JRVM
Journal of Research in Veterinary Medicine

RESEARCH ARTICLES
J Res Vet Med. 2020: 39(2) 82-92
DOI:10.30782/jrvm.743861

Determination of Opportunistic Pathogens and Antimicrobial Resistance Characterization Isolated From Rainbow Trout in Turkey

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Received 28-05-2020 Accepted 29-10-2020

Abstract

Aquaculture, by modern and technological methods, ensures effectual means for healthy fish production under "controllable" conditions. However, fish have disease problems due to a lack of imbalance with microbiota or opportunistic pathogen in culture systems. In the present study, we identified the opportunistic pathogens and some members of microbiota, which were obtained from our culture collection by culturable methods and characterized biochemically in addition to molecular analysis. We identified the isolates by partial gene sequencing and confirmed taxonomically in the bacterio.net database. The isolates were characterized based on antimicrobial susceptibilities by broth microdilution method analysis, and the resistance gene determinants were screened by PCR analysis. A total of 14 species were identified with high genetic similarity in the GenBank database. MIC results showed that bacteria have heterogeneous characteristics for the susceptibility of an agent into the genus, and species have high MIC values for sulfamethoxazole, trimethoprim, and ampicillin comparing to other agents. A total of 13 different resistance genes were determined in the bacteria, and some of them have multiple resistance genes up to five genes.

Keywords: Opportunistic pathogens, Microbial pathogenesis, Antimicrobial resistance genes, Microbiota

Introduction

Similar to the living organism, fish and other aquatic animals (farm or wild) tend to be disease with bacterial infections, when they are under stress conditions. Infections generally occur systemically with bacteremia or limited in a specific body area such as skin and gills. However, in any case opportunistic pathogens are found in environment and they are a part of the normal internal microbiota of an aquatic animal.[1] The fish bacteria can be divided into two groups; one is a dominant pathogenic bacteria, and the other one is opportunistic bacterial species that cause disease under stress conditions.

So far, there are many reports about mass fish deaths caused by pathogenic fish bacteria in cultured fish species which includes gram-negative bacteria such as; *Flavobacterium, Photobacterium, Aeromonas, Vibrio, Tenacibaculum, Yersinia ruckeri,* and *Pseudomonas* species, and also gram-positive bacteria such as; *Streptococcus, Lactococcus garvieae*, and *Renibacterium salmoninarum*.[2–6] In addition to the known bacterial fish pathogen, some of the bacterial species that inhabit the aquatic environment are essential to the balance of nature with no direct consequence in causing disease in fish. The bacteria were commonly reported as opportunistic fish pathogen under stress condition or together with primary pathogen especially, the various species of the genus *Chryseobacterium, Pseudoalteromonas, Shewanella, Arcobacter, Halomonas, Acine-*

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tobacter, Moraxella, Moritella, Citrobacter, Enterobacter, Plesiomonas, Hafnia, Myroides, Comamonas, and *Stenotrophomonas*.[1,7] Into these bacteria, some of *Citrobacter, Shewanella,* and *Chryseobacterium* species cause disease, and they were published as new emerging fish pathogen recently.[3,8,9] Many opportunistic pathogen or commensal microbiota are also used for the surrogate host model due to the probiotic effects.[10] There is still lack of the information about the status of these organisms—either as members of a healthy microbiome, or a latent step in disease establishment, or both. Under the healthy conditions, opportunistic pathogens seem to be present at low prevalence by suppressing with immune system.[7] One of the most crucial subjects for the opportunistic species is that they can act as a reservoir in the transmission of genetic information such as antimicrobial resistance genes. Researches showed that the occurrence of horizontal gene transfer (HGT) correlated with high microbial density.[11] The acquired antimicrobial resistance gene is mostly due to mutations, but the spreading of resistance is linked to HGT. In addition to the antimicrobial resistance, the alteration of an agent from opportunistic to primer pathogen is linked to spread of virulence factors via HGT.[12] For this reason, the determination of antimicrobial characteristics of opportunistic pathogens is crucial due to the reduction of antimicrobial pollution. These microorganisms also assumed that the indicator agents for monitoring of the antimicrobial pollutions, resistance gene transmission potency in addition to determining the risk of public health.

In the present study, we aimed at the identification of opportunistic pathogens isolated from rainbow trout with a molecular approach, biochemical and morphological such as growth conditions on different temperatures and salt concentration. Also, antimicrobial susceptibilities of these isolates were determined by broth microdilution methods (MIC), and resistance genes were characterized by PCR to determine whether the isolates are reservoir or not. The manuscript mainly emphasizes antimicrobial resistance spread, which is the other side of the environmental effects of opportunistic pathogens or members of microbiota.

Material and Methods

Phenotypic characterization of the bacteria isolates

The total of 37 bacteria, including seven *Acinetobacter* (n=7), seven *Myroides* (n=7), five *Hafnia* (n=7), three *Stenotrophomonas* (n=3), two of each *Comamonas, Enterobacter, Kluyvera,* and *Plesiomonas* (n=2), one of each *Curtobacterium, Exiguobacterium, Frigoribacterium, Prolinoborus, Psychrobacter, Shewanella* (n=1), in addition to an *E. coli* was used forthe control of antimicrobial studies, were analyzed obtained from the culture collection of aquatic animal disease laboratory isolated from previous studies. The information of isolates that were collected from rainbow trout (*Oncorhynchus mykiss*, Walbaum), aquarium fish (Discus, Symphysodon, Heckel, 1840), and pond water between 2013 to 2018 was presented in table 1. Samples were collected from the kidneys, liver, spleen, skin lesions, and ascites fluid of fish, and detailed isolate infor-

Table 1. Information about isolated bacteria

Bacteria code	*Sequence result*	Ident %	Fish species	Fish weight (g)	Isolation year	Isolation month	City	Region	Lesion
P37	*Acinetobacter johnsonii*	99.93	RT	0.3	2014	March	Kayseri	Central Anatolia	-
P68a	*Acinetobacter johnsonii*	99.85	RT	0.3	2014	February	Muğla	Aegean	-
P38b	*Acinetobacter lwoffii*	100	RT	0.3	2014	March	Kayseri	Central Anatolia	-
P39	*Acinetobacter lwoffii*	99.92	RT	0.7	2014	March	Kayseri	Central Anatolia	-
P40	*Acinetobacter lwoffii*	99.93	RT	0.7	2014	March	Kayseri	Central Anatolia	-
P41	*Acinetobacter lwoffii*	100	RT	0.7	2014	March	Kayseri	Central Anatolia	-
V54	*Acinetobacter* sp.	100	Water	Water	2014	April	Muğla	Aegean	-
P6	*Comamonas piscis*	100	RT	1.5	2013	December	Kayseri	Central Anatolia	-
P26	*Comamonas piscis*	99.86	RT	1.5	2013	December	Kayseri	Central Anatolia	-
P69	*Curtobacterium flaccumfaciens*	100	RT	0.4	2014	January	Kayseri	Central Anatolia	-
V51	*Enterobacter hormaechei**	93.69	Discuss	20	2015	May	Bursa	Marmara	Moribund fish
V71	*Enterobacter* sp.	100	RT	200	2013	July	Kayseri	Central Anatolia	Darkening in color, Exophthalmia
V83	*Exiguobacterium artemiae*	99.72	RT	7	2013	September	Kayseri	Central Anatolia	-
P70a	*Frigoribacterium* sp.	100	RT	0.4	2014	January	Kayseri	Central Anatolia	-
P33	*Hafnia alvei*	100	RT	10	2013	December	Kayseri	Central Anatolia	Dorsal lesion
V41a	*Hafnia alvei*	100	RT	20	2013	July	Kayseri	Central Anatolia	Tail, Pectoral and Dorsal fin erosion
V44	*Hafnia alvei*	100	RT	250	2013	Octeber	Kayseri	Central Anatolia	Tail, Pectoral and dorsal fin erosion
V93	*Hafnia alvei*	99.20	RT	250	2013	August	Kayseri	Central Anatolia	-
V94	*Hafnia alvei**	99.84	RT	20	2013	August	Kayseri	Central Anatolia	-
V46	*Kluyvera intermedia**	100	RT	300	2013	December	Kayseri	Central Anatolia	Dorsal lesion
V67	*Kluyvera intermedia**	100	RT	200	2013	August	Sivas	Central Anatolia	-
P60	*Myroides profundi*	98.96	RT	0.3	2014	February	Muğla	Aegean	-
P61-1	*Myroides profundi*	98.82	RT	0.3	2014	February	Muğla	Aegean	-
P61-2	*Myroides profundi*	98.82	RT	0.3	2014	February	Muğla	Aegean	-
P62	*Myroides profundi*	98.82	RT	0.3	2014	February	Muğla	Aegean	-
P63	*Myroides profundi*	98.96	RT	20	2014	February	Muğla	Aegean	Darkening in color
P64	*Myroides profundi*	98.86	RT	0.3	2014	February	Muğla	Aegean	-
P65	*Myroides profundi*	98.89	RT	0.3	2014	February	Muğla	Aegean	-
V56	*Plesiomonas shigelloides**	100	RT	0.7	2018	April	Kütahya	Aegean	Darkening in color, Exophthalmia, Dorsal fin erosion, Anemia
V87	*Plesiomonas shigelloides*	100	RT	200	2013	September	Kayseri	Central Anatolia	-
P36	*Prolinoborus fasciculus*	100	RT	0.3	2014	March	Kayseri	Central Anatolia	-
P74	*Psychrobacter cryohalolentis*	100	RT	0.3	2014	February	Kayseri	Central Anatolia	-
P159	*Shewanella putrefaciens*	99.78	RT	0.1	2017	January	Elazığ	Eastern Anatolia	-
P107	*Stenotrophomonas humi*	99.86	RT	0.1	2013	Jun	Kayseri	Central Anatolia	Moribund fish
P134	*Stenotrophomonas maltophilia*	100	RT	0.1	2017	January	Muğla	Aegean	-
V33a	*Stenotrophomonas* sp.	99.93	RT	0.3	2014	March	Kayseri	Central Anatolia	-

RT: Rainbow trout, -: No lesion, *: identification was made by rpoD gene region

Table 2. Biochemical characteristics of strains

Bacteria code	P	KOH	H	M	Ox	Cat	O/F	O129 (10ug)	O129 (150ug)	The growth rate on NaCl (%)										Growth on incubation temperature °C					PH
										0	1,5	3	4,5	6	7	8	9	10	4	25	37	42	45	
P37	-	ND	α	-	-	+	-/+	R	R	+	+++	+++	+	-	-	-	-		+	+	+	-	-	-
P68a	-	ND	-	-	-	+	+/+	R	R	+	+++	+++	+++	++	+	-	-			+	+	-	-	+
P38b	-	ND	-	-	-	+	+/+	R	R	+	+++	+++	+++	++	+	+	-		+	+	+	-	-	+
P39	-	ND	-	-	-	+	-/-	R	R	+	+++	+++	++	+	+	+	-		+	+	+	-	-	+
P40	-	ND	-	-	-	+	-/-	R	R	-	+++	+++	+	-	-	-	-		+	+	+	-	-	+
P41	-	ND	-		-	+	-/-	R	R	-	+++	+++	+	-	-	-	-		+	+	+	-	-	+
V54	-	ND	α	-	-	+	+/+	R	R	++	+	+	-	-	-	-	-		-	+	-	-	-	-
P6	-	ND	-	-	+	+	+/+	R	R	-	++	++	-	-	-	-	-		+	+	-	-	-	+
P26	-	ND	-	-	+	+	-/-	R	R	-	++	+	-	-	-	-	-		+	+	+	-	-	+
P69	-	ND		+	-	+	+/+	R	R	-	+++	++	++	++	+	+	+		+	+	+	+	-	+
V51	-	ND	-	+	-	+	+/+	R	S	+++	+++	+++	+++	++	++	++	+		-	+	+	+	+	-
V71	+	ND	β	+	-	+	+/+	R	R	+++	+++	++	+	+	-	-	-		-	+	+	-	-	-
V83	-	ND	-	+	-	+	+/+	R	S	++	++	++	++	+	+	+	+	+	-	+	+	-	-	-
P70a	-	ND	-	+	-	+	-/-	R	R	-	++	++	++	+	+	-	-		+	+	+	-	-	-
P33	-	ND	-	+	-	+	+/+	R	R	+	+++	+++	++	++	-	-	-		+	+	+	-	-	-
V41a	-	ND	-	+	-	+	+/+	R	R	+++	+++	+++	+++	+++	++	-	-		-	+	+	+	+	-
V44	-	ND	β	+	-	+	+/-	R	R	+++	+++	+++	++	+	+	-	-		-	+	+	+	-	-
V93	-	ND	-	+	-	+	+/+	R	R	++	+++	++	++	++	+	-	-		-	+	+	+	+	-
V94	-	ND	-	+	-	+	+/+	R	R	++	+++	+++	++	++	+	-	-		-	+	+	+	-	-
V46	-	ND	-	+	-	+	+/-	R	R	+++	+++	+++	+++	++	+	-	-		-	+	+	-	-	-
V67	-	ND	-	-	-	+	+/+	R	S	+++	+++	+++	++	+	-	-	-		-	+	+	-	-	-
P60	-	-	-	gliding	+	+	-/+	R	R	+	+++	+++	++	-	-	-	-		-	+	+	-	-	+
P61-1	-	+	-	gliding	+	+	+/+	R	R	+	+++	+++	+++	++	-	-	-		-	+	+	-	-	+
P61-2	+	-	β	gliding	+	+	+/+	R	R	+	+++	+++	++	+	-	-	-		-	+	+	-	-	+
P62	-	+	-	gliding	+	+	-/-	R	R	+	+++	+++	++	+	-	-	-		+	+	+	-	-	+
P63	-	+	-	gliding	+	+	-/-	R	R	+	+++	+++	++	+	-	-	-		-	+	+	-	-	+
P64	-	-	-	gliding	+	+	+/+	R	R	+	+++	+++	++		-	-	-		+	+	+	-	-	+
P65	-	-	-	gliding	+	+	-/+	R	R	+	+++	++	++	++	-	-	-		-	+	+	-	-	+
V56	-	ND	-	+	+	+	+/+	S	S	+++	+++	-	-	-	-	-	-		-	+	+	+	+	-
V87	-	ND	-	+	+	+	+/+	S	S	++	++	+								+	+	+	+	-
P36	-	ND	-	+	weak +	+	-/-	R	R	-	+++	++	+	+	+	+	-		+	+	+	-	-	-
P74	-	ND		-	+	+	-/-	R	R	-	+++	+++	++	++	+	-	-		+	+	+	-	-	-
P159	-	ND	α	+	+	+	+/+	R	R	+	++	++	++	-	-	-	-		+	+	+	-	-	+
P107	+	ND	α	+	-	+	-/-	R	R	+	++	++	+	-	-	-	-		-	+	-	-	-	+
P134	-	ND	α	+	-	+	-/+	R	R	+	+++	++	+	-	-	-	-		-	+	+	-	-	+
V33a	-	ND	β	+	-	+	-/-	R	R	+++	+++	+	-	-	-	-	-		-	+	-	-	-	-

P: Pellicle formation, KOH, flexirubin type pigment, H: Hemolysis on blood agar, M: Motility, Ox: Oxidase, Cat: Catalase, PH: Protease hydrolysis, ND: No data, +: positive, -: negative, R: Resistant, S: Sensitive The bacterial growth rate was shown from – to +++; -: no growth, +: weak growth, ++: rich growth, +++: very rich growth, grey cells show pellicle formation, *: identification was made by rpoD gene region

mation were given in table 1. The biochemical and morphological characteristics of all isolates were determined using conventional microbial tests such as gram staining, motility, oxidase, catalase, glucose fermentation on O/F test, growth on NaCl concentration 0-10%, and incubation temperature from 4°C to 45°C, the susceptibility of Vibriostatic agent, growth on Thiosulfate-citrate-bile salts-sucrose (TCBS) medium, hydrolysis for gelatin, Tween 20 and 80 (Table 2). All isolates were cultured on Tryptic Soy Agar (TSA, 22091, Sigma-Aldrich St. Louis, MO, USA) at 22°C for 24–48h, and pure cultures were supplemented with 20% glycerol and kept at -80°C.

Molecular identification

A spin column extraction kit was used to obtain the DNA of the bacteria from freshly cultured strains following the manufacturer's instructions (51306, Qiagen, Venlo, Netherlands). Before using the DNA samples, the amount and purity of each sample were measured at wavelengths of 260 nm and 260/280 nm using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Waltham, MA, USA). The primarily molecular identification was performed by using the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') on the 16S rRNA gene region;13 further identifications for some bacterial species due to low discriminatory result with 16S rRNA were performed using the housekeeping gene with rpoD 70F 5'-ACGACTGACCCGGTACGCATGTA-3' and 70R 5'-ATAGAAATAACCAGACGTAAGTT-3'.14 The PCR reactions were conducted according to previously used methods.3,15 All PCR products were confirmed by Sanger sequence analyses with double-stranded DNA by Macrogen Korea (Republic of Korea). Based on sequence similarities with 16S rRNA and rpoD gene region, the bacteria were identified as species-level showing the highest similarities of the reference genome (98-100%) in the GenBank 16S refSeq database and all of the sequences were deposited in GenBank; the taxonomic names were updated based on the LPSN database (http://www.bacterio.net/aeromonas.html).

Graphical analysis

The graphic and figures were constructed on Tableau Public: Free Data Visualization Software, the results were presented based on bacterial identification results comparing with growth temperature, NaCl concentration, fish weight, isolation year, month and observed lesion in addition to the sequence result.

Antibiotic Susceptibility Test

The antimicrobial characteristics of identified isolates were determined by broth microdilution methods for florfenicol (FFC; F1427), oxytetracycline (OTC; 1491004), trimethoprim (TRI; T7883), tetracycline (TET; 31741), sulfamethoxazole (SUL; S7507), sulfamethoxazole-trimethoprim (19:1), erythromycin (ERM; E5389), enrofloxacin (ENR,

17849), oxolinic acid (OXA, 67126), doxycycline (DO, 33429), ciprofloxacin (CIP; 17850), Gentamycin (GEN; G1264), chloramphenicol (CML; C0378), ampicillin (AMP; 10835242001) and amoxicillin (AMX, A8523) according to the CLSI document.16 All the antimicrobial standards were obtained from Sigma Aldrich (St. Louis, Missouri, United States). The antimicrobials were diluted with the suggested solution, and dilutions were concentrated between 0.008 to 256mg/L of each antimicrobial solution.17 The fresh culture of bacteria solution was prepared according to the VET 03/04-S2 guidelines and then mixed with antimicrobial solutions equal volume. The microplates were incubated at 28°C for 24±2h and observed in a spectrophotometer at 595nm wavelengths (Multiscan Go, Thermo).

Detection of Antimicrobial Resistance Genes

To determine the genetically resistance characteristics of isolates resistance genes including floR, tetA, tetB, tetC, tetD, tetG, tetM, tetL, tetS, tetE, tetH, ermA, ermB, sul1, sul2, sul3, cmlO1, int I, int II, int III, qnrA, qnrB, and qnrS were used in the PCR analysis. Primers and PCR reactions used to detect these genes were based on the published literature.3,18,19 PCR amplifications were conducted with the positive control of each gene region detected previously in our lab, and all detected resistance genes were confirmed by sequence analysis.

Results

Bacterial isolates and phenotypic characterization

The bacterial species used in this study were identified as species-level including the strains of seven *Myroides profundi,* five *Hafnia alvei,* four *Acinetobacter lwoffii*, two strains each of *Acinetobacter johnsonii, Kluyvera intermedia, Comamonas piscis,* one strain each of *Curtobacterium flaccumfaciens, Enterobacter hormaechei, Enterobacter* sp., *Exiguobacterium artemiae, Frigoribacterium* sp., *Plesiomonas shigelloides, Prolinoborus fasciculus, Psychrobacter cryohalolentis, Shewanella putrefaciens, Stenotrophomonas humi, Stenotrophomonas maltophilia, Stenotrophomonas* sp., *Acinetobacter* sp. were identified. Our isolates were commonly collected from 0.1 (yolk-sac fish) to 300g of fish. Also, *Enterobacter* sp., *Hafnia alvei, Kluyvera intermedia,* and *Plesiomonas shigelloides* were collected from rainbow trout weighing up to 200g while others commonly recovered from under 2g. While all bacteria were collected from rainbow trout, *Acinetobacter* sp. and *E. hormaechei* were obtained from water (pond water) and aquarium fish (Discus, Symphysodon, Heckel, 1840) and isolates commonly detected on 2013 and 2014, in fish farms located in Mugla and Kayseri town (Table 1). About 24% of all cases were reported with a symptom or health problem of fish species, including darkening color, exophthalmia, tail, pectoral and dorsal fin erosion, ascites, anemia also moribund fish.

The identified bacteria have typical characteristic seen in their genus as oxidase, catalase, motility, and glucose fermentation on the O/F basal medium. Hemolysis characteristics on blood agar with sheep blood (5%) are variable between species and strains, while some of them show α hemolytic, others are β hemolytic, and the rest of them are non-hemolytic. The yellow-pigmented bacteria, Myroides profundi, produce flexirubin type pigmented with KOH reagent, and gliding motility was observed. Nevertheless, most species characterized in the study were resistant for Vibriostatic agent both 10 and 150μg concentration; *E. hormaechei, E. artemiae* and *K. intermedia* showed susceptibility for Vibriostatic agent (10 μg) and *P. shigelloides* was determined susceptible to Vibriostatic agent both 10 and 150μg concentration.

Based on incubation temperature and salinity tolerance; most species can grow on 4°C, 25°C and 37°C except for *M. profundi, S. humi, S. maltophilia* (P60, P61-1, P61-2, P63, P65, P107 and P134) strains (growth was not observed on 4°C) and *C. piscis* and *S. humi* (P6 and P107, growth was not observed on 37°C). Besides, *C. flaccumfaciens* can grow on 42°C, *E. hormaechei, H. alvei,* and *P. shigelloides* also grown on both 42°C and 45°C. Detailed results were shown in table 2. Salt concentration based on NaCl concentration, most of the strains easily grown on 0-4.5% NaCl concentration, variable growth was observed into the species and genus for 6 to 9% NaCl concentration. Only *E. artemiae* (V83) can tolerate the highest NaCl concentration (10%) within the analyzed strains. The results were presented in table 2. Virulence characteristic of the strains in respect of protease is variable within the species and half of them hydrolyzed the protease.

Molecular Identification

The bacteria strains were biochemically characterized and identified as a genus level. Molecular identification results were confirmed with sequence similarity results on the NCBI database. All bacteria have high similarity values between 98.8-100% except for V51 (E. hormaechei) has 93.69% similarity values by the rpoD gene region. Almost all species have a good quality 16S rRNA gene sequence, and high similarity, V51, V94, V46, V67, and V56 do not show a proper quality sequence and electropherogram, so the identification was made by rpoD gene region which is a housekeeping gene. The sequence results, identity values and other details were shown in table 1. The 16S rRNA gene sequences were submitted to GenBank database with the accession numbers MT323124-MT323150.

Isolate Distribution and Data Comparison

Identification results were compared to isolation year and most of the isolates were recovered in 2013 and 2014. Only one isolate was detected in 2015, and no isolate was detected in 2016. Two isolates were collected from 2017 and one was from 2018 (Figure 1). Based on the comparison of the species, fish weight, and lesion, lesions were commonly observed on up to 10g of rainbow trout. Tail erosion, pectoral, and dorsal fin lesions were noted commonly in 20g and 250g fish by H. alvei (Figure 2). Most of the identified bacteria were isolated from rainbow trout fry weighing 1.5g, although they do not show any lesion (except for two isolates) (Figure 2). Monthly bacteria distribution were also screened, and no bacterial isolation was done on May, from the rest of the month, commonly one or two different bacterial species were isolated (Figure 3). The comparison of bacteria based on salinity tolerance, V83 *E. artemia* is the most salt tolerating bacteria, and each bacteria can tolerate 1.5% NaCl concentration. Most of them easily grow on up to 3% NaCl concentration, while some differences were observed between strains belonging to the same genus (Figure 4). Temperature tolerance values showed that *Plesiomonas, Hafnia,* and *Enterobacter* species are the most heat tolerant bacteria; they can grow at 45°C incubation temperature. The some of *Myroides* (P60, P61-1, P61-2, P63, P65, P107, P134) and *Stenotrophomonas* isolates do not show any growth at 4°C and *Comamonas piscis* (P6), and *Stenotrophomonas humi* (P107) have not tolerated 37°C also (Figure 5).

Results of Antibiotic Susceptibility Test

The MIC values for the standard reference strain of E. coli all fell within the acceptable range suggested by the CLSI guidelines (VET04-A2).16 The distribution of MIC values of the isolates for 14 antimicrobial agents is presented in table 3. There are too many differences in MIC values between species and genus, while almost all species have high MIC values for sulfamethoxazole, trimetoprim, and ampicillin. The lowest MIC values were seen for ENR and OXO. Because there are no specific breakpoints values in CLSI and EUCAST database for opportunistic fish pathogen and commensal organisms, the strains should not be evaluated as sensitive or resistant.

Detection of Antimicrobial Resistance Genes

The resistance genes of tetA, tetE, tetG, tetL, sul1, sul3, cmlO1, intII, intIII, and qnrS were not detected in the isolates. The most detected AMR genes are floR and ermA from nine and five isolates, respectively (Table 4). A to-

Table 3. MIC values distribution of bacteria

Bacteria code	Antibiotics and MIC values													
	TET	SUL	SXT 1/19	ENR	TRI	FLO	CIP	AMX	OXO	GEN	CML	AMP	DOX	ERM
P37	4	16	256	0,5	256	256	32	64	0,125	0,25	64	128	0,5	16
P68a	512	16	512	16	512	512	2	32	64	256	512	64	128	512
P38b	8	16	256	0,5	512	512	0,125	512	32	0,5	128	512	8	512
P39	16	16	256	0,008	256	4	2	128	0,128	0,5	8	256	1	4
P40	8	8	256	0,5	256	8	2	256	0,125	0,5	8	256	0,5	4
P41	8	8	256	0,5	256	8	2	512	0,5	0,5	16	128	0,125	4
V54	256	0,125	32	0,008	NG	4	NG	0,5	0,031	NG	NG	NG	16	16
P6	8	16	256	0,5	512	128	32	512	0,5	8	128	512	16	256
P26	32	16	128	0,008	256	32	32	512	0,5	4	64	512	0,5	16
P69	128	8	256	0,125	512	64	0,125	512	64	0,0625	64	512	16	128
V51	4	0,25	16	0,031	NG	16	NG	256	0,125	NG	NG	NG	0,5	64
V71	4	0,125	128	0,5	NG	>256	NG	>256	2	NG	NG	NG	4	32
V83	0,031	0,125	32	0,125	NG	1	NG	0,031	0,5	NG	NG	NG	0,008	16
P70a	0,5	8	128	0,125	512	0,5	2	0,031	16	0,031	0,5	0,031	0,031	0,008
P33	32	32	256	2	256	32	2	128	0,5	8	32	256	16	512
V41a	64	0,25	125	0,008	NG	128	NG	64	0,062	NG	NG	NG	32	64
V44	32	0,5	128	0,008	NG	32	NG	128	0,062	NG	NG	NG	32	64
V93	32	0,125	128	0,008	NG	64	NG	128	0,031	NG	NG	NG	32	64
V94	64	0,125	128	0,008	NG	32	NG	128	0,031	NG	NG	NG	32	64
V46	8	0,125	0,125	0,125	NG	16	NG	256	0,5	NG	NG	NG	16	32
V67	8	0,125	0,25	0,031	NG	16	NG	256	0,125	NG	NG	NG	16	32
P60	16	16	256	0,5	512	64	32	512	16	512	32	512	8	16
P61-1	512	16	256	16	512	512	64	512	64	512	512	512	128	512
P61-2	512	16	256	16	512	256	32	128	512	512	512	128	64	256
P62	512	16	256	1	512	128	64	256	8	512	128	256	64	256
P63	512	16	256	1	512	128	64	256	16	512	128	256	64	256
P64	512	16	256	32	512	512	32	128	512	512	512	512	64	256
P65	512	16	256	0,5	512	0,5	64	256	4	512	128	256	32	128
V56	8	0,25	32	0,008	NG	64	NG	256	0,062	NG	NG	NG	0,5	32
V87	16	0,125	0,125	0,008	NG	256	NG	128	0,031	NG	NG	NG	8	32
P36	4	8	256	0,5	256	256	32	512	0,32	0,25	512	256	0,5	256
P74	64	8	128	2	256	32	0,5	16	16	0,031	32	32	16	1
P159	512	512	512	0,5	512	256	0,125	512	2	1	128	512	4	256
P107	32	8	256	0,125	512	4	8	512	0,5	64	4	512	0,5	64
P134	256	8	128	1	512	512	2	512	1	128	256	512	32	128
V33a	2	0,125	128	0,125	NG	4	NG	>256	2	NG	NG	NG	0,125	16
E. coli	0,5	0,125	0,5	0,008	ND	8	ND	8	0,031	ND	ND	ND	0,5	32

TET: Tetracycline; SUL: Sulfonamide; SXT: Trimetoprim+Sulfamethoxazole; ENR: Enrofloxacin; TRI: Trimetoprim; FLO: Florfenicol; CIP: Ciprofloxacin; AMX: Amoxicillin; OXO: Oxolinic acid; GEN: Gentamycin; CML: Chloramphenicol; AMP: Ampicillin; DOX: Doxycycline; ERM: Erythromycin; NG: No Growth

Table 4. Distribution of Resistance gene

Bacteria code	Resistance Genes												
	*flo*R	*tet*A	*tet*C	*tet*D	*tet*M	*tet*S	*tet*H	*erm*A	*erm*B	*sul*2	*int* I	*qnr* A	*qnr* B
P68a	+	-	-	-	-	-	-	-	-	+	-	-	-
P38b													
V54	-	-	-	-	-	+	+	-	-	-	-	-	-
P6	-	-	-	+	-	-	-	-	-	-	+	-	-
P26	-	-	-	+	-	-	-	-	-	-	+	+	-
P70a	-	-	-	+	-	-	-	-	-	-	-	-	-
P33	-	-	-	-	-	-	-	+	-	-	-	-	-
V41a	-	-	-	-	-	-	-	+	-	-	-	-	-
V44	-	-	-	-	-	-	-	+	-	-	-	-	-
V93	-	-	-	-	-	-	-	+	-	-	-	-	-
V94	-	-	-	-	-	-	-	+	-	-	-	-	-
V67	+	-	-	-	-	-	-	-	-	-	-	-	-
P60	-	-	-	-	-	+	-	-	-	-	-	-	-
P61-1	+	-	-	-	+	-	-	-	-	-	-	-	-
P62	+	-	-	-	+	-	-	-	-	-	-	-	-
P63	+	-	-	-	+	-	-	-	-	-	-	-	-
P64	+	-	-	-	-	-	-	-	-	-	-	-	-
P65	+	-	-	-	-	-	-	-	-	-	-	-	-
V56	+	+	+	-	-	-	-	-	-	+	-	-	+
V87	-	+	-	-	-	-	-	-	-	+	-	-	+
P36	-	-	-	-	-	-	-	-	+	-	-	-	-
P74	+	-	-	-	-	+	-	-	-	-	-	-	-
P134	-	-	+	-	-	-	-	-	-	-	-	-	-

Only determined resistance genes and isolates were presented, positive results were marked

tal of 13 antimicrobial resistance genes was found in the species including *A. johnsonii, A. lwoffii, Acinetobacter* sp., *C. piscis, Frigoribacterium* sp., *H. alvei, K. intermedia, M. profundi, P. shigelloides, P. fasciculus, P. cryohalolentis,* and *S. maltophilia*. At the same time, no resistance gene was detected in *C. flaccumfaciens, E. hormaechei, Enterobacter* sp., *E. artemiae*, and *S. putrefaciens*. All of *M. profundi* and *P. shigelloides* were detected as a carrier of at least one resistance gene.

Discussion

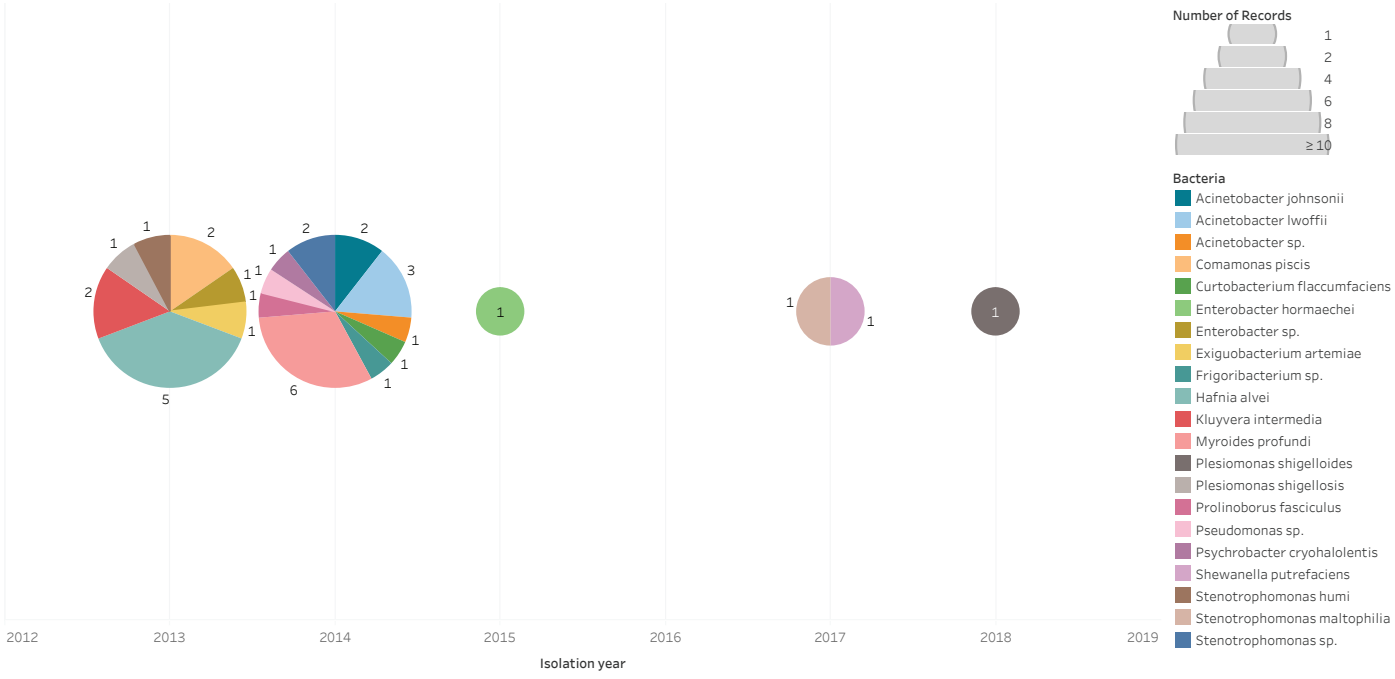
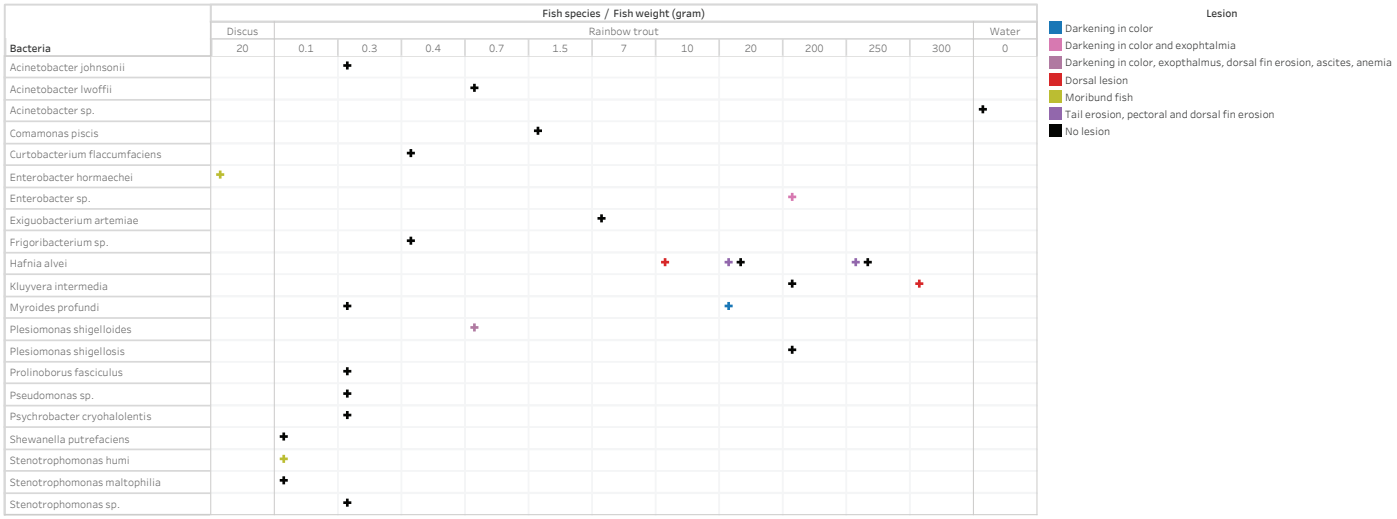
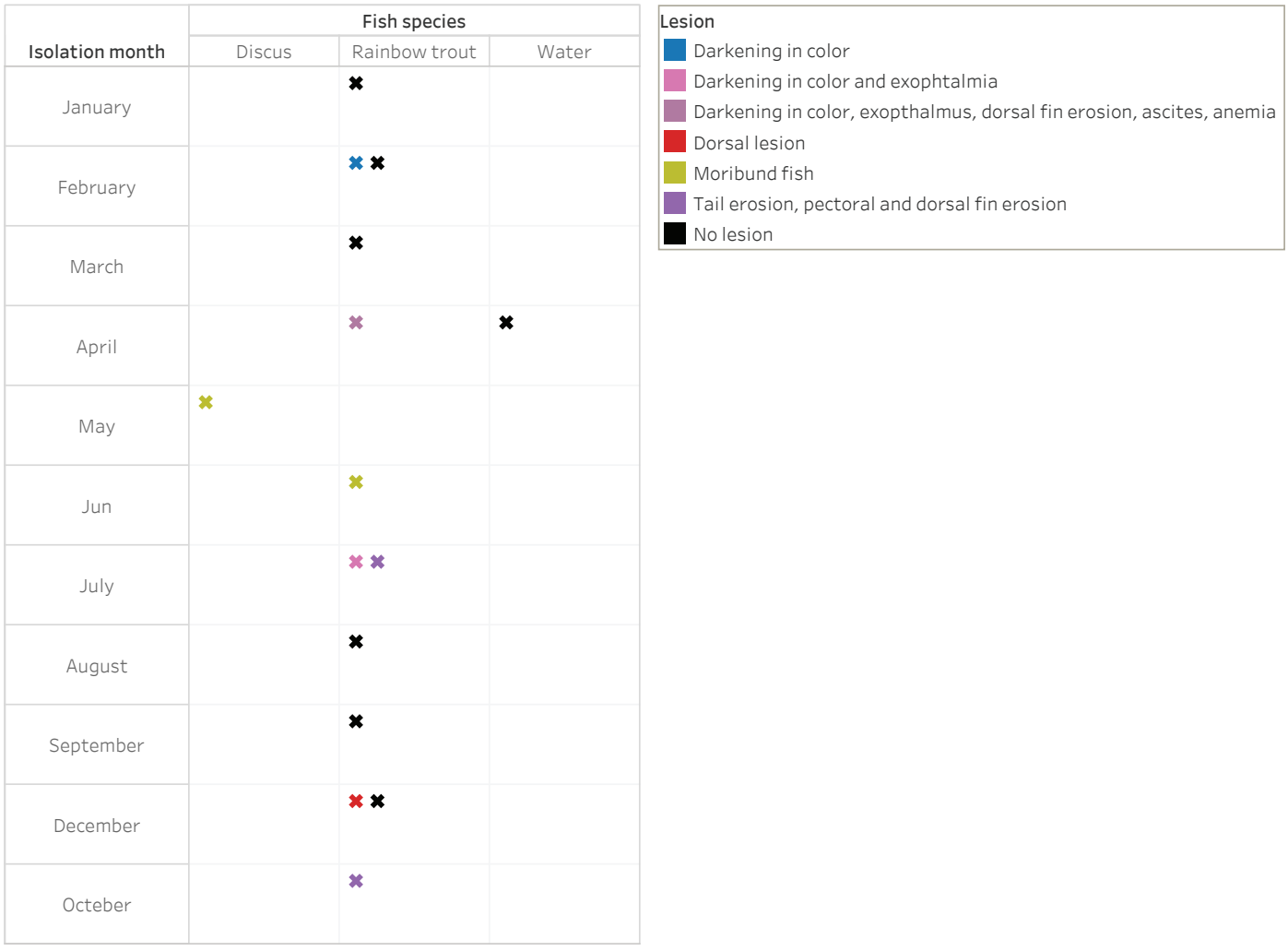


Figure 1. Bacteria, isolation month and year statics



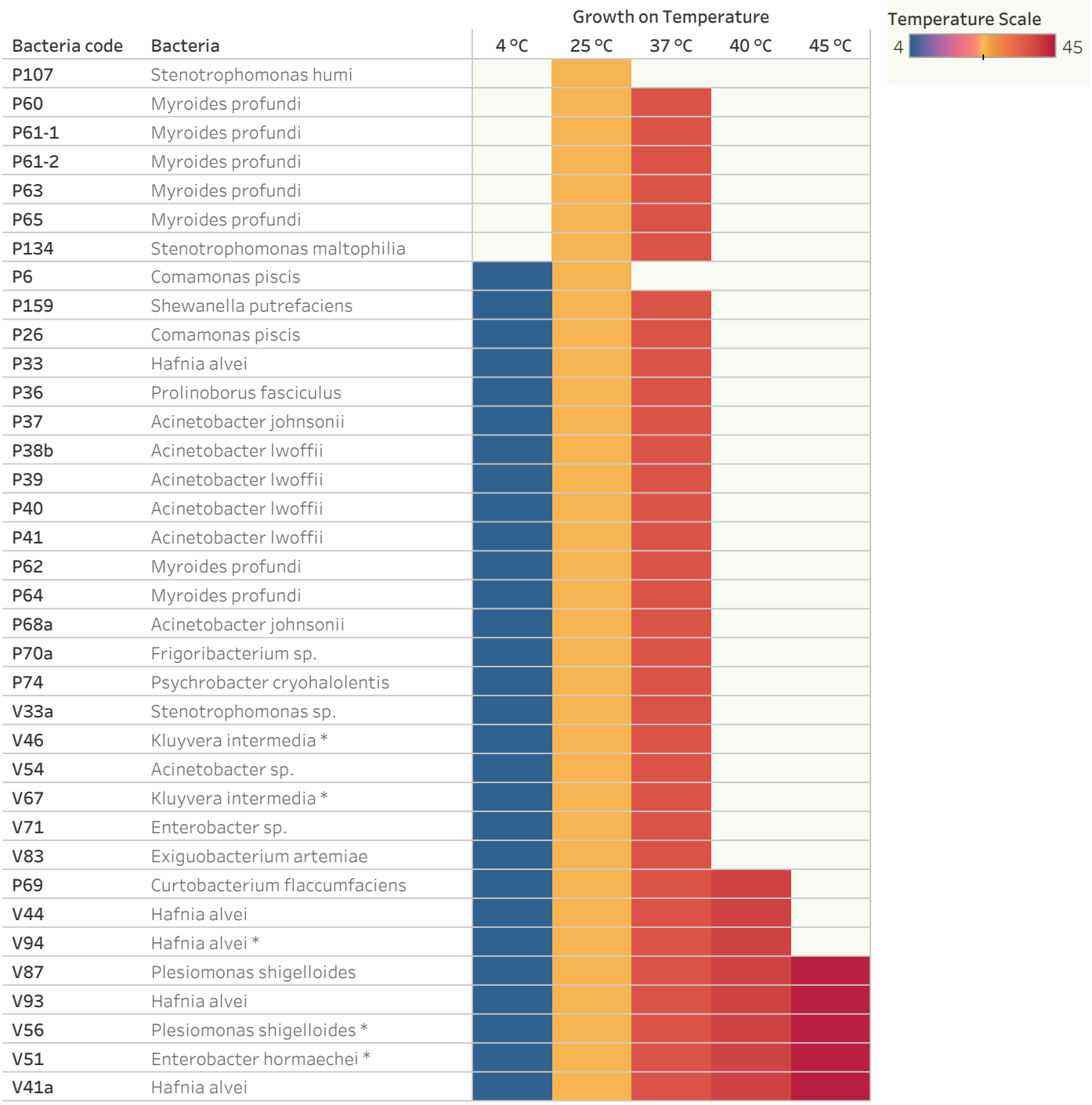
Lesion (color) broken down by Fish species and Fish weight (gram) vs. Bacteria.

Figure 2. Bacteria, fish weight and lesion correlation



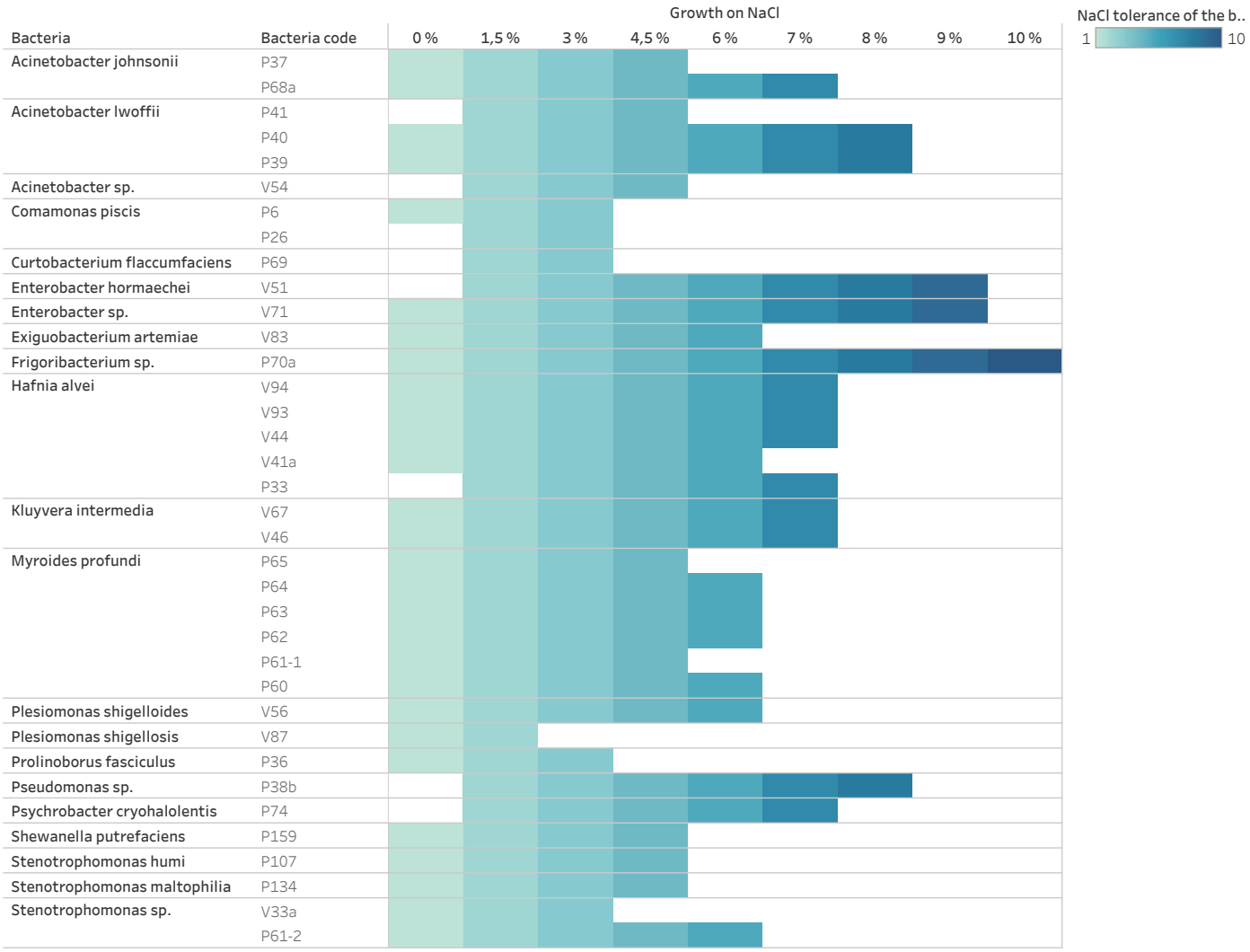
Lesion (color) broken down by Fish species vs. Isolation month.

Figure 3. Bacteria, fish species and isolation month correlation



Sum of Temperature tolerance of the bacteria (color) broken down by Growth on Temperature vs. Bacteria code and Bacteria.

Figure 4. Bacteria growth ranges at incubation temperature



Sum of NaCl tolerance of the bacteria (color) broken down by Growth on NaCl vs. Bacteria and Bacteria code.

Figure 5. Bacteria growth ranges on salinity conditions

In the present study, we found that about 18 different bacterial species were isolated from fish seemingly healthy or ones showing symptoms. These bacteria were generally assumed as opportunistic pathogens or organisms of microbiota in fish or environment. Thus, the salt and temperature tolerance of them showed that species might be pathogenic or zoonotic under stress conditions or immune suppression because most of them grow from 4-37°C, also up to 10% NaCl concentrations. In addition, antimicrobial characteristics of these species showed that these opportunistic pathogens or members of microbiota could be highly potential for antimicrobial resistance gene transmitter.

Intensive aquaculture ensure excellent medium for growth of heterotrophic or opportunistic bacteria by feeding fish that is added at high concentration to water and this will affect growth of microorganisms in the aquaculture condition. In the natural ecosystem, many living species coexist and host millions of different microorganisms in each species microflora. In the aquatic ecosystem, the vast majority of these microorganisms spread to the environment and other aquatic animals through water. These natural microorganisms are needed for the continuity of nature and life, and the smallest imbalance in these microorganisms will cause their life to deteriorate. As it can be seen in our study, nearly 18 bacterial species were isolated in all fish sizes from 0.1g to 300g, especially pond water. It is stated that the majority of these bacteria do not have a primary pathogen effect under normal conditions. We observed that a large number of isolated bacterial species caused tail and dorsal fin erosion or mild lesions such as darkening in color rather than mortality, which shows that these bacterial species do not cause disease under normal conditions similar to reported studies.[7,20]

There is a balance that exists between fish and bacteria, yet the exact relationship is unknown about the establishment of healthy microflora in larvae or early immunity.[21] The vast majority of these microorganisms contribute to the effective development of the immune system in fish. This situation was supported in our study by the fact that microorganisms detected from fish below 7g were isolated

from fish that showed almost no symptoms. In the artificial conditions, it was assumed that the culturable bacteria represent 0.01–10% of the viable bacterial population of fish or the aquatic environment and, thus, the sheer number of bacteria still unknown or not isolated yet.[21] In this respect, we supposed that isolated species reflect up to 10% of all opportunistic pathogens or microbiota in our study. When the natural condition changed, the members of microbiota may become pathogenic that named as the Rasputin effect.[7] This is the first possible hazard of microbiota for living organisms, especially for cultured fish. It is reported that the majority of bacterial species similar to our study cause disease in different fish species; such as *Enterobacter* species cause disease in bullhead (*Amiurus bebulosus*) fish; *Acinetobacter* sp., *A. johnsonii*, and *A. Iwoffii* species cause "*Acinetobacter disease*" in carp and rainbow trout; *H. alvei* causes "hemorrhagic septicemia" in salmon and rainbow trout; *Myroides* species cause disease in grey mullet; and *Plesiomonas* species cause disease in African catfish, and rainbow trout.[1] In addition to that, *Enterobacter, Stenotrophomonas, Pantoe,* and *Morganella* species were recovered from cultured fish species and assumed as the potential new fish pathogen.[15,19] Due to the bacterial agents determined in our study were isolated from lesioned fish revealed that these agents were possible pathogens as a result of possible changes in environmental conditions or suppression of the immune systems.

Besides the hazardous for fish, another important concern is that these pathogens can be potential pathogens for humans. The majority of the agents identified in our study were evaluated as fish borne bacterial zoonoses, and they were reported to cause significant infections in humans.[22–25] Duman et al. (2019) also showed that the agents were recovered from live and shocked fish tissues, and therefore they are more likely to reach people with fish consumption. In our study, almost all of the agents determined were able to grow at 37°C and high salinity, showing that these strains have high temperature and tolerance levels. Due to some isolates grow up to 45°C has confirmed that species could be easily transferred to people in areas with consumption of raw/undercooked fish (sushi) and that they can survive at body temperature. Members of natural microbiota are an indispensable part of the survival. However, this benefit has significant harmful effects is that these species are reservoirs of antimicrobial resistance genes. Generally, veterinarians apply no treatment for diseases caused by opportunistic pathogens in fish farms.[15] Diseases caused by the species often occur due to stress, the presence of a primary pathogen, or an environmental change, so diseases can often be treated by the elimination of environmental conditions or primary pathogens. In this respect, while no specific antimicrobial treatment is applied for these bacteria, it is reported that species carry the antimicrobial resistance gene, and many of them have become resistant to the antimicrobials used in aquaculture. While antimicrobial resistance in human pathogens is primarily the consequence of inappropriate use of antimicrobials in human medicine, there is growing evidence that their use in terrestrial agriculture has also contributed to the emergence of resistant foodborne pathogens.[26,27] Associations between reported antimicrobial use and bacterial resistance have been demonstrated for specific aquaculture production environments, and similar associations might exist at the higher aquaculture production levels, such as country of production.[26,27]

In the reported studies, it has been shown that opportunistic pathogens isolated from fish carry many resistance genes, and these resistance genes have a very close genetic relationship with genes isolated in humans pathogens.[15] In our study, we determined antimicrobial resistance genes from more than 13 different bacterial species with 30 antimicrobial resistance genes. Determined resistance genes revealed that the agents potentially serve as a reservoir for resistance genes and can spread genes continuously to the aquatic environment. Therefore, more studies should be carried out to determine the potential hazardous of resistance genes in cultured fish by analyzing with an opportunistic pathogen or member of natural microbiota. In conclusion, we revealed that many opportunistic pathogens that we isolated from rainbow trout might be organisms that may threaten human health due to temperature and salinity tolerance characteristics and the resistance genes isolated in these species pose an important risk for transferring to the environment or humans by consumption of fish.

Acknowledgments

This research was supported by the Scientific and Technological Research Council of Turkey (TUBITAK) (No: 118O420).

Conflict of interest: None declared.

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