo fish

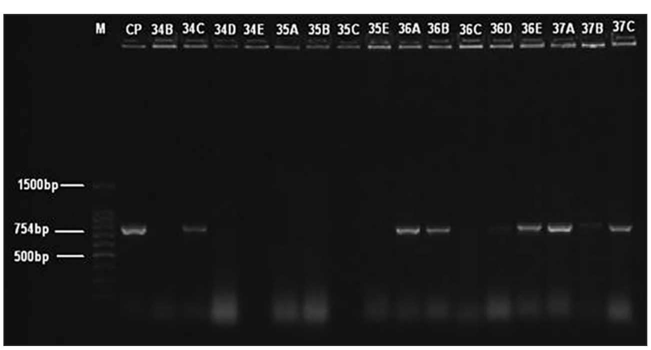

Figure 3: Representative polymerase chain reaction amplified fragments of the blaCTX-M gene (amplicon size 754 bp) on 1.5% Agarose gel electrophoresis of *Escherichia coli* isolates isolated from milkfish imported from Vietnam. Lane M: Bench Top 100 bp DNA ladder (Promega, USA), Lane coat protein: In-house positive control, Lane 34C: Positive isolate, Lane 34D to35E: Negative isolates, Lane 36A and 36B: Positive isolates, Lane 36C: Negative isolate, Lane 36E to 37C: Positive isolates

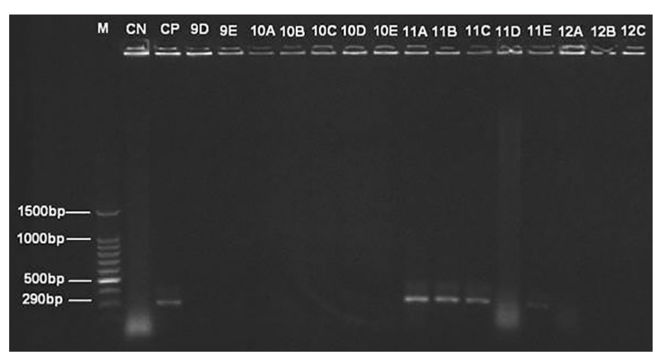

Figure 4: Polymerase chain reaction amplified fragments of the blaSHV gene (amplicon size 293 bp) on 1.5% Agarose gel electrophoresis of *Escherichia coli* isolates isolated from catfish imported from Thailand. Lane M: Bench Top 100 bp DNA ladder (Promega, USA), Lane CN: Negative control, Lane coat protein: In-house positive control, Lane 9D to 10E: Negative isolates: Lane 11A to 11E: Positive isolates

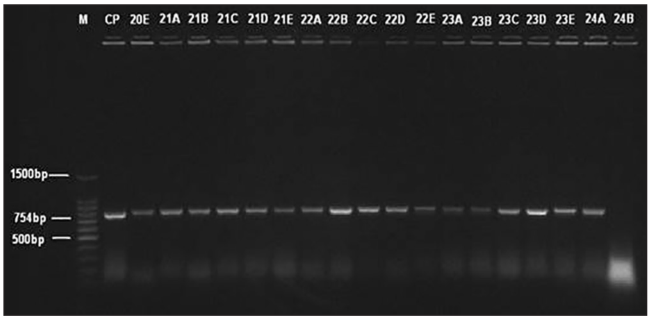

Figure 5: Representative polymerase chain reaction amplified fragments of the blaCTX-M gene (amplicon size 754 bp) on 1.5% Agarose gel electrophoresis of *Escherichia coli* isolates isolated from catfish fish imported from Thailand. Lane M: Bench Top 100 bp DNA ladder (Promega, USA), Lane coat protein: In-house positive control, Lane 20E to 24A: Positive isolates, Lane 24B: Negative isolate

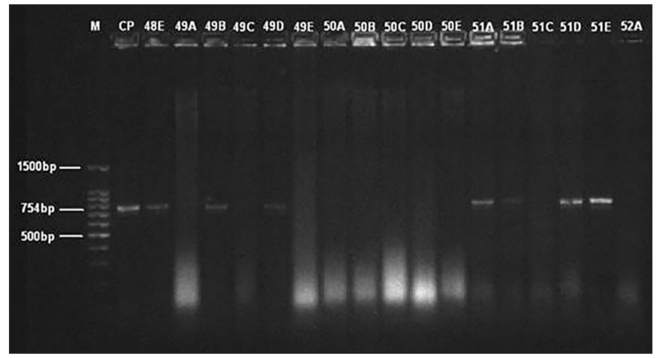

Figure 6: Representative polymerase chain reaction amplified fragments of the blaCTX-M gene (amplicon size 754 bp) on 1.5% Agarose gel electrophoresis of *Escherichia coli* isolates isolated from tilapia fish imported from India. Lane M: Bench Top 100 bp DNA ladder (Promega, USA), Lane coat protein: In-house positive control, lane 48E, 49B, 49D51A, 51B, 51D, and 51E: Positive isolates, lane 49A, 49C, 49E to 50E, 51C and 52A: Negative isolates

The present study showed that the prevalence rate of ESBL producing *E. coli* in the frozen freshwater fish imported into the Eastern Province of Saudi Arabia was higher than that in published data.[3] The high degree of ESBL colonization with ESBL producing *E. coli* was found in milkfish (54.3%) imported from Vietnam [Figure 3], catfish (49.2%) imported from Thailand [Figure 5], and tilapia (37.3) imported from India [Figure 6], respectively [Table 1]. A recent study from China demonstrated the existence of high levels of mobile genes conferring reduced susceptibility to fluoroquinolones, as well as the presence of ESBL genes, in the fish produced.[3]

The level of antibiotic resistance observed in this study was higher than the study conducted in China by Jiang *et al*.[3] which investigated the prevalence of β-lactamase in a fish farm in China. In the present study, the rates of multi-resistant patterns to antimicrobial agents were observed in *E. coli* isolates, isolated from catfish, mrigal, and tilapia imported from Thailand and milkfish imported from Vietnam. Our results may indicate that the increased prevalence of ESBL producing *E. coli* with multi-antibiotic resistant in the fish imported to Saudi Arabia is associated with the very high usage of antibiotics in fish farms in these countries. This high level of usage of antibiotics in fish farming has promoted the growth of several bacterial diseases, which has led to an increase in the use of antimicrobial agents.[17] The different classes of antibiotics used as growth promoters in aquaculture worldwide is very difficult to control due to the regulations and policies in different countries of the types and amount of antibiotics to be used in aquaculture systems.[8] The presence of antibiotic-resistant bacteria in aquaculture products may pose a potential health threat due to plasmid carrying antibiotic resistant genes being transferred to other bacteria.[7,9]

CONCLUSION

The results confirmed that imported frozen freshwater fish is pool reservoir of antibiotic resistance and ESBL producing *E. coli*. Contamination of retailed imported frozen freshwater fish with antibiotic resistant bacteria can be a major threat to public health, as the antibiotic resistance genes can be transferred to other bacteria of human clinical significance. Therefore, further studies are needed to investigate further trends of ESBL frequencies and monitor the other imported fish to Saudi Arabia are of major importance.

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Conflicts of interest

There are no conflicts of interest.

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