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

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**Running head:** Fecal contaminants in farmed rainbow trout

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ABSTRACT

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 *gyrB* housekeeping gene regions. The sequencing results obtained from the 16S rRNA and *gyrB* 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 *gyrB* 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 *floR*, *tetC*, *tetD*, and *tetE*.

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.
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 produce toxins in chilled seafood. And this bacteria
have 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 (1975) 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'-GG G CGG WGT GTA CAA GGC-3') [30]; further identifications were performed using the housekeeping gene with gyrB 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 gyrB primers, and the sequences were used to create a phylogenetic tree with Molecular Evolutionary Genetics Analysis Version 7.0 (MEGA7) [32]. The gyrB 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 (1000 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 [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; CLSI, 2017).

Polymerase chain reaction amplification and antimicrobial resistance gene sequencing

To determine the presence of antimicrobial resistance genes, we analyzed the floR gene for FFC resistance; the tetA, tetB, tetC, tetD, and tetE genes for tetracycline resistance;
and sul1, sul2, and sul3 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 gluonate, malic acid, citric acid (CIT), and trisodium citrate but tested negative for urea and adipic acid. Contrastingly, *E. cloacae* isolates showed different biochemical characteristics.

**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 *gyrB* 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 *gyrB* gene region. However, S117 showed low similarity (92%) with the *gyrB* gene region; this isolate should be examined in detail using other genotyping methods (such as MLST). *E. asburiae* was 100% identical in the 16S rRNA gene region, but the isolate was 97% similar based on the *gyrB* 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 *gyrB* primer pairs; this gave three main genogroups with different levels of similarity (Figure 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 gyrB gene, though it also had distant genogroup similarities with other strains of Pseudomonas spp. E203 had high similarity in the gyrB 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 sulI, sulII, sulIII, tetA, and tetB resistance genes were not detected in the all isolates, whereas E. cloacae (E166) carried two resistance genes (floR and tetD), E. cloacae (E167) carried two resistance genes (tetD and tetE), and Pseudomonas spp. (P22) carried the tetC resistance gene (Table 3). While E201, E167, E200, E203, P22, and P23 isolates were phenotypically FFC resistant, none of them carried the floR resistance gene. More interestingly, only phenotypically susceptible isolates (E166) carried the floR resistance gene. All isolates were phenotypically susceptible to TET, but E166, E167, and P22 carried the tetC,
tetD, and tetE 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 floR-tetD and tetD-tetE 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 gyrB 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, 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 [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 gyrB gene region was also used in this study. A dendrogram was constructed to identify the gyrB 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 floR gene showed high similarity (at least 99%) to other floR 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 tetC gene showed high similarity (at least 99%) to other tetC 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 *tetE* gene showed high similarity (at least 99%) to other *tetE* 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 *floR*, *tetC*, *tetD*, and *tetE* 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” 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.

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Conflict of Interest

We have no conflicts of interest to declare.
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Figure legends

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

- **S117** - *S. maltophilia* (KY971297)
- **S. maltophilia** (HQ434495.1) Sweden
- **P23** - *Pseudomonas sp.* (KY971294)
- **E167** - *E. cloacae* (KY971299)
- **E68** - *E. asburiae* (KY971295)
  - *E. asburiae* (CP007546.1) Malaysia
- **E200** - *E. cloacae* (KY971300)
- **E201** - *E. cloacae* (KY971301)
  - **E166** - *E. cloacae* (KY971298)
  - *E. cloacae* (CP006580.1) USA
- **M94** - *M. psychrotolerans* (KY971296)
- *M. psychrotolerans* (HM122065.1) India
  - **E203** - *E. cloacae* (KY971302)
  - **P22** - *Pseudomonas sp.* (KY971293)
  - *Pseudomonas sp.* (HE603570.1) Belgium
Table 1. Isolate information and identification results

| Bacterial code | Fish weight | Isolation organ | Region and date | Sequence result (similarity with gyrB) | 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           | Central Anatolia (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)        | Uncultured 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 brackets. Negative isolates gave negative results in PCR.*
Table 2. Biochemical characteristics of isolates

| Biochemical characteristic | S. maltophilia | P. marginalis | M. psychrotolerans | E. asburiae | E. cloacae | E. cloacae | E. cloacae | 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 | floR | 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   | 0.128   | 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: sulfamethoxazole; TET: tetracycline; ENR: enrofloxacin; CIP: ciprofloxacin; GEN: gentamycin; AMX: amoxicillin; DOX: doxycycline; SXT: sulfamethoxazole-trimethoprim (1/5); OXO: oxolinic acid; E: erythromycin