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Original Article

Molecular and phenotypic characteristics of isolated *Escherichia coli* from the skin, gills, and intestine of rainbow trout in retail stores of Kerman, Iran

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Abstract

Background: *Escherichia coli* is not naturally present in fish microbiota but can be acquired from sewage-contaminated aquatic environments. **Aims:** This study was performed to isolate and characterize the *E. coli* strains in rainbow trout fish marketed for human consumption. **Methods:** A total number of 166 fish were randomly collected from different retail settings in Kerman, Iran. The fish samples were analyzed to detect *E. coli* isolates. Antimicrobial resistance genes, Shiga toxin virulence subtypes and phylogenetic sequences were screened by PCR. **Results:** Prevalence of *E. coli* isolates on the skin, in the gills and intestine were 76.5% (127/166), 6.6% (11/166), and 3% (5/166), respectively. The most prevalent antimicrobial resistance phenotypes were observed against florfenicol (86.61%), erythromycin (83.46%), flumequine (82.67%), and oxytetracycline (81.88%); and 98.42% of the isolates were multi-drug resistant. The most frequent resistance gene was bla_{TEM} (14.17%), followed by *qnrA* (10.23%), *tetB* (9.44%), *sul2* (8.66%), bla_{SHV} (7.87%), *sul1* (7.87%), *dhfr1* (3.93%), $bla_{CTX\text{-}M}$ (3.14%), *dhfrV* (1.57%), bla_{OXA} (0.78%). Totally, 8.66% of isolates were categorized into three pathotypes including STEC, EPEC and EHEC. The *stx* subtypes including *stx1a*, *stx1c*, *stx1d*, *stx2c*, *stx2d*, *stx2e* and *stx2f* were identified in *stx*-positive strains. The *E. coli* isolates were classified into five phylogenetic groups including A (23.62%), B2 (3.93%), D (2.36%), F (9.44%) and cryptic clade I (11.81%). **Conclusion:** The study revealed that the skin of retail rainbow trout marketed in Kerman may be one of the potential passive carriers of multi-drug resistant and virulent *E. coli* strains.

Key words: *Escherichia coli*, Drug resistance, Fish, Phylogenetic groups, Virulence

Introduction

Fish is a very important protein source on a global scale, accounting for about 17% of animal protein and 7% of total world protein consumption in 2017 (Andreoli *et al*., 2021). Fish and fishery products may be a source of some food-borne infections for humans, which can be caused by bacteria, viruses, and algae (WHO, 2014). Some examples of important foodborne pathogens include *Vibrio* spp., *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium botulinum*, *Shigella* spp., *Aeromonas* spp., and *Escherichia coli* (Austin, 2006). Some bacteria are members of the fish microbiota, while others are acquired from the environment. Acquired microorganisms are typically transient and are temporarily found in different parts of the fish body, such as the skin, digestive, and respiratory systems. For example, *E. coli* is not naturally present in fish microbiota but can be acquired from sewage-contaminated aquatic environments. *E. coli* has been reported in the digestive system of rainbow trout (*Oncorhynchus mykiss*) as a transient pathogen (Austin, 2006). *E. coli* is an indicator of fecal contamination of water and fish that may lead to food-borne infections such as diarrhea in consumers (Cardozo *et al*., 2018), which depends on different factors of the bacterium including antimicrobial resistance, virulence, phylogenetics, etc.

Recently, we screened *E. coli* isolates and their antimicrobial resistance (AMR) on the skin of a small sample size of fish marketed in various retail stores in Kerman. According to our results, there was a potentially high prevalence of multi-drug resistant (MDR) isolates (Mohseni *et al*., 2023), which required further molecular investigations with a larger sample size. Therefore, the

present study aimed to investigate the prevalence, antimicrobial resistance (phenotypic and genotypic), Shiga toxin virulence genes, and phylogenetic background of *E. coli* strains on the skin, in the gills and intestine of rainbow trout available in Kerman, Southeast of Iran.

Materials and Methods

Sampling, culture and *E. coli* isolation

A total of 166 rainbow trout fish were randomly collected from different retail settings in Kerman, Iran, between February 2020 and February 2021 at two-week intervals. The fish samples were placed in polyethylene bags and immediately transferred to the Veterinary Microbiology Laboratory of Shahid Bahonar University of Kerman at 4°C. Totally, 498 swabs were obtained from the skin (n=166), gills (n=166), and intestine (n=166) using sterile dissecting instruments. These swab samples were cultured on MacConkey agar (Merck, Germany) and incubated at 37°C for 24 h. Suspected *E. coli* colonies (smooth and pink) were selected for the next step; five suspect *E. coli* colonies were selected from each plate and biochemical confirmation including indole, methyl-red-Voges-Proskauer, citrate tests were performed on the suspect colonies. After biochemical confirmation, only one colony was randomly selected for further analysis.

Phenotypic assessment of *E. coli* strains

Antimicrobial susceptibility testing was performed using disc diffusion method against the following antibiotic discs according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2018): chloramphenicol (C; 30 µg), amoxicillin (AMX; 30 µg), nitrofurantoin (FM; 30 µg), trimethoprim (TMP; 30 µg), oxytetracycline (T; 30 µg), erythromycin (E; 30 µg), flumequine (FLM; 30 µg), ceftazidime (CAZ; 30 µg), ceftazidime-clavulanate (CZA; 30 µg), cefotaxime-clavulanate (CTC; 30 µg), cefotaxime (CTX; 30 µg), ciprofloxacin (CP; 30 µg), tetracycline (TE; 30 µg), florfenicol (FF; 30 µg), and trimethoprim sulphamethoxazole (SXT; 30 µg) (Hudzicki, 2009). Isolates were classified as susceptible (S), intermediate (I), and resistant (R) based on zone diameters of bacterial growth inhibition in MH agar presented in CLSI; ESBL producers were identified by ≥5 mm increase in the inhibition zone diameter around cefotaxime-clavulanate and/or ceftazidime-clavulanate disks compared with the zone diameter of cefotaxime and/or ceftazidime, respectively (CLSI, 2018). The MAR index for the bacterial isolates was calculated as follow: (Fernandes *et al*., 2024).

$$\mathrm{MARindex} = \frac{a}{b}$$

Which,

a: The number of antibiotics to isolates were resistant

b: The total number of antibiotics tested

Genotypic assessment of AMR

For genotypic evaluation, DNA of *E. coli* isolates was first extracted by boiling method. The presence of antimicrobial resistance genes including bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}15}$, bla_{OXA}, *sulI*, *sulII*, *dhfrI*, *dhfrV*, *cat1*, *aadA*, *qnrA*, *qnrB*, *tetA* and *tetB* were examined (Naderi *et al*., 2016). Clinical *E. coli* strains 17DN for *sulI* and *sulII*; 21DN for *qnrB*; 25DN for *tetA*, *tetB* and *cat1*; 170DN for *dhfrI* and *dhfrV* were used as a positive control (Naderi *et al*., 2016). Distilled water was used as the negative control.

Detection and subtyping of Shiga toxin virulence genes

Four virulence genes (VGs) including *stx1*, *stx2*, *eaeA* and *hlyA* were screened using PCR described by Paton and Paton (1998) for pathotyping (Paton and Paton, 1998, 2002) to detect EPECs (*eaeA*+, *stx1*- and *stx2*-), STECs (*stx1*+ and/or *stx2*+ and *eaeA*-) and EHECs (*stx1*+ and/or *stx2*+ and *eaeA*+).

All strains may be positive or negative for hemolysin encoded by *hlyA*. The *E. coli* strains of Sakaï was used as positive controls for *stx1*, *stx2*, *eaeA* and *hlyA* genes. *E. coli* strain MG1655 was used as a negative control.

All *stx*-positive strains were subtyped via an accredited molecular technique described by Scheutz *et al*. (2012) which includes a multiplex-PCR to find *stx1a*, *stx1c* and *stx1d* subtypes; and also, seven simplex PCR to detect *stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f* and *stx2g* subtypes.

PCR for phylo-grouping

The distribution of phylogenetic groups, including A, B1, B2, C, D, E, F, and Clade I, among *E. coli* isolates was determined using the Clermont method (Clermont *et al*., 2013). In the first step, the sequences *arpA* (400 bp), *chuA* (288 bp), *yjaA* (211 bp), and *TspE4.C2* (152 bp) were amplified using specific primers. If the strains could not be classified into phylogenetic groups A, B1, B2, or F, a secondary PCR was performed to detect groups C, D, and E based on the *arpA*-group E (301 bp) and *trpA*-group C (219 bp) sequences. Strains that did not fall into any of the above phylogenetic groups were classified as unknown (U). All PCR products were visualized by electrophoresis on a 1.5% agarose gel.

Results

E. coli isolation

E. coli isolates were obtained from 28.71% of the swabs (143/498). Among the 143 *E. coli* positive swabs, almost 90% (127/143) belonged to skin but from less than 10% belonged to gills (11/143) and intestinal (5/143) swabs (Table 1). Also, the prevalence of *E. coli* isolates on the skin, in the gills and intestine of 166 fish were 76.5% (127/166), 6.6% (11/166) and 3% (5/166), respectively. Therefore, statistical analysis of genetic variables was performed on the skin strains due to their higher prevalence than gill and gut strains.

Table 1: Pattern of *E. coli* isolation from different sites of swabbing

E. coli detection from different swab sites in each fish			No. of fish	Percentage (%)
Skin	Gill	Intestine		
+	-	-	116	69.9
+	+	-	6	3.6
+	+	+	4	2.4
-	+	-	1	0.6
+	-	+	1	0.6
-	-	-	38	22.9
Total			166	100
Prevalence of *E. coli*-positive swabs (%)				Total
Skin	Gill	Intestine		
127 (88.8)	11 (7.7)	5 (3.5)		143 (100)

Phenotypic antimicrobial resistance

All 127 skin isolates, 11 gill and 5 intestinal *E. coli* isolates (100%) showed phenotypic resistance to at least one of the studied antibiotics. Among the skin strains (n=127), resistance to the antibiotics including florfenicol (86.61%), erythromycin (83.46%), flumequine (82.67%) and oxytetracycline (81.88%) were the most prevalent (Table 2, Fig. 1). There were 91 various phenotypic resistance profiles in the skin strains. Some profiles involved just one antibiotic resistance (AR) while some others showed co-resistance against up to 13 antibiotics (Table 3). Of the 91 profiles, 89 were resistant to three or more antibiotics from different antimicrobial families (MDR). Totally 98.42% (125/127) of skin isolates had MDR profiles and MAR (multiple antibiotic resistance) index ≥0.2 (Table 3). The most common resistance pattern was related to FF/E/FLM/T/CP/C/SXT/TE/TMP which were detected in 12.60% (95% CI: 7.3%-19.67%) of isolates (Table 3).

Table 2: Prevalence of resistance phenotypes and genes

Antibiotic family	AR gene	No. of positive isolates	Prevalence (%)	95% CI	AR phenotype	No. of positive isolates	Prevalence (%)	95% CI
β-Lactam	bla_{TEM}	18	14.17	8.6%-21.47%	AMX	45	35.43	27.15%-44.41%
	bla_{SHV}	10	7.87	3.8%-14%	CAZ	4	3.14	0.08%-7.8%
	bla_{CTX-M}	4	3.14	0.08%-7.8%	CTX	24	18.89	12.50%-26.80%
	bla_{OXA}	1	0.78	0%-4.3%	-	-	-	-
Tetracycline	*tetA*	0	0	0%-2.8%	TE	75	59.05	49.9%-67.70%
	tetB	12	9.44	4.9%-15.92%	T	104	81.88	74.08%-88.16%
Amphenicol	*cat1*	0	0	0%-2.8%	FF	110	86.61	79.43%-92.01%
	floR	0	0	0%-2.8%	C	88	69.29	60.49%-77.17%
Fluoroquinolones	*qnrA*	13	10.23	5.5%-16.87%	CP	95	74.8	66.33%-82.08%
	qnrB	0	0	0%-2.8%	FLM	105	82.67	74.96%-88.81%
Sulphonamide	*sul1*	10	7.87	3.8%-14%	SXT	77	60.62	51.57%-69.18%
	sul2	11	8.66	4.4%-14.97%	-	-	-	-
Trimethoprim	*dhfr1*	5	3.93	1.2%-8.9%	TMP	70	55.11	46.04%-63.95%
	dhfrV	2	1.57	0.01%-5.5%	-	-	-	-
Aminoglycoside	*aadA*	0	0	0%-2.8%	-	-	-	
Macrolides	-	-	-	-	E	106	83.46	75.84%-89.46%
Others	-	-	-	-	FM	20	15.74	9.8%-23.27%

Table 3: Phenotypic resistance pattern of *E. coli* strains isolated from skin of retail rainbow trout

Phenotypic resistance pattern	MDR	No. of antibiotics	No. of isolates	MAR index
FF/E/FLM/T/CP/C/SXT/TE/TMP/AMX/CTX/FM/CAZ	MDR	13	1	1
FF/E/FLM/T/CP/C/SXT/TE/TMP/AMX/CTX	MDR	11	1	0.84
FF/E/FLM/T/CP/C/SXT/TE/TMP/AMX/FM	MDR	11	1	0.84
FF/E/FLM/T/CP/C/SXT/TE/TMP/CTX/SAZ	MDR	11	1	0.84
FF/E/FLM/T/CP/C/SXT/TE/TMP/CTX	MDR	10	6	0.76
FF/E/FLM/T/CP/C/SXT/TE/TMP/FM	MDR	10	3	0.76
FF/E/FLM/T/CP/C/SXT/TE/TMP/AMX	MDR	10	1	0.76
FF/E/FLM/T/CP/C/SXT/TE/TMP/AMX	MDR	10	1	0.76
FF/E/FLM/T/CP/C/SXT/TE/TMP	MDR	9	16	0.69
FF/E/FLM/T/CP/C/SXT/TE/AMX	MDR	9	1	0.69
FF/E/FLM/T/CP/C/SXT/TMP/AMX	MDR	9	1	0.69
FF/E/FLM/T/C/SXT/TE/TMP/CTX	MDR	9	1	0.69
FF/FLM/T/CP/C/SXT/TE/TMP/FM	MDR	9	1	0.69
E/FLM/T/CP/C/SXT/TE/TMP/CTX	MDR	9	1	0.69
FF/E/FLM/T/CP/C/SXT/FM	MDR	8	2	0.61
FF/E/FLM/T/CP/C/TE/TMP	MDR	8	1	0.61
FF/E/FLM/T/CP/C/TE/AMX	MDR	8	1	0.61
FF/E/FLM/T/CP/C/TE/CTX	MDR	8	1	0.61
FF/E/FLM/T/CP/C/AMX/FM	MDR	8	1	0.61
FF/E/FLM/T/CP/SXT/TE/AMX	MDR	8	1	0.61

FF/E/FLM/T/CP/TE/TMP/FM	MDR	8	1	0.61
FF/E/FLM/T/CP/TE/AMX/CTX	MDR	8	1	0.61
FF/E/FLM/T/C/SXT/TE/TMP	MDR	8	1	0.61
FF/E/FLM/CP/C/SXT/CTX/FM	MDR	8	1	0.61
FF/E/FLM/CP/C/TE/AMX/FM	MDR	8	1	0.61
FF/E/FLM/CP/C/TE/CTX/FM	MDR	8	1	0.61
FF/E/T/CP/C/SXT/TE/TMP	MDR	8	2	0.61
FF/E/T/CP/C/SXT/TE/AMX	MDR	8	1	0.61
FF/E/T/CP/C/SXT/TMP/CTX	MDR	8	1	0.61
FF/E/T/CP/C/SXT/TMP/AMX	MDR	8	1	0.61
FF/E/T/C/SXT/TE/AMX/FM	MDR	8	1	0.61
FF/FLM/T/CP/C/SXT/TE/TMP	MDR	8	3	0.61
FF/FLM/T/CP/C/SXT/TMP/FM	MDR	8	1	0.61
E/FLM/T/CP/TE/AMX/CTX/CAZ	MDR	8	1	0.61
FF/E/FLM/T/CP/C/SXT	MDR	7	1	0.53
FF/E/FLM/T/CP/C/TE	MDR	7	2	0.53
FF/E/FLM/T/CP/C/AMX	MDR	7	3	0.53
FF/E/FLM/T/CP/SXT/TMP	MDR	7	1	0.53
FF/E/FLM/T/C/SXT/AMX	MDR	7	3	0.53
FF/E/FLM/T/C/TE/TMP	MDR	7	1	0.53
FF/E/FLM/T/C/TE/FM	MDR	7	1	0.53
FF/E/FLM/CP/C/SXT/TMP	MDR	7	1	0.53
FF/E/T/CP/C/TE/AMX	MDR	7	1	0.53
FF/E/T/CP/C/TE/CAZ	MDR	7	1	0.53
FF/E/T/C/SXT/TE/TMP	MDR	7	1	0.53
FF/E/T/CP/SXT/TMP/AMX	MDR	7	1	0.53
FF/E/CP/C/SXT/TE/TMP	MDR	7	1	0.53
FF/E/CP/C/TMP/AMX/CTX	MDR	7	1	0.53
FF/FLM/T/C/SXT/TE/TMP	MDR	7	1	0.53
E/FLM/T/CP/C/SXT/FM	MDR	7	1	0.53
E/FLM/T/CP/SXT/TE/TMP	MDR	7	2	0.53
E/FLM/T/CP/SXT/TMP/AMX	MDR	7	1	0.53
FF/E/FLM/T/CP/TE	MDR	6	2	0.46
FF/E/FLM/T/CP/TMP	MDR	6	1	0.46
FF/E/FLM/T/CP/AMX	MDR	6	1	0.46
FF/E/FLM/T/C/AMX	MDR	6	1	0.46
FF/E/FLM/T/TMP/AMX	MDR	6	1	0.46
FF/E/FLM'/T/AMX/CTX	MDR	6	1	0.46
FF/E/FLM/CP/C/TE	MDR	6	1	0.46
FF/E/FLM/C/TE/AMX	MDR	6	1	0.46
FF/E/TE/CP/C/AMX	MDR	6	1	0.46
FF/FLM/T/CP/CTX/FM	MDR	6	1	0.46
FF/FLM/T/C/SXT/TMP	MDR	6	1	0.46
FF/FLM/T/C/TE/AMX	MDR	6	2	0.46
FF/T/C/SXT/TE/TMP	MDR	6	3	0.46
E/FLM/T/SXT/TMP/AMX	MDR	6	1	0.46
E/T/SXT/TE/TMP/AMX	MDR	6	1	0.46
E/CP/C/TMP/AMX/FM	MDR	6	1	0.46
FLM/T/CP/C/TE/AMX	MDR	6	1	0.46
FLM/CP/C/TE/TMP/CTX	MDR	6	1	0.46
FF/E/FLM/T/CP	MDR	5	1	0.38
FF/E/FLM/T/TMP	MDR	5	1	0.38
FF/E/FLM/T/AMX	MDR	5	1	0.38
FF/E/FLM/CP/SXT	MDR	5	1	0.38
FF/E/FLM/CP/AMX	MDR	5	1	0.38
FF/E/FLM/SXT/AMX	MDR	5	1	0.38
FF/E/FLM/SXT/CTX	MDR	5	1	0.38
FF/E/T/TE/AMX	MDR	5	1	0.38
FF/FLM/T/CP/TE	MDR	5	1	0.38
FLM/T/CP/SXT/TMP	MDR	5	1	0.38
FF/E/FLM/T	MDR	4	1	0.31
FF/E/CP/AMX	MDR	4	1	0.31
FF/FLM/T/CP	MDR	4	1	0.31
E/FLM/T/CP	MDR	4	1	0.31
E/FLM/CP/CTX	MDR	4	1	0.31
E/FLM/SXT/CTX	MDR	4	1	0.31
FF/E/CP	MDR	3	1	0.23

FF/FLM/AMX	MDR	3	1	0.23
E/TMP/FM	MDR	3	1	0.23
E/FLM	-	2	1	0.15
FLM	-	1	1	0.07
Total	-	-	127	-

Table 4: Distribution pattern of various resistance gene profiles among different phylo-groups

Resistance profile	Phylo-groups [No. of isolates in each phylo-group (% among 127 *E. coli* isolates)]						Prevalence of profile (%)	ESBL-positive
	A	B2	D	F	U	Clade I		
	[30 (23.6)]	[5 (3.9)]	[3 (2.3)]	[12 (9.4)]	[62 (48.9)]	[15 (11.9)]		
	Prevalence of resistance gene profiles; No. in each phylo-group							
bla$_{TEM}$/tetB/dhfr1	1	-	-	-	-	-	1 (0.78)	-
bla$_{SHV}$/sul2/qnrA	-	-	-	1	-	-	1 (0.78)	-
bla$_{CTX}$/sul1/sul2	-	-	-	-	1	1	2 (1.57)	-
bla$_{CTX}$/sul2/qnrA	-	-	-	-	1	-	1 (0.78)	-
bla$_{TEM}$/bla$_{OXA}$	-	-	-	-	-	1	1 (0.78)	-
bla$_{TEM}$/tetB	-	-	-	1	1	-	2 (1.57)	-
bla$_{TEM}$/sul2	-	-	-	-	2	-	2 (1.57)	1
bla$_{TEM}$/qnrA	1	-	-	-	-	-	1 (0.78)	-
bla$_{SHV}$/qnrA	-	-	-	-	1	-	1 (0.78)	-
bla$_{CTX}$/sul1	-	-	-	-	1	1	2 (1.57)	-
tetB/dhfr1	1	-	-	-	-	-	2 (1.57)	-
tetB/dhfr5	1	-	-	-	-	-	1 (0.78)	-
sul1/sul2	-	-	-	-	1	1	2 (1.57)	1
sul2/qnrA	-	-	-	1		1	2 (1.57)	-
bla$_{TEM}$	3	-	-	1	5	-	9 (7.08)	2
bla$_{SHV}$	2	-	-	-	4	2	8 (6.29)	1
bla$_{CTX}$	-	-	1		1	-	2 (1.57)	1
tetB	1	-	1	-	4	-	6 (4.72)	1
sul1	1	1	-	-	4	-	6 (4.720	2
sul2	2	1	-	-	-	1	3 (2.36)	2
dhfr1	1	-	-	2	-	-	3 (2.36)	-
dhfr5	1	-	-	-	-	-	1 (0.78)	-
qnrA	1	-	-	-	4	2	7 (5.51)	-
Total	16	2	2	6	30	10	66 (52)	11
No resistance gene	14	3	1	6	31	6	61 (48.03)	5

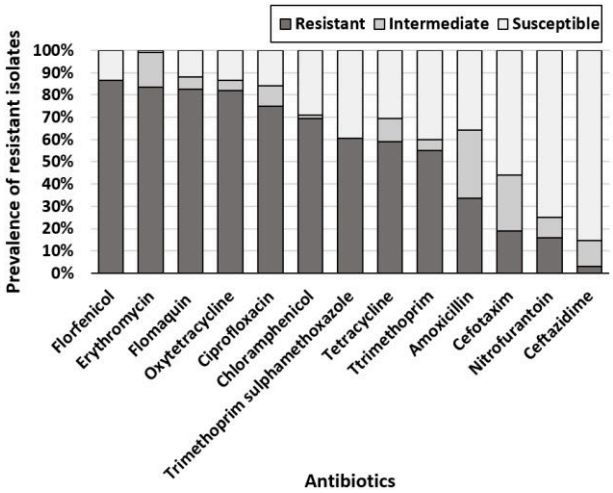

Fig. 1: Prevalence of resistant, susceptible, and intermediate *E. coli* strains against each studied antibiotic

Antimicrobial resistance genes

Totally, 51.96% (66/127) of the skin isolates, 45.45% (5/11) of the gill isolates and 60% (3/5) of the intestinal isolates were positive for at least one of the studied resistance genes. Among the 127 skin isolates, the genes associated with resistance to β-Lactams had the highest prevalence (for example *bla$_{TEM}$* with 14.17% frequency). Some other resistance genes with considerable prevalence were *tetB*, *qnrA*, *sul1* and *sul2* with 7% to 10%. *dhfr1* and *dhfrV* had low frequency and *tetB*, *cat1*, *floR*, *qnrB* and *aadA* had no prevalence (Table 2). There were 23 various resistance gene profiles in the skin isolates. Some profiles had just one gene and some others had up to three resistance genes simultaneously (Table 4).

Pathotypes, VGs and *stx* subtypes

Only 11 of 127 *E. coli* skin isolates were positive for at least one of the VGs. The 11 VG-positive skin isolates were categorized into three pathotypes including STEC (6/127 isolates; 4.72%), EPEC (6/127 isolates; 3.14%) and EHEC (1/127 isolate; 0.78 %). Among gill *E. coli* isolates, only one isolate harbored *stx1a* virulence gene and was STEC. None of the intestinal isolates were positive for VGs.

The prevalence rate of VGs in skin isolates was various without any significant difference, including: 4/127 positive-isolates for *stx1* (3.14%; 95% CI: 0.08%-7.8%), 2/127 for *stx2* (1.57%; 95% CI: 0.01%-5.5%), 5/127 for *eae* (3.93%; 95% CI: 1.2%-8.9%) and 1/127 for *ehly* (0.78%; 95% CI: 0%-4.3%). Subtyping of *stx1* and *stx2* genes revealed that *stx1* could be subtyped to *stx1a* (2 isolates), *stx1c* (3 isolates) and *stx1d* (1 isolate). Also, *stx2* gene was subtyped into *stx2c*, *stx2d*, *stx2e* and *stx2f* (1 isolate for each subtype) (Fig. 2). Virulence gene

Table 5: Distribution pattern of various virulence gene profiles among different phylo-groups

Resistance profile	Pathotype	Phylo-groups [No. of isolates in each phylo-group (% among 127 *E. coli* isolates)]						Prevalence of profile (%)
		A	B2	D	F	U	Clade I	
		[30 (23.6)]	[5 (3.9)]	[3 (2.3)]	[12 (9.4)]	[62 (48.9)]	[15 (11.9)]	
		Prevalence of resistance gene profiles; No. in each phylo-group						
eae/ehly	EPEC	-	-	-	-	1	-	1 (0.78)
eae/stx1d	EHEC	-	-	-	-	-	1	1 (0.78)
stx1a/stx1c	STEC	-	-	-	-	-	1	1 (0.78)
stx2d/stx2f	STEC	-	-	-	-	1	-	1 (0.78)
stx2c/stx2e	STEC	-	-	-	-	1	-	1 (0.78)
eae	EPEC	-	-	-	1	2	-	3 (2.3)
stx1c	STEC	2	-	-	-	-	-	2 (1.57)
Total	-	2	0	0	1	5	2	10 (7.87)
No. resistance gene	-	28	5	3	11	57	13	117 (92.12)

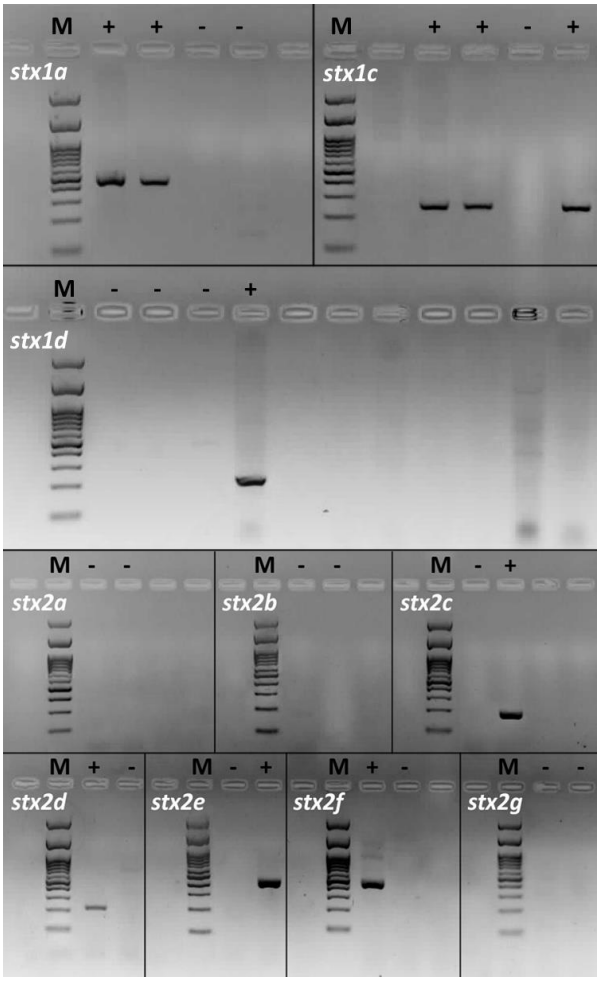

Fig. 2: Electrophoresis image of PCR products for *stx* subtypes: ***stx1a*** (478 bp), ***stx1c*** (252 bp), ***stx1d*** (203 bp), ***stx2a*** (349 or 347 bp), ***stx2b*** (251 bp), ***stx2c*** (177 bp), ***stx2d*** (179, 235, or 280 bp), ***stx2e*** (411 bp), ***stx2f*** (424 bp), and ***stx2g*** (573 bp). Lane M: 100 bp marker. lane +: Positive sample, and lane -: Negative sample

profiles are shown in Table 5.

Prevalence of phylo-groups

Totally, 65 of 127 skin *E. coli* isolates (51.1%), 4 of 11 gill isolates (36.3%) and 3 of 5 intestinal isolates (60%) were phylo-typed into groups A, B2, and F in the first step (based on *arpA*, *chuA*, *yjaA*, and *TspE4.C2* genes), and group D (based on *arpA*-group E and *trpA*-group C) in the second step of phylo-typing. The remaining isolates were classified as unknown (U). Among the 127 *E. coli* isolates from skin, five phylo-groups were identified as A (30 isolates; 23.6%; 95% CI: 16.5%-31.9%), B2 (5 isolates; 3.9%; 95% CI: 1.2%-8.9%), D (3 isolates; 2.3%; 95% CI: 0.04%-6.7%), F (12 isolates; 9.4%; 95% CI: 4.9%-15.9%), and cryptic clade I (15 isolates; 11.9%; 95% CI: 6.7%-18.7%). Sixty-two (48.9%; 95% CI: 39.8%-57.8%) isolates had unknown (U) phylogenetic background (Tables 4 and 5).

Investigation of AR, VGs and phylo-group in relation to each other

The strains that had at least one resistance and/or virulence gene mostly belonged phylo-group A, followed by F and clade I; a significant part of strains was phylogenetically unknown (Tables 4 and 5). These results indicate the commensal nature of the *E. coli* strains. Odds ratio between AR genes and phenotypes was evaluated. This study showed that some positive isolate for a specific phenotype significantly carried ($P<0.05$) a resistance gene many times more than negative isolates for that specific phenotype. Eight significant phenotype-gene relations were found including CTX-*qnrA*, CAZ-*sul1*, CTX-*dhfrI*, FF-*sul2*, E-*dhfrI*, FM-*dhfrI*, FM-*dhfrV*, FF-*tetB* (Fig. 3). Also, investigation of positive correlation between two factors indicates the presence of high positive correlation among phenotypic antimicrobial resistance factors (Fig. 4).

Discussion

In this study, *E. coli* strains were isolated from more than a quarter of fish samples. *E. coli* was significantly found on the skin samples compared with gill and intestinal samples. In a study (2016), *E. coli* was found in 80.7% of fish samples collected from retail markets in Kolkata (Dutta and Sengupta, 2016). Alttai *et al*. (2023) showed that only 29.9% of fish samples were *E. coli*-positive, which is similar to our study. The presence of coliforms such as *E. coli* in fish could be an indicator of sewage-pollution in various stages, including: fish farming, processing, storage, and distribution (Ahmed *et al*., 2023). Untreated wastewater from urban areas, hospitals, animal breeding facilities, slaughterhouses, processing plants, and agricultural industries can lead to

microbial contamination of groundwater and rivers used by fish farms as water sources (Some *et al*., 2021). The hands of employees during the processing and preparation of fish and contaminated ice with bacteria in the stages of storage and distribution may be the cause of secondary contamination of fish skin with *E. coli* (Sheng and Wang, 2021).

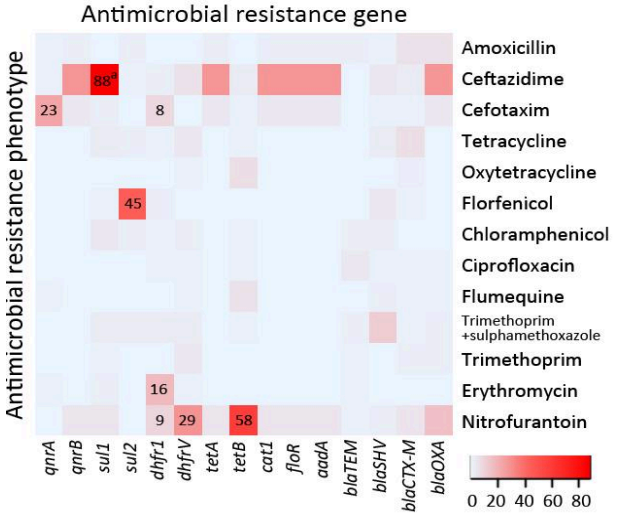

Fig. 3: Heat map of odds ratio between *AR* genes and phenotypes. [a] The isolates showing resistance against ceftazidime were 88 times more likely to the positive for *sul1* gene than the isolates which were susceptible to ceftazidime. The odds ratios with P-value <0.05 are marked with number

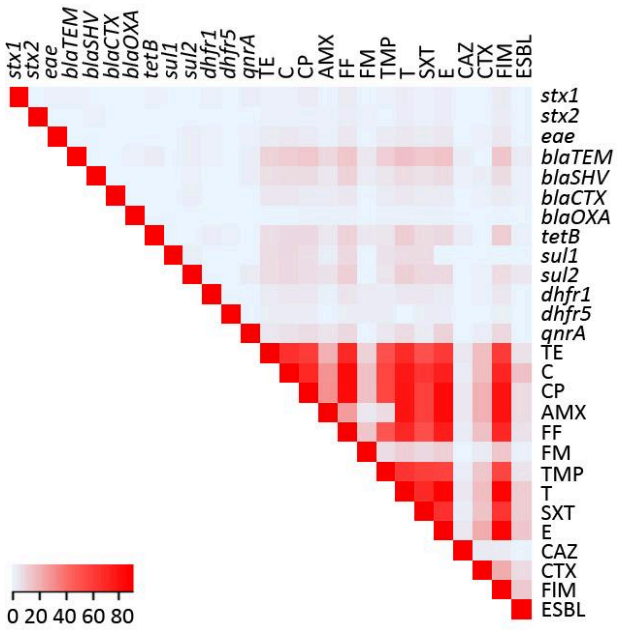

Fig. 4: Correlation matrix showing association between resistance and virulence variables; blue and red colors represent low to more positive correlation between two factors

In modern fish farming systems, the use of antibiotics is a common practice with the aim of treating and preventing infectious disease and increasing production. The indiscriminate use of antimicrobial drugs in fish farming has led to the emergence and spread of antibiotic-resistant microbial agents. The results of this study indicated that the prevalence of ESBL (extended-spectrum β-lactamase)-producing *E. coli* strains was less than one-tenth of the all isolates. This prevalence is lower compared with studies conducted in Cambodia (53.1%) (Nadimpalli *et al*., 2019) and India (92.6%) (Saharan *et al*., 2020). Among the β-lactam antibiotics, the highest prevalence was observed for AMX, CTX and CAZ, respectively. These findings align with the prevalence rates reported from Nigeria (Odumosu *et al*., 2021). The frequency of CAZ in some countries was much higher than the present study (Tran *et al*., 2018; Nadimpalli *et al*., 2019). The occurrence of phenotypic resistance in bacteria is due to the genes encoding resistance factors. In this study, the frequency of some important resistance genes was investigated. About β-lactam resistance genes, order of frequencies was bla_{TEM}>bla_{SHV}>bla_{CTX-M}>bla_{OXA}; this finding is not consistent with some previous studies (Tran *et al*., 2018; Prisca Aleru, 2022) reporting bla_{CTX-M} as the most frequent β-lactam resistant gene. Our results on the bla_{CTX-M} were close to the results obtained from pangasius fillets and shrimp in Danish retail imported from Asia (Ellis-Iversen *et al*., 2020).

The present study revealed that the phenotypic prevalence of tetracycline-resistant *E. coli* strains ranged from 59% to 82%. This prevalence is comparable to findings in different countries; for instance, the prevalence of tetracycline-resistant isolates in Ethiopia was reported as 55.6% (Yohans *et al*., 2022). Additionally, the prevalence of oxytetracycline-resistant strains in Nigeria was 100% (Odumosu *et al*., 2021). In this study, two tetracycline resistance genes, *tetA* and *tetB*, were examined that frequency of the *tetB* was higher than that of *tetA* but lower compared to other studies conducted in China (Liao *et al*., 2021) and India (Divya *et al*., 2020). In those studies, the frequencies of *tetB* ranged between 22% and 40%.

In the present study, a high prevalence of amphenicol-resistant *E. coli* strains was observed, specifically to amphenicols including FF and C. Interestingly, this prevalence was higher than the results reported from Tunisia (Hassen *et al*., 2020) and India (Saharan *et al*., 2020). Chloramphenicol is one of the widely used antibiotics in aquaculture for the treatment and prevention of infectious diseases (Schar *et al*., 2020). Despite the high prevalence of phenotypic resistance to amphenicols, it is interesting to note that the frequency of specific genes, such as *cat1* and *floR* was zero. This differs from findings reported in China (Liao *et al*., 2021), Denmark (Ellis-Iversen *et al*., 2020) and Pakistan (Shah *et al*., 2012) where various percentages were detected, ranging from less than 1% to more than 20%.

The prevalence of fluoroquinolone resistance, particularly against ciprofloxacin, was higher as compared with studies conducted in Ethiopia during 2020-2022 (Tilahun and Engdawork, 2020; Yohans *et al*., 2022). However, our findings align with the prevalence reported from Egypt (Al Qabili *et al*., 2022)

and Nigeria (Tran *et al*., 2018). *qnrA* was found to be one of the most common genes, in consistent with the findings reported from Brazil (14.7%) (Lima *et al*., 2022) and Algeria (9.1%) (Brahmi *et al*., 2018).

The prevalence of trimethoprim-sulfamethoxazole resistance phenotype was considerable which is higher than that reported from Turkey (Onmaz *et al*., 2020) and Algeria (Brahmi *et al*., 2018), where the prevalence exceeded 50%. In contrast, studies conducted in the Republic of Korea (Ryu *et al*., 2012) and Egypt (Ishida *et al*., 2010) reported lower prevalence rates, below 10%. About trimethoprim, the incidence of phenotypic resistance is similar to the findings in Nigeria (88.6%) (Odumosu *et al*., 2021) and India (76.5%) (Saharan *et al*., 2020). However, the present study found higher resistance rates compared with the findings in Ethiopia (Tilahun and Engdawork, 2020). Regarding the genes responsible for sulfonamide resistance, the frequencies of *sul1* and *sul2* were in consistent with studies in Pakistan (Shah *et al*., 2012), and India (Divya *et al*., 2020), where *sul1* and *sul2* frequencies ranged from 25% to 50%. About *sul2* gene, our frequency was lower than the results in China (Liao *et al*., 2021). Also, the frequency of trimethoprim resistance genes (*dhfr1* and *dhfrV*) was lower than the findings in Tanzania (Shah *et al*., 2012).

Resistance to macrolides (based on erythromycin) was high (exceeding 80%). Similar prevalence rates have been observed in Ethiopia (Yohans *et al*., 2022). The level of phenotypic resistance to nitrofurantoin was below 20%. This contrasts with the findings in Sri Lanka (Jagoda *et al*., 2014) which reported a higher frequency of nitrofurantoin resistance among various bacterial agents in fish.

β-lactams, tetracyclines, sulfonamides, amino-glycosides, amphenicols, and nitrofurantoin are commonly used in aquaculture with an average usage of 500 g per ton of produced salmon (Done *et al*., 2015; Schar *et al*., 2020). The use of antibiotics can cause the emergence of microorganisms with multiple resistance. In this study, approximately all of the isolates exhibited MDR and MAR index ≥0.2, which is consistent with the results reported from Algeria (Brahmi *et al*., 2018), showing a higher prevalence as compared with findings from Nigeria (Kusunur *et al*., 2022); The MAR index is commonly used to assess the level of antibiotic resistance in bacterial populations. An MAR index ≥0.2 indicates a high-risk source of contamination, often linked to environments where multiple antibiotics are used extensively. In our study, the MAR index values suggest that the bacterial isolates may have originated from such high-risk environments, which further highlights the need for strict antibiotic stewardship and monitoring of resistance patterns in these settings. The presence of untreated sewage water, industrial effluents, and medical waste discharged into water ecosystems plays a significant role in the emergence of MDR bacteria in aquatic animals (Addae-Nuku *et al*., 2022).

In this study, some pathotypes of *E. coli* were screened; PCR results indicated that less than one-tenth of the strains belonged to STEC (Shiga toxin-producing *E. coli*), EPEC (Enteropathogenic *E. coli*), and EHEC (Enterohemorrhagic *E. coli*) pathotypes. The prevalence rates of virulence genes had no significant differences. Further analysis of the *stx1* and *stx2* genes showed that *stx1* could be subtyped into *stx1a*, *stx1c*, and *stx1d*, while the *stx2* gene could be subtyped into *stx2c*, *stx2d*, *stx2e*, and *stx2f*. Interestingly, the isolates were found to possess multiple subtypes of *stx* genes simultaneously. The observed combinations of *stx* subtypes are almost rare and may contribute to increased pathogenicity of *E. coli*. Some studies suggest that the contamination of rivers with virulence gene-positive *E. coli* strains may be influenced by the presence of ruminants and their associated fecal matter in the vicinity (Tumacácori, 2019).

In this study, various phylo-groups of *E. coli* were identified including A, B2, D, E, and Clade I; phylo-group A had the highest frequency. No significant relationship was observed between virulence and resistance in the phylogenetic assessment of the isolates. In a study on *E. coli* isolates from water and fish, phylo-group A was the most prevalent, accounting for 69.8% and 81.2% in water and fish, respectively. B1 in the mentioned study was the next most common, comprising 23.6% (water) and 13.7% (fish), and the occurrence of B2 and D was rare (<5%) (Kouadio-N'gbesso *et al*., 2016). Phylo-grouping of *E. coli* strains may help to understand the evolutionary relationships among microorganisms. The prevalence of phylo-groups could be influenced by various factors such as nutrition, host species, sex, age, body mass, climate, geographic location, and the composition of the gut microflora.

In conclusion, the skin of retail rainbow trout may be one of the potential passive carriers of environmental MDR *E. coli* strains. The prevalence of *E. coli* strains on the fish skin was significantly higher than in gill and intestine. The frequency of antibiotic-resistant *E. coli* strains was observed with high rates for florfenicol, erythromycin, flumequine and oxytetracycline. Also, this study showed the occurrence of MDR strains on fish skin. The low prevalence of virulence factors in *E. coli* strains and identification of group A as the most prevalent phylo-group show the commensal nature of the isolates. Overall, this study emphasizes the need for proper hygiene practices, water quality management, and responsible antibiotic use in fish farming to reduce the dissemination of antibiotic-resistant bacteria and ensure food safety.

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Conflict of interest

The authors have no conflicts of interest to declare.

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