Full Paper Bioscience of Microbiota, Food and Health Vol. 38 (2), 41–48, 2019

Molecular characterization and antimicrobial resistance profile of fecal contaminants and spoilage bacteria that emerge in rainbow trout (*Oncorhynchus mykiss*) farms

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Received April 4, 2018; Accepted November 6, 2018; Published online in J-STAGE December 7, 2018

Fecal contaminants are a major public concern that directly affect human health in the fish production industry. In this study, we aimed to determine the fecal coliform, spoilage bacteria, and antimicrobial-resistant bacterial contamination in rainbow trout (*Oncorhynchus mykiss)* farms. Fish were sampled from rainbow trout farms that have a high production capacity and are established on spring water, stream water, and dammed lakes in six different regions of Turkey. A total of seven *Enterobacter* subspecies, two strains of *Pseudomonas* spp., and one isolate each of *Morganella* and *Stenotrophomonas* were characterized based on biochemical and molecular methods, including the 16S rRNA and *gyr*B housekeeping gene regions. The sequencing results obtained from the 16S rRNA and *gyr*B gene regions were deposited in the GenBank database and compared with isolates from different countries, which were registered in the database. Resistance to 10 different antimicrobial compounds was determined using the broth microdilution method, and molecular resistance genes against florfenicol, tetracycline, and sulfamethoxazole were identified by PCR. All detected resistance genes were confirmed by sequencing analyses. *E. cloacae*, *E. asburiae*, *Pseudomonas* spp., *S. maltophilia,* and *M. psychrotolerans* were identified using the *gyr*B housekeeping gene, while isolates showed different biochemical characteristics. All isolates were found to be phenotypically resistant to sulfamethoxazole, and some isolates were resistant to tetracycline, florfenicol, amoxicillin, and doxycycline; the resistance genes of these isolates included *flo*R, *tet*C, *tet*D, and *tet*E. We showed that fecal coliforms, spoilage bacteria, and antimicrobial resistant bacteria were present in farmed rainbow trout, and they pose a threat for human health and must be controlled in the farming stage of fish production.

Key words: fecal contaminants, spoiled bacteria, *Enterobacter cloacae*, molecular characterization, antimicrobial resistance genes

INTRODUCTION

Aquaculture fishery products are becoming increasingly important due to overfishing of wild fish stocks. Aquaculture production of aquatic organisms, including fish, crustaceans, and mollusks is the fastest growing food industry globally; while wild capture fisheries have become relatively stable in recent years, aquaculture production has increased by about 9% per year since 1985 [1]. Both capture fisheries and aquaculture have caused much public concern due to their impact on sustainability and their influence on the environment [2]. Water pollution is one of the most important global issues; in particular, water supplies are significantly decreasing because of global warming and the increasing human population. Beside these dangers, water supplies are polluted by discharging wastes and sewage into inland waters or seas [3]. Fish farms are generally established on streams or dammed lakes, regardless of whether the water supply is mixed with waste water, urban water. Aquaculture waters can be polluted when urban wastes mix with farm water, resulting in contamination with coliforms and other pollutants [2]. Both fresh and sea water fishes can harbor human/animal pathogenic bacteria, particularly those of the coliform group [4–6]. Fecal coliforms, such as *Enterobacter cloacae*, are not among the normal intestinal flora of fish and usually originate from the feces of warm-blooded animals; hence their presence in infected fish or aquaculture water supplies indicates environmental pollution [6, 7]. This pollution results in food or fish product spoilage [8, 9].

Human consumption of fish farmed in coliform-infected waters could lead to the transfer of these bacteria to consumers, especially if the fish is consumed undercooked. In addition to enteric contamination, spoilage flora like *Pseudomonas* spp., *Aeromonas* spp., and *Morganella psychrotolerans* contribute to the decomposition of fresh fish products (stored at low temperatures) [10]. These psychrotolerant isolates (*M. psychrotolerans*) produce histamine at 0 to 5°C and appear to be produce toxins in chilled seafood. And this bacteria have

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specifically shown evidence of causing histamine poisoning in humans via fish [11, 12].

S. maltophilia, formerly named *Xanthomonas maltophilia* and *Pseudomonas maltophilia*, is an important and widespread environmental opportunistic pathogen associated with nosocomial infection in humans [13]. *S. maltophilia* infection causes septicemia, meningitis, urinary tract and gastrointestinal infections, and several other conditions in humans [14, 15]. *S. maltophilia* was isolated from goats with lymphadenitis, sea bream with gill disease, and horses with lung disease; however, the pathogenesis of this bacterium is unclear [16–18].

Troast identified antibodies against *E. cloacae* and *Escherichia coli* in brown bullhead catfish (*Ameiurus nebulosus*) caught in coliform-contaminated waters and suggested that the fish was infected with these bacteria [4]. Additionally, the isolation of *E. agglomerans* from the kidneys of infected dolphinfish (*Coryphaena hippurus* Linnaeus) showed the pathogenicity of this enteric bacterium in fish [19]. Recently, *E. cloacae* and *Pseudomonas* spp. infections were reported in rainbow trout, mussels, and rainbow trout meat in the same geographical region as the one included in the present study [20–23]. Fecal coliforms and other spoilage flora could be transferred between terrestrial and aquatic ecosystems by rainbow trout, fish meat, mussels, and water; thus, humans could easily be infected by these bacteria [24–26].

In the present study, we determined whether fecal coliforms and other bacteria from aquacultured fish pose a health risk to humans. Whereas a number of research in this field has determined the food quality of fish meat or fish storage conditions, few studies have focused on the bacteria that infect fishes and the bacterial species that pose a risk to humans through aquaculture. We also checked for antimicrobial resistance and resistance genes in order to understand how coliform and spoilage pathogens could potentially be altered as a result of antimicrobial pollution in aquaculture.

MATERIAL AND METHODS

Sample collection and phenotypic identification

Fish were sampled from rainbow trout farms with high production capacities established using spring water, stream water, and dammed lake water in six different regions of Turkey (Aegean, Central Anatolia, Black Sea, Mediterranean Sea, Marmara, and Eastern Anatolia). About 100 fish were randomly collected from either subclinical, clinical, or moribund fishes monthly during each sampling period from 2013 to 2014. Fishes were grouped based on condition, region, cohort, weight, and month. Samples were taken from the kidneys, liver, spleen, and ascites fluid of fish with a sterile loop and swab and plated on tryptic soy agar (TSA), nutrient agar, and blood agar (BA; with 5% sheep blood). Sampling was carried out according to the guidelines for the diagnosis of fish diseases and considering international guidelines for animal welfare [27]. The biochemical characteristics of all isolates were determined using conventional microbial tests such as colony morphology, color upon Gram staining, oxidase and catalase activities, oxidative fermentation (O/F), growth on different media, and different temperature conditions [28, 29]. Detailed biochemical characteristics of isolates were determined by using API ID 20NE (bioMérieux, Marcy l'Etoile, France) according to the user's manual. All isolates were cultured on TSA (Sigma-Aldrich St. Louis, MO, USA) at 22°C for 24–48 hr, and pure cultures were supplemented with 20% glycerol and kept at −80°C.

Molecular identification

DNA was extracted from the colon with a spin purification kit according to the manufacturer's instructions (51306, Qiagen, Venlo, Netherlands) The amount and purity of DNA in each sample were measured at wavelengths of 260 nm and 260/280 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA).

Identification was performed for the 16S rRNA gene region using the universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′) [30]; further identifications were performed using the housekeeping gene with *gyr*B 3F 334–354 (5′-TCC GGC GGT CTG CAC GGC GT-3′) and 14R 1464–1444 (5′-TTG TCC GGG TTG TAC TCG TC-3′) [31]. Polymerase chain reaction (PCR) was performed in a total volume of 25 µl containing 2.5 µl 10x PCR Buffer, 5 µl 5x Q-Solution, 0.5 µl 0.2 mM dNTP mix (10 mM of each), 25 pmol of each gyrA primer, 37.5 pmol of each gyrB primer, 1 µl 25 mM MgCl2, 0.125 µl HotStarTaq DNA Polymerase (Qiagen, Venlo, Netherlands), 10 ng DNA template, and DNase/RNase-Free Distilled Water. The PCR reactions were conducted according to previously used methods [30, 31]. All PCR products were confirmed by Sanger sequence analyses with double-stranded DNA by Macrogen Korea (Republic of Korea). After all bacteria species were named based on the highest sequence similarity (98–100%) in the GenBank database and checked with reference sequences deposited in GenBank, the taxonomic names were updated based on the LPSN database (http://www.bacterio.net/aeromonas.html).

Molecular characterization

Each bacterial isolate was sequenced with *gyr*B primers, and the sequences were used to create a phylogenetic tree with Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA7) [32]. The *gyr*B sequences were deposited in GenBank (the accession numbers are provided in Fig. 1).

Evolutionary history was inferred using the neighbor-joining method [33]. The optimal tree with the sum of branch lengths = 17.16288443 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown above the branches [34]. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method

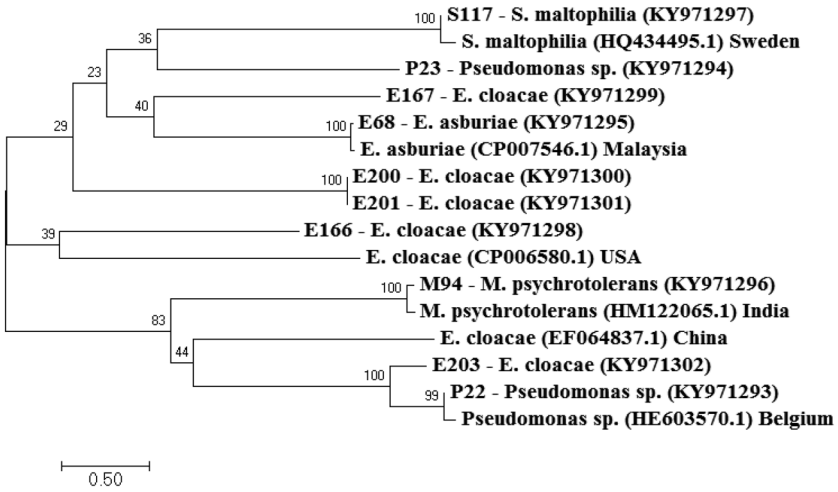

Fig. 1. Comparison of isolates with the GenBank database, with accession numbers given in brackets.

[35] and expressed as the number of base substitutions per site. The analysis involved 16 nucleotide sequences. The codon positions included were 1st, 2nd, 3rd, and noncoding positions. All positions containing gaps and missing data were eliminated. There were a total of 723 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 [32].

Antimicrobial susceptibility test

The minimum inhibitory concentrations (MICs) were determined using broth dilution methods according to CLSI (2017) for florfenicol (FFC), tetracycline (TET), sulfamethoxazole (SUL), sulfamethoxazole-trimethoprim (5:1 SXT), oxolinic acid (OXO), enrofloxacin (ENR), doxycycline (DOX), gentamicin (GEN), erythromycin (E), amoxicillin (AMX), and ciprofloxacin (CIP), which were all obtained from Sigma-Aldrich (St. Louis, MO, USA; product numbers F1427, 31741, S7507, T7883 [trimethoprim], 67126, 17849, 33429, G1914, E5389, A8523, and 17850, respectively), with dilutions of 0.008 to 256 µg/ml of E. coli ATCC 25922 used as the quality control (QC) strains.

After incubation at 22°C for 24–48 hr, the plates were measured at a wavelength of 595 nm using a microplate reader (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA), and MICs were defined as the lowest concentration of antibiotic that inhibited visible growth (turbidity) [36].

Polymerase chain reaction amplification and antimicrobial resistance gene sequencing

To determine the presence of antimicrobial resistance genes, we analyzed the *flo*R gene for FFC resistance; the *tet*A, *tet*B, *tet*C, *tet*D, and *tet*E genes for tetracycline resistance; and *sul*1, *sul*2, and *sul*3 for SUL resistance using specific primers and polymerase chain reaction (PCR) conditions modified according to primer melting temperatures and kit protocols (HotStarTaq DNA Polymerase; Qiagen, Venlo, Netherlands) [37–40]. PCR analysis was performed using the positive control genes used in our laboratory. The amplification products were screened with an ultraviolet (UV) transilluminator after agarose gel electrophoresis (1–2%) with ethidium bromide, which was run at 100 V for 100 min. After PCR analysis of antimicrobial resistance genes, the sequences were deposited in GenBank.

RESULTS

Phenotypic identification

A total of seven *Enterobacter* subspecies, two strains of *Pseudomonas* spp., and one isolate each of *Morganella* and *Stenotrophomonas* were isolated; information on the isolates is provided in Table 1. Isolates were detected from the livers and spleens of rainbow trout weighing 0.8 to 200 g. Some bacterial species were recovered from more than two fishes within the same group, although E166, E167, E200, and E203 were recovered from only one fish. Thus, bacteria species isolated in the same year were sometimes collected in different sampling periods. While only P23 (*Pseudomonas* sp.) was isolated from diseased rainbow trout, other bacteria species were isolated from seemingly healthy (possible carrier) or moribund fish. Sampling was performed for about 75% of moribund fish and 25% of healthy (possibly carrier) fish in a fish population. The M94, S117, and P22 were isolated from only moribund fishes which had no disease lesions. All strains were Gram negative, catalase positive, O/F fermentative, and Vibriostat (O/129) resistant. *Pseudomonas* spp. and *Stenotrophomonas* isolates are oxidase positive, while *Morganella* and other *Enterobacter* species are oxidase negative; however; *E. cloacae* (E200, E201, and E203) are weakly positive for oxidase. All isolates were positive for nitrate reductase for fermentation of glucose, 4-nitrophenyl-β D-galactopyranoside, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, potassium

Table 1. Isolate information and identification results

Bacterial code	Fish weight	Isolation organ	Region and date	Sequence result (similarity with *gyr*B)	Sequence result (similarity with 16S rRNA)
E201	8 g	Spleen	Central Anatolia (June 2013)	*E. cloacae* (99%) (KY971301)	*E. cloacae* (99%) (KY983967)
E166	10 g	Spleen	Aegean (August 2013)	*E. cloacae* (99%) (KY971298)	-
E167	100 g	Liver	Aegean (September 2013)	*E. cloacae* (99%) (KY971299)	Uncultured bacterium (99%) (KY983965)
M94	200 g	Kidney	Central Anatolia (September 2013)	*M. psychrotolerans* (95%) (KY971296)	*M. psychrotolerans* (95%) (KY971298)
E191	100 g	Liver	Aegean (January 2013)	*E. cloacae* (99%) (KY971297)	-
E200	100 g	Liver	Aegean (March 2013)	*E. cloacae* (99%) (KY971300)	*E. cloacae* complex (99%) (KY983963)
E203	100 g	Liver	Aegean (May 2013)	*E. cloacae* (99%) (KY971302)	*E. cloacae* complex (99%) (KY983964)
E68	0.2 g	Spleen	Aegean (October 2014)	*E. asburiae* (97%) (KY97295)	*E. asburiae* (100%) (KY983971)
S117	200 g	Spleen	Aegean (May 2014)	*S. maltophilia* (92%) (KY971297)	Uncululred *Enterobacter* clone (99%) (KY983970)
P22	0.8 g	Liver	Aegean (November 2014)	*Pseudomonas* sp. (98%) (KY971293)	-
P23	250 g	External lesion	Aegean (November 2014)	*Pseudomonas* sp. (98%) (KY971294)	*Pseudomonas* sp. (99%) (KY983969)

Accession numbers are given in parenthesis. Negative isolates gave negative results in PCR.

gluconate, malic acid, citric acid (CIT), and trisodium citrate but tested negative for urea and adipic acid. Contrastingly, *E. cloacae* isolates showed different biochemical characteristics (Table 2).

Molecular identification and characterization

Biochemically grouped isolates were differentiated into three large groups, namely, *Enterobacter*, *Morganella,* and *Pseudomonas/Aeromonas/Stenotrophomonas*. These isolates were not differentiated based on biochemical tests alone, because isolates showed weakly positive results for some tests or differences in biochemical characteristics. Therefore, the isolates were identified using PCR and sequencing analyses, with two different gene regions. Some isolates had negative or poor sequencing profiles (poor quality) in the 16S rRNA analyses. However, six isolates identified as *E. cloacae* (at least 99% similarity), two were identified as *Pseudomonas* spp. (at least 98% similarity), one was identified as *S. maltophilia* (92% similarity), one was identified as *M. psychrotolerans* (95% similarity), and one was identified as *E. asburiae* (97% similarity) in the *gyr*B housekeeping gene analysis. While E167 and S117 were identified as uncultured bacteria, with high 16S rRNA similarities in the GenBank database, the bacteria were identified as *E. cloacae* and *S. maltophilia* at the species level using the *gyr*B gene region. However, S117 showed low similarity (92%) with the *gyr*B gene region; this isolate should be examined in detail using other genotyping methods (such as MLST). *E. asburiae* was 100% identic in the 16S rRNA gene region, but the isolate was 97% similar based on the *gyr*B gene region. The relevant accession numbers were deposited in the GenBank database (KY971293–KY971302). A dendrogram of the PCR product was generated using the MEGA7 software and *gyr*B primer pairs; this gave three main genogroups with different levels of similarity (Fig. 1). The sequences were compared with isolates in the GenBank database from Europe, Asia, and America. Although S117 had low similarity in the 16S rRNA region, the isolate showed 100% similarity with the *S. maltophilia* (HQ434495.1) strain in the GenBank database. P23 was 98% similar to the *gyr*B gene, though it also had distant genogroup similarities with other strains of *Pseudomonas* spp. E203 had high similarity in the *gyr*B gene region but showed close genogroup similarities with *Pseudomonas* spp. and *E. cloacae* (EF064837.1) even though it was distant to other *E. cloacae* isolates. These isolates, which were close to other bacterial species, should be further studied in detail using genetic methods to determine if they are new species or if some isolates were mistakenly recorded in the database.

Minimum inhibitory concentration, resistance genes, and sequencing

The MICs of isolates are provided in Table 3. All isolates were resistant to SUL; E201 and P23 were resistant to FFC and AMX; E167 was resistant to FFC, AMX, and DOX; M94 was resistant to AMX and DOX; S117 and E68 were resistant to DOX; E191 was resistant to AMX; and P22, E200, and E203 were resistant to FFC. All isolates were susceptible to TET, ENR, CIP, GEN, SXT, and OXO. QC control values of standard strains are as suggested by the CLSI.

The *sul*I, *sul*II, *sul*III, *tet*A, and *tet*B resistance genes were not detected in the all isolates, whereas *E. cloacae* (E166) carried two resistance genes (*flo*R and *tet*D), *E. cloacae* (E167) carried two resistance genes (*tet*D and *tet*E), and *Pseudomonas* spp. (P22) carried the *tet*C resistance gene (Table 3). While E201, E167, E200, E203, P22, and P23 isolates were phenotypically FFC resistant, none of them carried the *flo*R resistance gene. More interestingly, only phenotypically susceptible isolates (E166) carried the *flo*R resistance gene. All isolates were phenotypically susceptible to TET, but E166, E167, and P22 carried the *tet*C, *tet*D, and

Table 2. Biochemical characteristics of isolates

Biochemical characteristic	S117 *S. maltophilia*	P23 *P. marginalis*	M94 *M. psychrotolerans*	E68 *E. asburiae*	E191 *E. cloacae*	E200 *E. cloacae*	E201 *E. cloacae*	E203 *E. cloacae*
Gram Color	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+
OX	+	+	-	-	-	-*	-*	-*
CAT	+	+	+	+	+	+	+	+
O/F	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
O/129	R	R	R	R	R	R	R	R
NO3	+	+	+	+	+	+	+	+
TRP	-	+	-	-	-	-	+	-
GLU	+	+	+	+	+	+	+	+
ADH	-	+	-	+	-	-	+	-
URE	-	-	-	-	-	-	-	-
ESC	-	+	-	+	-	+	-	+
GEL	-	+	-	-	+	-	+	-
PNG	+	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+
ARA	-	+	+	+	-	+	-	+
MNE	+	+	+	+	+	+	+	+
MAN	+	+	+	+	+	+	+	+
NAG	+	+	+	+	+	+	+	+
MAL	+	+	+	+	+	+	+	+
GNT	+	+	+	+	+	+	+	+
CAP	-	+	-	-	-	-	+	-
ADI	-	-	-	-	-	-	-	-
MLT	+	+	+	+	+	+	+	+
CIT	+	+	-	+	+	+	+	+
PAC	-	-	+	+	-	+	-	+

OX: oxidase (*weakly positive); CAT: catalase; O/F: oxidation/fermentation; NO3: potassium nitrate; TRP: L-tryptophan; GLU: D-glucose; ADH: L-arginine; ESC: esculin; GEL: gelatin; PNG: 4-nitrofenil-β D-galaktopiranosid; ARA: L-arabinose; MNE: D-mannose; MAN: D-mannitol; NAG: N-acetil-glucosamine; MAL: D-maltose; GNT: potassium gluconate; CAP: capric acid; ADI: adipic acid; MLT: malic acid; CIT: trisodyum citrate; PAC: fenilasetic acid. Grey cells indicate interspecies differences.

Table 3. Isolate antimicrobial resistance genes*

Bacterial code	FFC MIC	*flo*R	SUL MIC	*sul*I	*sul*II	*sul*III	TET MIC	*tet*A	*tet*B	*tet*C	*tet*D	*tet*E	ENR MIC	CIP MIC	GEN MIC	AMX MIC	DOX MIC	SXT MIC	OXO MIC	E MIC
E201	64	-	256<	-	-	-	2	-	-	-	-	-	0.032	0.008	0.016	16	0.128	0.032	0.064	0.512
E166	1	+	256<	-	-	-	0.256	-	-	-	+	-	0.016	0.008	0.016	0.256	0.032	0.256	0.128	0.512
E167	16	-	256<	-	-	-	0.512	-	-	-	+	+	0.008	0.008	0.064	16	128	0.256	0.128	16
M94	0.512	-	256<	-	-	-	0.256	-	-	-	-	-	0.008	0.008	0.064	16	32	0.256	0.008	256
S117	1	-	256<	-	-	-	0.128	-	-	-	-	-	0.008	0.008	0.064	0.256	32	0.128	0.032	0.512
E68	0.032	-	256<	-	-	-	0.512	-	-	-	-	-	0.064	0.008	0.128	0.512	16	0.128	0.032	0.256
P22	16	-	256<	-	-	-	0.512	-	-	+	-	-	0.032	0.008	0.032	1	0.512	0.512	1	1
P23	64	-	256<	-	-	-	0.512	-	-	-	-	-	0.032	0.016	0.008	16	0.512	0.512	0.256	0.256
E191	4	-	256<	-	-	-	1	-	-	-	-	-	0.008	0.008	0.064	16	1	0.512	0.032	0.512
E200	16	-	256<	-	-	-	4	-	-	-	-	-	0.064	0.008	0.016	0.256	0.032	0.256	0.128	0.512
E203	32	-	256<	-	-	-	4	-	-	-	-	-	0.064	0.008	0.016	4	0.064	0.128	0.512	0.256
E. coli ATCC 25922	2	-	256	-	-	-	0.128	-	-	-	-	-	0.008	0.008	0.064	0.128	0.128	0.064	0.008	0.128

*Grey cells indicate resistant values. FFC: florfenicol; SUL: sulfamethoxsazole; TET: tetracycline; ENR: enrofloxacin; CIP: ciprofloxacin; GEN: gentamycin; AMX: amoxicillin; DOX: doxycycline; SXT: sulfamethoxazole-trimethoprim (1/5); OXO: oxolinic acid; E: erythromycin.

*tet*E resistance genes respectively. In addition, no resistance genes were detected in all SUL resistance isolates.

All resistance genes were confirmed by sequence analysis. The sequences were deposited in GenBank with accession numbers KY971303–KY971306. The *flo*R-*tet*D and *tet*D-*tet*E resistance genes were detected together in *E. cloacae* (E166 and E167; multiple antimicrobial resistances). In addition, none of the resistance genes were detected in the reference strain; these isolates showed only phenotypic SUL resistance.

DISCUSSION

In the present study, *E. cloacae, E. asburiae*, *Pseudomonas* spp., *M. psychrotolerans*, and *S. maltophilia* were isolated from the internal organs of rainbow trout and identified through biochemical and molecular methods, including 16S rRNA and *gyr*B gene region sequencing; we found fecal coliform contamination and spoilage flora in rainbow trout at the farming stage. Additionally, the phenotypic and genotypic antimicrobial resistance of contaminants and spoilage bacteria were examined, and the resistance genes were similar to those found in pathogens of warm-blooded animals and humans.

In this study, we showed that there are fecal contamination and spoilage bacteria in farmed rainbow trout; the findings suggest that sewage or warm-blooded animal wastes reached the aquaculture water supply. Our suspicions were supported by data on the prevalence of local diseases indicating that *E. cloacae* in nosocomial infections occurred at the same time and in the same regions [24, 26, 41, 46, 47]. The sources of these nosocomial infections, which were caused by enterobacterial agents, were identified in some fish meat, bivalves, and other aquaculture products during the same period in regions where fish were aquacultured [42–45, 48–50]. Additionally, it has been reported by Duman *et al.* [40] that *Yersinia ruckeri* was observed frequently in samples of these fish in the same sampling period. Thus this study showed that there was contamination in aquaculture products and that coliform contaminants and spoilage bacteria were likely transferred from aquacultured fish to humans. During our project sampling period, we observed (in the field study) the following: (I) Aquaculture pond water was used to fill fish shocking tanks without sterilization. (II) Contaminated fish tank water containing fish blood was then discharged into a pool. (III) Careless personnel threw dead fish into a dammed lake; spoiled bacteria could easily grow on dead fish in the aquaculture pools, and herons could potentially eat these fish and defecate into pools again. (IV) Warm-blooded animals walked in the pools (cats and dogs). (V) At some farms, other terrestrial animals (e.g., cows) used the same water and defecated in the water. All these things suggested how contaminant bacteria and antimicrobial-resistant bacteria transfer from animals to fish and then to humans. The samples were collected in the Aegean region in which farm constructed on water source that used both ten different rainbow trout farms (constructed on the same water supply was published on the Ministry of Food, Agriculture and Livestock website). Also Herons and other warm-blooded animals were observed more in the Aegean region than in the other regions during the sampling period.

Isolation was performed continuously during the sampling period, and rainbow trout were sampled over the course of two years. The Ministry of Food, Agriculture and Livestock was notified when we isolated these bacteria. If contaminant bacteria are isolated repeatedly, the Ministry of Food, Agriculture and Livestock in orders to involved farms to close until appropriate precautionary measures are taken. We started sampling again after precautionary measures were taken and were not able to isolate the species thereafter.

Bacterial pathogens are not identified by using merely biochemical methods, especially in the case of large families like *Enterobacteriaceae*. Similarly, some isolates in this study could mistakenly have been assigned to closed genetic groups with only biochemical characterization [31]. Therefore, molecular tools such as housekeeping genes need to be used to identify the most important bacterial agents [31]. In the present study, some isolates belonged to complex genogroups and were identified as uncultured bacteria by using the 16S rRNA gene region; however, this identification is not sufficient, therefore the *gyr*B gene region was also used in this study. A dendrogram was constructed to identify the *gyr*B sequences with similar, comparable isolates in the GenBank database; however, some isolates had close relationships with other bacteria despite being highly identic. Both highly identic bacteria showing closeness to other groups in the dendrogram and poorly identified isolates should be identified in the future by using molecular methods (e.g., MLST) for the accurate identification of fish-disease agents, and determination of contaminants in human food sources. Easily transmitted fecal, spoilage, and opportunistic bacteria have not been commonly reported as disease agents in aquaculture [51]. Thus farmers do not use antimicrobials directly to treat these agents. We determined that some resistance genes could be found in mobile genetic elements (such as plasmids and integrons) and they might contribute to spread of antimicrobial resistant bacterial pollution in aquaculture. When resistance genes were compared with other records of isolated bacteria from humans and other animals, we found that

- the *flo*R gene showed high similarity (at least 99%) to other *flo*R resistance genes deposited in the GenBank database, such as bacterial genomes isolated in human sputum (CP020704.1), the domestic cow *Bos taurus* (AP017617.1), human stools (CP015598.1), eel pond water (CP019441.1), chicken meat (KY019259.1), human patients (KY019258.1), and the lungs of sick pigs (KX966395.1);
- the *tet*C gene showed high similarity (at least 99%) to other *tet*C resistance genes deposited in the GenBank database, such as bacterial genomes isolated in human and bovine bacterial isolates (CP019898.1), hospital effluents (CP013115.1), seawater (CP017782.1), healthy adults (NG_048181.1), and ventilator-associated pneumonia patients (CP012582.1);

» the *tet*E gene showed high similarity (at least 99%) to other *tet*E resistance genes deposited in the GenBank database, such as bacterial genomes isolated in perirectal surveillance culture (CP014774.1), compost piles (CP007518.2), laboratory worker fecal flora (NG_048186.1), aquaculture sources (EF471995.1), and sewage from environmental samples (KU544465.1).

The results of this study also revealed that there was a low linkage between the results of the phenotypic and genetic antimicrobial susceptibility tests. This reason for this may be that antimicrobial resistance is encoded by multiple genes or that the genes, although present in the bacteria genotype, were not expressed, as was shown by the antimicrobial resistance analysis.

Misuse of antimicrobial agents causes genotypic antimicrobial resistance in the environment and opportunistic pathogens that disseminate these genetic elements to terrestrial ecosystems and humans. Thus, we found that some fecal contaminants and spoilage bacteria carried the *flo*R, *tet*C, *tet*D, and *tet*E resistance genes, although most of them were phenotypically susceptible to florfenicol and tetracycline.

In conclusion, for the benefit of human health, water and live, farmed fish must not be contaminated with fecal coliform and spoilage bacteria, especially in the initial steps of fish production. Sewage and aquaculture output waters should be sterilized to prevent transfer of bacteria and resistance genes between ecosystems. Finally, all fish products should be controlled in terms of fecal coliforms, spoilage flora, and antimicrobial resistance genes by following the "One Health approach"1 from farm to table. Follow-up studies should be conducted to investigate transmission of other contaminants, spoilage bacteria, and resistance genes to humans or terrestrial animals via aquaculture.

Conflict of interest

We have no conflicts of interest to declare.

ACKNOWLEDGEMENTS

This research was supported by the Scientific Researcher Department through the KUAP(V)-2014/7 project and by the General Directorate of Agricultural Research and Policies via the TAGEM/14/AR-GE/26 project. This research was approved by the Local Ethics Commission (report 2012-14-04). We thank Prof. Dr. Stefan Schwarz for his assistance with the antimicrobial resistance genes.

REFERENCES

1. Food and Agriculture Organization of the United Nations (FAO). 2005 Yearbook of Fishery Statistics. FAO, Rome.
2. Goldburg R, Triplett T. 1997. Murky Waters: Environmental effects of aquaculture in the United States. Environmental Defense Fund, Washington (DC).
3. Glassmeyer S, Furlong E, Kolpin D, Cahill J, Zaugg S, Werner S, Meyer M, Kryak D. 2005. Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. USGS Staff–Published Research, p. 66.
4. Troast JL. 1975. Antibodies against enteric bacteria in brown bullhead catfish (*Ictalurus nebulosus*, LeSueur) inhabiting contaminated waters. Appl Microbiol 30: 189–192. [Medline]
5. Sarter S, Nguyen HNK, Hung LT, Lazard J, Montet D. 2007. Antibiotic resistance in Gram-negative bacteria isolated from farmed catfish. Food Control 18: 1391–1396. [CrossRef]
6. Sekar VT, Santiago TC, Vijayan KK, Alavandi SV, Raj VS, Rajan JJS, Sanjuktha M, Kalaimani N. 2008. Involvement of *Enterobacter cloacae* in the mortality of the fish, *Mugil cephalus*. Lett Appl Microbiol 46: 667–672. [Medline] [CrossRef]
7. Geldreich EE, Clarke NA. 1966. Bacterial pollution indicators in the intestinal tract of freshwater fish. Appl Microbiol 14: 429–437. [Medline]
8. Kaneko S. 1971. Microbiological study of fresh fish. New Food Industry 13: 76–80.
9. Mandal SC, Hasan M, Rahman MS, Manik MH, Mahmud ZH, Islam MS. 2009. Coliform bacteria in Nile Tilapia, *Oreochromis niloticus* of shrimp-Gher, pond and fish market. World J Fish Marine Sci 1: 160–166.
10. Gram L, Huss HH. 1996. Microbiological spoilage of fish and fish products. Int J Food Microbiol 33: 121–137. [Medline] [CrossRef]
11. Dalgaard P, Madsen HL, Samieian N, Emborg J. 2006. Biogenic amine formation and microbial spoilage in chilled garfish (*Belone belone belone*)—effect of modified atmosphere packaging and previous frozen storage. J Appl Microbiol 101: 80–95. [Medline] [CrossRef]
12. Emborg J, Dalgaard P, Ahrens P. 2006. *Morganella psychrotolerans* sp. nov., a histamine-producing bacterium isolated from various seafoods. Int J Syst Evol Microbiol 56: 2473–2479. [Medline] [CrossRef]
13. Swings J, De Vos P, den Mooter MV, De Ley J. 1983. Transfer of *Pseudomonas maltophilia* Hugh 1981 to the Genus *Xanthomonas* as *Xanthomonas maltophilia* (Hugh 1981) comb. nov. Int J Syst Evol Microbiol 33: 409–413.
14. Denton M, Kerr KG. 1998. Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clin Microbiol Rev 11: 57–80. [Medline] [CrossRef]
15. Looney WJ, Narita M, Mühlemann K. 2009. *Stenotrophomonas maltophilia*: an emerging opportunist human pathogen. Lancet Infect Dis 9: 312–323. [Medline] [CrossRef]
16. Johnson EH, Al-Busaidy R, Hameed MS. 2003. An outbreak of lymphadenitis associated with *Stenotrophomonas* (*Xanthomonas*) *maltophilia* in Omani goats. J Vet Med B Infect Dis Vet Public Health 50: 102–104. [Medline] [CrossRef]
17. Kapetanovic D, Kurtovic B, Vardic I, Teskeredtzic E, Teskeredzic Z. 2006. Gill disease in a gilthead sea bream (*Sparus aurata* L.). Med Welt 62: 1239–1241.
18. Albini S, Abril C, Franchini M, Hüssy D, Filioussis G. 2009. *Stenotrophomonas maltophilia* isolated from the airways of animals with chronic respiratory disease. Schweiz Arch Tierheilkd 151: 323–328. [Medline] [CrossRef]
19. Hansen GN, Raa J, Olafsen JA. 1990. Isolation of *Enterobacter aglomerans* from dolphin fish, *Coryphaena hippurus* L. J Fish Dis 13: 93–96. [CrossRef]
20. Akar S. 2009. İzmir İç Körfezi'nde, kıyı sularında ve kara midyelerde (*Mytilus galloprovincialis* Lamarck, 1819) fekal koliform bakterilerin izlenmesi (Doctoral dissertation, DEÜ Fen Bilimleri Enstitüsü).
21. Kayis S, Capkin E, Altinok I. 2009. *Bacteria in rainbow trout* (Oncorhynchus mykiss) *in the southern Black Sea Region of Turkey-A survey*. Israeli J Aquac – Bamigdeh 61: 339–344.
22. Öztürk RC, Altınok I. 2014. Bacterial and viral fish diseases in Turkey. Turk J Fish Aquat Sci 14: 275–297.
23. Yildirim S, Ozer S. 2010. Mersin İli Çağlarca Köyündeki Gökkuşağı Alabaliği (*Oncorhynchus mykiss*, Walbaum, 1792) Kuluçkahanelerinde *Flavobacterium* spp. Varliği. J Fisheries Sci 4: 112–122.
24. Cirit OS, Muderris T, Mizrakli AU, Vurupalmaz Y, Baris A. 2014. Yara kültürlerinden izole edilen aerop bakteriler ve antibiyotik duyarlılıkları. Turk Mikrobiyol Cem Derg 44: 149–157.
25. Saticioglu IB, Duman M, Altun S. 2018. Antimicrobial resistance and molecular characterization of *Pantoea agglomerans* isolated from rainbow trout (*Oncorhynchus mykiss*) fry. Microb Pathog 119: 131–136. [Medline] [CrossRef]
26. Şimşek-Yavuz S, Şensoy A, Kaşıkçıoğlu H, Çeken S, Deniz D, Yavuz A, Koçak F, Midilli K, Eren M, Yekeler İ. 2015. Infective endocarditis in Turkey: aetiology, clinical features, and analysis of risk factors for mortality in 325 cases. Int J Infect Dis 30: 106–114. [Medline] [CrossRef]
27. OIE, Fish Diseases Commission. 2000. Diagnostic Manual for Aquatic Animal Diseases.
28. Brenner DJ, Farmer JJ. 1984. *Enterobacteriaceae*. Bergey's Manual of Systematics of Archaea and Bacteria.
29. Austin B, Austin DA. 2007. Bacterial Fish Pathogens, Diseases of Farmed and Wild Fish, 4th ed. Springer-Praxis, Godalming.

30. Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D, Wade WG. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64: 795–799. [Medline]
31. Yáñez MA, Catalán V, Apráiz D, Figueras MJ, Martínez-Murcia AJ. 2003. Phylogenetic analysis of members of the genus *Aeromonas* based on *gyrB* gene sequences. Int J Syst Evol Microbiol 53: 875–883. [Medline] [CrossRef]
32. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870–1874. [Medline] [CrossRef]
33. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425. [Medline]
34. Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791. [Medline] [CrossRef]
35. Tamura K, Nei M, Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101: 11030–11035. [Medline] [CrossRef]
36. Clinical and Laboratory Standards Institute (CLSI) guidelines, Performance standards for antimicrobial susceptibility testing, 2017.
37. Schmidt AS, Bruun MS, Dalsgaard I, Larsen JL. 2001. Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes among motile aeromonads from a fish farming environment. Appl Environ Microbiol 67: 5675–5682. [Medline] [CrossRef]
38. Van TTH, Chin J, Chapman T, Tran LT, Coloe PJ. 2008. Safety of raw meat and shellfish in Vietnam: an analysis of *Escherichia coli* isolations for antibiotic resistance and virulence genes. Int J Food Microbiol 124: 217–223. [Medline] [CrossRef]
39. Wang N, Yang X, Jiao S, Zhang J, Ye B, Gao S. 2014. Sulfonamide-resistant bacteria and their resistance genes in soils fertilized with manures from Jiangsu Province, Southeastern China. PLoS One 9: e112626. [Medline] [CrossRef]
40. Duman M, Altun S, Cengiz M, Saticioglu IB, Buyukekiz AG, Sahinturk P. 2017. Genotyping and antimicrobial resistance genes of Yersinia ruckeri isolates from rainbow trout farms. Dis Aquatic Organ 125: 31–44. [Medline] [CrossRef]
41. WHO (World Health Organization). 2018. Guidelines for Drinking-water Quality, Incorporating 1st and 2nd Addenda, Volume 1, Recommendations, 3rd ed. WHO, Geneva.
42. Yang FC, Yan JJ, Hung KH, Wu JJ. 2012. Characterization of ertapenem-resistant *Enterobacter cloacae* in a Taiwanese university hospital. J Clin Microbiol 50: 223–226. [Medline] [CrossRef]
43. Falagas ME, Kastoris AC, Vouloumanou EK, Rafailidis PI, Kapaskelis AM, Dimopoulos G. 2009. Attributable mortality of *Stenotrophomonas maltophilia* infections: a systematic review of the literature. Future Microbiol 4: 1103–1109. [Medline] [CrossRef]
44. Mezzatesta ML, Gona F, Stefani S. 2012. *Enterobacter cloacae* complex: clinical impact and emerging antibiotic resistance. Future Microbiol 7: 887–902. [Medline] [CrossRef]
45. Altun S, Duman M, Buyukekiz AG, Ozyigit MO, Karatas S, Turgay E. 2013. Isolation of *Citrobacter braakii* from rainbow trout (*Oncorhynchus mykiss*). Isr J Aquacult Bamidgeh 65: 915–922.
46. Capkin E, Terzi E, Altinok I. 2015. Occurrence of antibiotic resistance genes in culturable bacteria isolated from Turkish trout farms and their local aquatic environment. Dis Aquat Organ 114: 127–137. [Medline] [CrossRef]
47. Tosun M, Ozdes EK, Yanik K, Kokcu A, Ozhan E. 2016. Which antibiotoc is better to select empirically for lower urinary tract infections in pregnant women? Int J Clin Exp Med 9: 12039–12045.
48. Sipahi N, Mutlu C, Akkan T. 2013. Giresun İlinde Tüketime Sunulan Bazı Balıklardan İzole Edilen *Enterobacteriaceae* Üyelerinin Antibiyotik ve Ağır Metal Dirençlilik Düzeyleri. Gıda Dergisi 38: 343–349.
49. Matyar F. 2016. Isolation, Identification and Antibacterial Agents Resistance Among *Enterobacteriaceae* Spp. In Fish of The Eastern Mediterranean. In The 2016 WEI International Academic Conference Proceedings, Barcelona, pp. 18–21.
50. Seker D, Ocak F. 2016. Isolation of the *Pseudomonas* spp. to cause of microbial spoilage of fishes. J Appl Biol Sci 10: 22–25.
51. Al-Bahry SN, Mahmoud IY, Al-Belushi KIA, Elshafie AE, Al-Harthy A, Bakheit CK. 2009. Coastal sewage discharge and its impact on fish with reference to antibiotic resistant enteric bacteria and enteric pathogens as bio-indicators of pollution. Chemosphere 77: 1534–1539. [Medline] [CrossRef]