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Genotyping and antimicrobial resistance genes of *Yersinia ruckeri* isolates from rainbow trout farms

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ABSTRACT: In this study, we compared 142 *Yersinia ruckeri* isolates collected between 2013 and 2016 from 6 different regions in Turkey. A total of 18 different genogroups were found, though most of the isolates clustered into the same genogroup as serotype O1. As immunization of fish with inactivated *Y. ruckeri* by injection, immersion, or feeding provide minimal protection against *Y. ruckeri* infection in Turkey, many fish producers use antimicrobials unrestrictedly, resulting in antimicrobial resistance in aquatic pathogens. Accordingly, we investigated resistance to the antimicrobials most commonly used to treat yersiniosis. More than 80% of the *Y. ruckeri* isolates were susceptible to sulfamethoxazole-trimethoprim (SXT), florfenicol (FFC), and tetracycline, whereas none were susceptible to sulfamethoxazole. The most commonly used antimicrobials (SXT and FFC) can be effectively administered because the resistance levels to these drugs are the lowest among those reported for agents used to control enteric red mouth disease (12.6 and 14.7%, respectively). In conclusion, to the best of our knowledge, this study is the first characterization of the antimicrobial resistance genes *floR, sulI, tetC, tetD*, and *tetE* in *Y. ruckeri* isolates from aquaculture. Additionally, we detected the *sulII* gene but not the *tetA, tetB, tetM, tetS*, or *sulIII* genes.

KEY WORDS: Enteric red mouth disease · *Oncorhynchus mykiss* · Turkey · Genetic relationship · Resistance gene

INTRODUCTION

Yersiniosis, also called enteric red mouth disease (ERM) is an important disease of fish and is caused by *Yersinia ruckeri*. *Y. ruckeri* affects predominantly species of salmonid fish (genera *Oncorhynchus* and *Salmo*), but it has also been isolated from other fish species and other animals (e.g. pigs, birds, cows, horses, dogs, and cats) (Furones et al. 1993, Danley et al. 1999). *Y. ruckeri* was first isolated and identified in 1956 from a fish farm in Hagerman Valley, Idaho, USA, and was therefore named the Hagerman strain (Ross et al. 1966, Rucker 1966). The bacterium has also been isolated in Australia, South Africa, Europe, and Turkey (Bullock et al. 1978, Bragg & Henton 1986, Cagirgan & Yurekliturk 1991, Stevenson et al. 1993) and was likely disseminated following the expansion and development of aquaculture facilities and international trade of fish and/or eggs. *Y. ruckeri* infections are easily transmitted by direct fish to fish contact with carriers (asymptomatic) or overtly infected animals.

Y. ruckeri is a Gram-negative bacillus, commonly motile and oxidase-negative, and belongs to the family *Enterobacteriaceae*. *Y. ruckeri* strains can be classified serotypically and phenotypically by outer membrane protein type. However, whole-cell serological reactions have been used to differentiate

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Y. ruckeri into 6 different serovars to date. The genetic structure of *Y. ruckeri* has been investigated using randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE). The O1a strains of *Y. ruckeri* show a high level of homogeneity in regard to their genetic structure (Schill et al. 1984, Huang et al. 2013). Antigenic and genetic differences of *Y. ruckeri* species have been determined between biotypes 1 and 2 by using genotyping methods, especially enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and (GTG)5-PCR and 16S rRNA gene region sequencing (Tinsley et al. 2011). Huang et al. (2013) used a combination of different typing methods and suggested that the ERIC-PCR and PFGE methods had the highest discriminatory power.

The most commonly used antimicrobials to treat yersiniosis include oxytetracycline, amoxicillin, florfenicol, and sulfadiazine in combination with trimethoprim (Michel et al. 2003). *In vitro* testing has demonstrated that *Y. ruckeri* can develop resistance to antimicrobials like oxytetracycline, oxolinic acid, and potentiated sulfonamides (Rodgers 2001). Sulfonamide resistance is most common, and *Y. ruckeri* resistance to both tetracycline and a combination of sulfamethoxazole+trimethoprim has been reported but is limited (Balta et al. 2010, Altun et al. 2013, Huang et al. 2013, Orozova et al. 2015, Ture & Alp 2016). Although numerous investigations have assessed the phenotypic resistance of *Y. ruckeri*, investigations of antimicrobial resistance genes in *Y. ruckeri* isolates from aquaculture are limited.

The present study was conducted between 2013 and early 2016 and was designed to (1) characterize the prevalence of *Y. ruckeri* and its genotypic variability in different regions of Turkey compared with isolates from other countries deposited in the GenBank database and (2) determine the resistance phenotype and genotype of *Y. ruckeri* and the prevalence of antimicrobial resistance genes in aquaculture. To our knowledge, this study is the first report of the identification of the *floR, sulI, tetC, tetD*, and *tetE* antimicrobial resistance genes in *Y. ruckeri* isolates from aquaculture.

MATERIALS AND METHODS

Phenotypic identification of *Yersinia ruckeri*

The areas in Turkey where bacterial isolates were obtained are indicated in Fig. 1 (see Table 3 for a list of all isolates used in this study). The isolates used were collected from the Aegean Sea, Central Anatolia, Black Sea, Marmara, Mediterranean Sea, and Eastern Anatolia regions between 2013 and

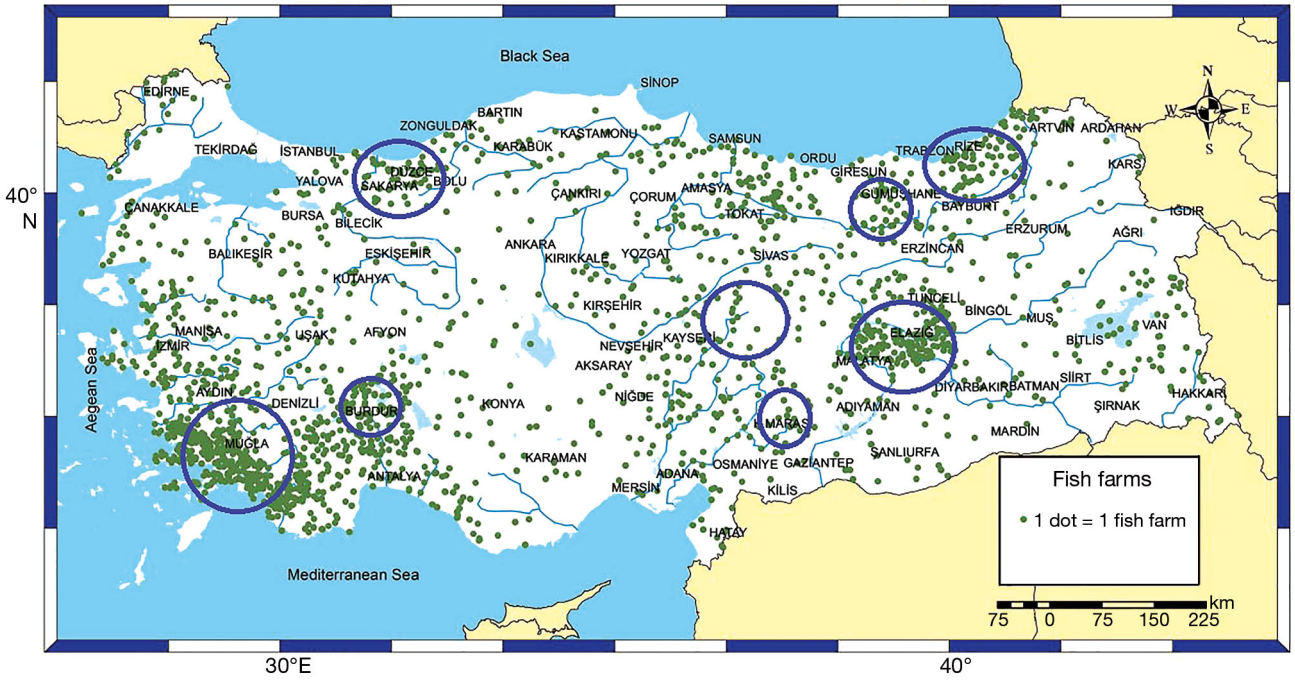

Fig. 1. Areas in Turkey where *Yersinia ruckeri* isolates were collected in 2013 to 2016. Blue rings indicate areas of monthly sampling. Green points mark individual rainbow trout farms (Balci Akova 2015)

2016. Altogether, 137 *Y. ruckeri* isolates were collected from clinical, subclinical, and reservoir fish cases originating in different populations, fish of different weights, and in different months and regions. These bacteria were isolated from seemingly healthy (disease symptoms were not observed) rainbow trout *Oncorhynchus mykiss* and from those suffering from ERM outbreaks. Of the 137 Turkish isolates, 136 were sampled from rainbow trout in trout hatcheries and farms, and 1 isolate was sampled from rainbow trout farm water (water and sediment samples were collected almost every month). Sampling was performed following the guidelines for the diagnosis of fish diseases and the international and national guidelines for animal welfare (OIE 2000). Additional isolates were obtained from the National Collections of Type Culture (NCTC, Central Public Laboratory Service, London, UK). The identification of each *Y. ruckeri* isolate was aided by classic phenotypic tests, such as colony morphology, color upon Gram staining, oxidase and catalase activities, oxidative fermentation (O/F), and growth on MacConkey agar (Merck). All isolates were cultured on trypticase soy agar (TSA; Merck) at 22°C for 24 to 48 h, and pure cultures were stored in tryptic soy broth containing 20% glycerol at −80°C (Austin & Austin 2007).

Molecular identification

DNA was extracted using a spin column filtration kit according to the manufacturer's instructions (QIAamp DNA mini kit). The DNA quantity and purity were measured at 260 nm and 260/280 nm using a spectrophotometer (Multiscan Go, Thermo).

This phenotypic identification was confirmed by standard PCR using previously described primers (Table 1) and a PCR kit (Qiagen 201205), resulting in the production of a 575 bp amplicon (Gibello et al. 1999).

Molecular characterization of *Y. ruckeri* using ERIC-PCR

The ERIC-PCR analysis was performed to determine the genetic diversity of all isolates according to the methods of Versalovic et al. (1991) and Huang et al. (2013) using PCR kits and ERIC-2 primers (Qiagen 201205) (Table 1). The amplification products were separated by electrophoresis in 1.5% agarose gels at 100 V for 100 min, and amplicons were visualized by UV transillumination of the ethidium bromide-stained gel. To determine the repeatability of the ERIC-PCR analysis, different DNA obtained from the same isolates (different times) was analyzed at least 3 times (Versalovic et al. 1991, Altun et al. 2013, Huang et al. 2013). A dendrogram was constructed using the Gelj program according to the unweighted pair group method with arithmetic mean (UPGMA) (Heras et. al. 2015).

For confirmation of bacterial identification and to determine similarities between our isolates and those in GenBank, representative isolates of each different ERIC-PCR pattern were sequenced with the Yer8–Yer10 primers that amplified the 16S rRNA region, and the sequences were used to create a phylogenetic tree with MEGA7 (Kumar et al. 2016). The Yer8–Yer10 sequences were deposited in GenBank (see Fig. 3 for accession numbers). The evolutionary history was inferred using the UPGMA method. The

Table 1. PCR primers and conditions for identification and genotyping of *Yersinia ruckeri* isolates; na: not applicable

Target gene	Primer name	Primer set	Amplicon size (bp)	PCR conditions			Reference
				No. of cycles	Temp. (°C)	Time (min)	
16S-rDNA	Yer8 (F)	5′-GCGAGGAGGAAGGGTTAAGTG-3′	575	1	92	5	Gibello (1999)
	Yer10 (R)	5′-GAAGGCACCAAGGCATCTCTG-3′		25	92	1	
					57	1	
					72	1	
				1	72	5	
16S-rDNA	ERIC-2	5′-AAGTAAGTGACTGGGGTGAGCG-3′	na	1	94	2	Versalovic et al. (1991)
				40	94	1	
					42	0.67	
					72	2	
				1	72	10	

tree was drawn to scale with the branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the maximum composite likelihood method, and the results are shown as the number of base substitutions per site. The analysis involved 32 nucleotide sequences. The codon positions were included as 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. The final dataset included a total of 370 positions. MEGA7 was used for the evolutionary analyses (Kumar et al. 2016).

Susceptibility testing

Minimum inhibitor concentrations (MIC) were determined using broth dilution methods according to CLSI (2014) for florfenicol (FFC; Sigma, F1427), tetracycline (TET; Sigma, 31741), sulfamethoxazole (SUL; Sigma, S7507), and SUL+trimethoprim 1:19 (SXT; combined with Sigma T7883) with 0.008 to 256 mg ml^{-1} dilutions. After incubation at 22°C for 24 to 48 h, plates were measured at a wavelength of 595 nm in a microplate reader (Multiskan Go, Thermo), and MICs were defined as the lowest concentration of antibiotic that inhibited visible growth (turbidity) (CLSI 2014). NCTC strains of *Y. ruckeri* were used as controls for antimicrobial susceptibility. *Escherichia coli* ATCC 25922 was used in this study as a quality control strain according to the CLSI (2014).

PCR 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, tetE, tetM*, and *tetS* genes for TET resistance and the *sulI*, *sulII*, and *sulIII* genes for SUL resistance using the specific primers and PCR conditions provided in Table 2, with some modifications (Ng et. al. 2001, Schmidt et al. 2001, Van et al. 2008, Nawaz et al. 2011, Wang et al. 2014). The PCR analysis was performed using positive control genes designed for our laboratory. The amplification products were separated by electrophoresis in 1–2% agarose gels at 100 V for 100 min, and bands were observed by UV transillumination of the ethidium bromide-stained gel. Sequences were deposited in GenBank under accession numbers KY001929 to KY001934.

RESULTS

Phenotypic and molecular identification

In total, we collected 137 isolates and 5 reference strains of *Yersinia ruckeri* between 2013 and 2016 from different sources (136 samples from rainbow trout weighing between 0.1 and 3000 g and 1 water sample). Most of these (n = 141) were from 6 different geographical regions of Turkey: 38 isolates from Central Anatolia, 68 isolates from the Aegean, 10 isolates from Marmara, 8 isolates from the Black Sea, 10 isolates from the Mediterranean Sea, and 2 isolates from the Eastern Anatolia regions. We also included 1 strain from Denmark, 1850621-1116B for comparison (Table 3, Fig. 2).

Positive isolates were Gram-negative, motile, oxidase-negative, and oxidative fermentative, exhibited growth on MacConkey agar, and presented a 575 bp band by PCR using primers Yer8–Yer10.

Molecular characterization of *Y. ruckeri*

The *Y. ruckeri* isolates were separated into 4 different genogroups according to the 72% similarity coefficients, which included 18 different RAPD fingerprinting patterns (Fig. 3). A total of 117 of the 141 Turkish *Y. ruckeri* isolates (82.3%) exhibited a pattern similar to that of the NCTC 12266 strain (serotype O1) (100%), and 4 (2.8%) exhibited a pattern (100%) similar to that of NCTC 12270 (serotype O7). No similarity to the patterns of NCTC 12267, NCTC 12268, and NCTC 12269 was found (Fig. 4). The Aegean isolates, which were isolated in 2013, exhibited the lowest similarity (54%) with the other isolates, whereas 124 *Y. ruckeri* isolates (87.3%) showed at least 90% similarity. The 2013 and 2014 isolates displayed great heterogeneity, but the 2015 isolates were more homogenous than the other isolates. All information regarding genogroups and isolates is provided in Table 3. A RAPD dendrogram was constructed based on representative isolates of these genogroups.

Every PCR product was sequenced and deposited in GenBank under accession numbers KY001929 to KY001934. A dendrogram of the PCR products was generated in MEGA7 for the Yer8–Yer10 primers. The Yer8 and Yer10 sequences yielded 3 different genogroups with different similarities (Fig. 4). The sequences were compared with *Y. ruckeri* isolates in GenBank from Europe, Asia, and the USA. All isolates exhibited at least 80% sequence similarity, and

Table 2. PCR primers and conditions for antimicrobial resistance genes

Target gene	Primer set	Amplicon size (bp)	PCR conditions			Reference
			No. of cycles	Temp. (°C)	Time (min)	
floR	F. 5′-TAT CTC CCT GTC GTT CCA G-3′	399	1	94	4	Van et. al. (2008)
	R. 5′-AGA ACT CGC CGA TCA ATG-3′		30	94	0.5	
				55	0.5	
				72	1	
			1	72	7	
tetA	F. 5′-GCT ACA TCC TGC TTG CCT TC-3′	210	1	94	5	Ng et. al. (2001)
	R. 5′-CAT AGA TCG CCG TGA AGA GG-3′		30	94	1	
				55	1	
				72	1.5	
			1	72	10	
tetB	F. 5′-CTC AGT ATT CCA AGC CTT TG-3′	416	1	95	3	Schmidt et al. (2001)
	R. 5′-CTA AGC ACT TGT CTC CTG TT-3′		25	95	0.5	
				59	0.5	
				72	3	
			1	72	5	
tetC	F. 5′-AAC AAT GCG CTC ATC GT-3′	1138	1	94	4	Frech & Schwarz (2000)
	R. 5′-GGA GGC AGA CAA GGT AT-3′		35	94	1	
				62	2	
				72	3	
			1	72	7	
tetD	F. 5′-ACA CTG CTG GAC GCG AT-3′	1121	1	94	4	Frech & Schwarz (2000)
	R. 5′-CTG ATC AGC AGA CAG AT-3′		35	94	1	
				62	2	
				72	3	
			1	72	7	
tetE	F. 5′-GTG ATG ATG GCA CTG GTC AT-3′	1180	1	95	4	Schmidt et al. (2001)
	R. 5′-CTC TGC TGT ACA TCG CTC TT-3′		25	95	0.5	
				62	0.5	
				72	0.75	
			1	72	7	
tetM	F. 5′-GTT AAA TAG TGT TCT TGG AG-3′	657	1	95	5	Nawaz et al. (2011)
	R. 5′-CTA AGA TAT GGC TCT AAC AA-3′		30	95	0.75	
				55	0.75	
				72	1	
			1	72	7	
tetS	F. 5′-ATC AAG ATA TTA AGG AC-3′	573	1	94	4	Nawaz et al. (2011)
	R. 5′-TTC TCT ATG TGG TAA TC-3′		30	94	0.5	
				55	0.5	
				72	1	
			1	72	7	
sulI	F. 5′-CGG CGT GGG CTA CCT GAA CG-3′	433	1	94	4	Wang et al. (2014)
	R. 5′-GCC GAT CGC GTG AAG TTC CG-3′		30	94	0.5	
				60	0.5	
				72	1	
			1	72	7	
sulII	F. 5′-GCG CTC AAG GCA GAT GGC ATT-3′	293	1	94	4	Wang et al. (2014)
	R. 5′-GCG TTT GAT ACC GGC ACC CGT-3′		30	94	0.5	
				55	0.5	
				72	1	
			1	72	7	
sulIII	F. 5′-TCA AAG CAA AAT GAT ATG AGC-3′	787	1	94	4	Wang et al. (2014)
	R. 5′-TTT CAA GGC ATC TGA TAA AGA C-3′		30	94	0.5	
				55	0.5	
				72	1	
			1	72	7	

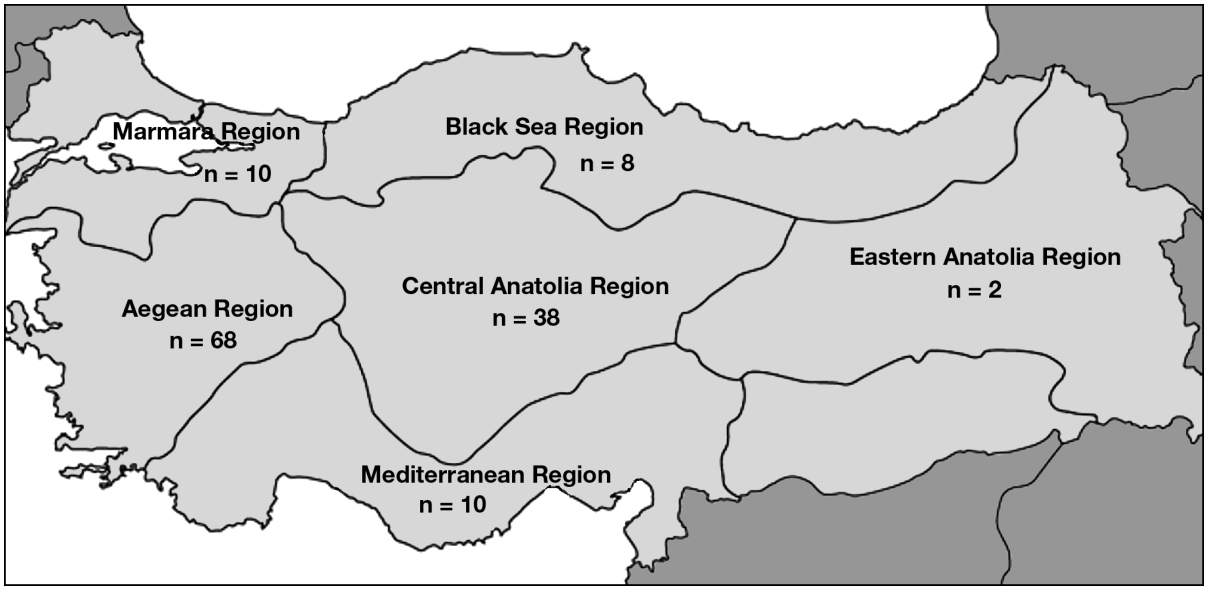

Fig. 2. Geographic distribution of *Yersinia ruckeri* isolates collected in 2013 to 2016 (n = number of isolates). An additional isolate was from Denmark

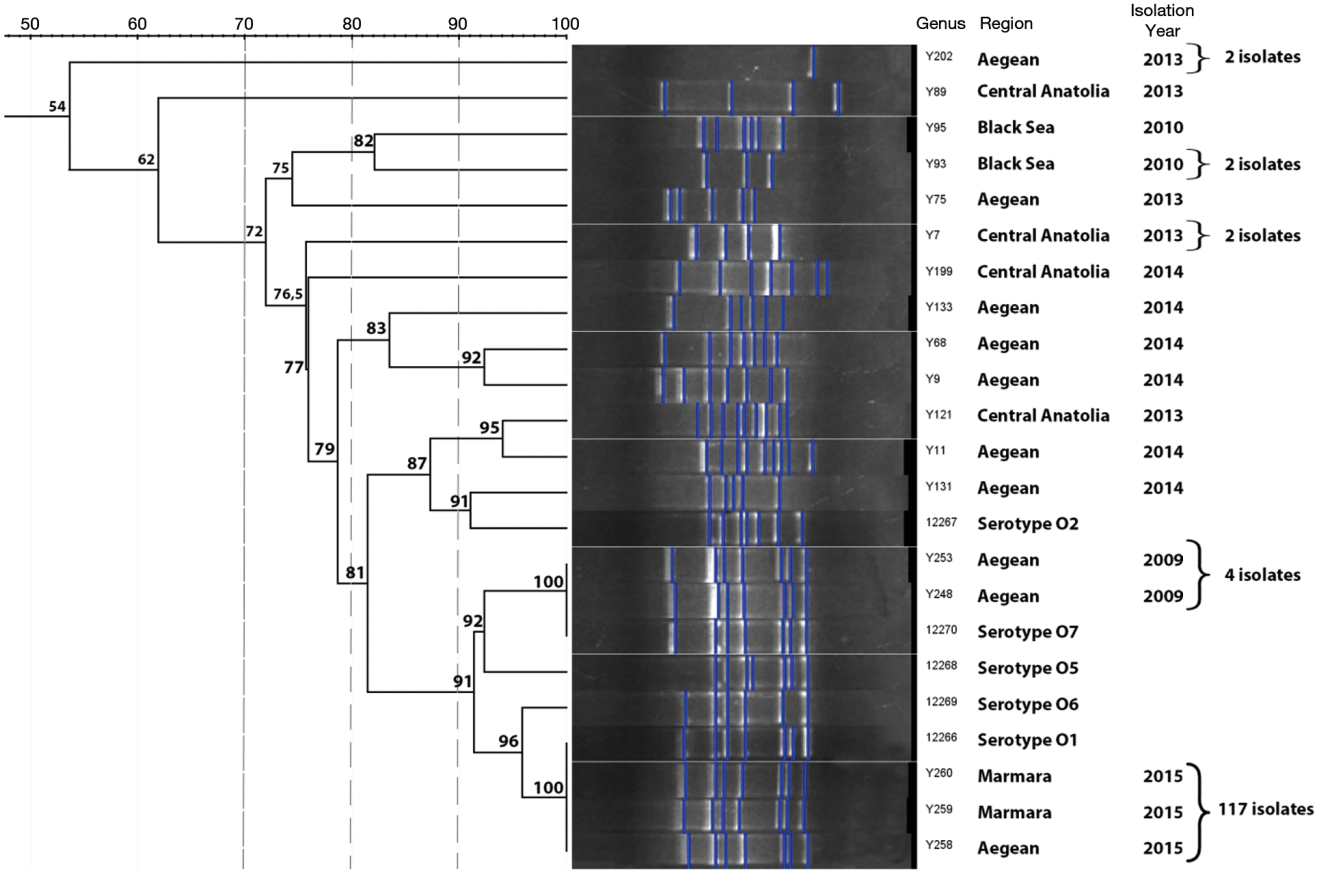

Fig. 3. RAPD-PCR images of 141 *Yersinia ruckeri* isolates based on enterobacterial repetitive intergenic consensus-PCR. The isolate from Denmark is not included in this dendrogram. Dendrogram was constructed with GelJ software, and 4 distinct genogroups were seperated at the 72% bootstrap

Table 3. *Yersinia ruckeri* isolates and genogroups. RT: rainbow trout; NCTC: National collection of type cultures (UK); blank: no data

Genogroup	Isolate ID no.	Fish species and weight (g)	Isolation year	Isolation month	Region
A	Y 32	RT (150)	2013	March	Marmara
I	Y 202	RT (5)	2013	August	Aegean
F	Y 75	RT	2013	August	Aegean
A	Y 122	RT (15)	2013	September	Central Anatolia
M	Y 121	RT (250)	2013	September	Central Anatolia
G	Y 89	RT (10)	2013	September	Central Anatolia
A	Y 13	RT (10)	2013	November	Central Anatolia
A	Y 14	RT (15)	2013	November	Central Anatolia
D	Y 10	RT (3000)	2013	November	Central Anatolia
D	Y 7	RT (3000)	2013	November	Central Anatolia
A	Y 201	RT (40)	2013	December	Central Anatolia
A	Y 200	RT (180)	2014	January	Central Anatolia
J	Y 199	RT (30)	2014	January	Central Anatolia
A	Y 173	RT (8)	2014	February	Aegean
A	Y 175	RT (8)	2014	February	Aegean
A	Y 183	RT (10)	2014	February	Central Anatolia
A	Y 186	RT (150)	2014	February	Central Anatolia
A	Y 190	RT (40)	2014	February	Central Anatolia
A	Y 193	RT (10)	2014	February	Central Anatolia
A	Y 174	RT (3000)	2014	February	Central Anatolia
A	Y 176	RT (3000)	2014	February	Central Anatolia
A	Y 167	RT (8)	2014	March	Aegean
A	Y 171	RT (8)	2014	March	Aegean
A	Y 165	RT (220)	2014	March	Aegean
A	Y 169	RT (20)	2014	March	Aegean
A	Y 146	RT (0.3)	2014	April	Central Anatolia
A	Y 147	RT (0.3)	2014	April	Central Anatolia
A	Y 127	RT (8)	2014	April	Aegean
A	Y 130	RT (5)	2014	April	Aegean
A	Y 132	RT (60)	2014	April	Aegean
A	Y 139	RT (8)	2014	April	Aegean
L	Y 131	Water	2014	April	Aegean
K	Y 133	RT (200)	2014	April	Aegean
A	Y 152	RT (120)	2014	April	Central Anatolia
A	Y 156	RT (300)	2014	April	Central Anatolia
A	Y 161	RT (200)	2014	April	Central Anatolia
A	Y 119	RT (10)	2014	May	Aegean
I	Y 110	RT (150)	2014	May	Aegean
A	Y 120	RT (200)	2014	May	Aegean
A	Y 111	RT (200)	2014	May	Central Anatolia
A	Y 118	RT (200)	2014	May	Central Anatolia
A	Y 86	RT (2)	2014	June	Aegean
A	Y 91	RT (2)	2014	June	Aegean
A	Y 51	RT (200)	2014	July	Central Anatolia
A	Y 66	RT (200)	2014	July	Central Anatolia
A	Y 2	RT (8)	2014	July	Aegean
A	Y 25	RT (12)	2014	July	Aegean
A	Y 3	RT (15)	2014	July	Aegean
O	Y 68	RT (0.3)	2014	July	Aegean
A	Y 53	RT (100)	2014	July	Central Anatolia
A	Y 64	RT (100)	2014	July	Central Anatolia
A	Y 71	RT (15)	2014	August	Aegean
A	Y 79	RT (15)	2014	August	Aegean
A	Y 61	RT (0.2)	2014	August	Aegean
A	Y 56	RT (10)	2014	September	Aegean
A	Y 57	RT (20)	2014	September	Aegean
A	Y 60	RT (150)	2014	October	Marmara
A	Y 78	RT (5)	2014	October	Aegean
A	Y 81	RT (14)	2014	October	Aegean

(continued on p. 38 and 39)

Table 3 (continued)

Genogroup	Isolate ID no.	Fish species and weight (g)	Isolation year	Isolation month	Region
A	Y 114	RT (250)	2014	October	Aegean
A	Y 58	RT (8)	2014	November	Mediterranean Sea
A	Y 73	RT (20)	2014	November	Mediterranean Sea
A	Y 77	RT (8)	2014	November	Mediterranean Sea
A	Y 106	RT (3)	2014	November	Aegean
A	Y 107	RT (5)	2014	November	Aegean
C	Y 11	RT (2)	2014	November	Aegean
E	Y 9	RT (0.1)	2014	November	Aegean
A	Y 40	RT (250)	2014	December	Eastern Anatolia
A	Y 43	RT (250)	2014	December	Eastern Anatolia
A	Y 44	RT (10)	2014	December	Central Anatolia
A	Y 45	RT (10)	2014	December	Central Anatolia
A	Y 29	RT (1)	2014	December	Aegean
A	Y 37	RT (50)	2014	December	Aegean
A	Y 42	RT (40)	2014	December	Central Anatolia
A	Y 47	RT (150)	2014	December	Central Anatolia
A	Y 38	RT (1)	2015	January	Aegean
A	Y 39	RT (7)	2015	January	Aegean
A	Y 36	RT (150)	2015	January	Aegean
A	Y 255	RT (250)	2015	February	Mediterranean Sea
A	Y 256	RT (250)	2015	February	Mediterranean Sea
A	Y 6	RT (300)	2015	February	Central Anatolia
A	Y 212	RT (250)	2015	March	Aegean
A	Y 213	RT (250)	2015	March	Aegean
A	Y 217	RT (30)	2015	March	Central Anatolia
A	Y 214	RT (200)	2015	April	Marmara
A	Y 215	RT (250)	2015	April	Marmara
A	Y 224	RT (6)	2015	April	Aegean
A	Y 206	RT (3)	2015	April	Aegean
A	Y 229	RT (3)	2015	April	Aegean
A	Y 207	RT (250)	2015	April	Aegean
A	Y 228	RT (250)	2015	May	Black Sea
A	Y 216	RT (12)	2015	May	Aegean
A	Y 232	RT (12)	2015	May	Aegean
A	Y 231	RT (10)	2015	May	Aegean
A	Y 259	RT (2000)	2015	Jun	Marmara
A	Y 260	RT (2000)	2015	Jun	Marmara
A	Y 257	RT (200)	2015	Jun	Aegean
A	Y 258	RT (200)	2015	Jun	Aegean
A	Y 210	RT (250)	2015	Jun	Aegean
A	Y 225	RT (250)	2015	Jun	Aegean
A	Y 227	RT (8)	2015	Jun	Aegean
A	Y 211	RT (5)	2015	Jun	Aegean
A	Y 223	RT (30)	2015	Jun	Aegean
A	Y 226	RT (5)	2015	Jun	Aegean
A	Y 209	RT (250)	2015	Jun	Central Anatolia
A	Y 94	RT	2015		Black Sea
A	Y 96	RT	2015		Black Sea
H	Y 92	RT	2015		Black Sea
L	Y 93	RT	2015		Black Sea
A	Y 233	RT			Aegean
A	Y 234	RT			Aegean
A	Y 235	RT			Aegean
A	Y 236	RT			Central Anatolia
A	Y 238	RT			Marmara
A	Y 239	RT			Central Anatolia
A	Y 240	RT			Denmark 1850621-1116b
A	Y 242	RT			Aegean
A	Y 243	RT			Marmara

Table 3 (continued)

Genogroup	Isolate ID no.	Fish species and weight (g)	Isolation year	Isolation month	Region
A	Y 244	RT			Central Anatolia
A	Y 245	RT			Aegean
A	Y 246	RT			Central Anatolia
A	Y 247	RT			Marmara
A	Y 249	RT			Aegean
A	Y 250	RT			Black Sea
A	Y 251	RT			Black Sea
A	Y 252	RT			Aegean
A	Y 254	RT			Aegean
B	Y 237	RT			Marmara
B	Y 241	RT			Aegean
B	Y 248	RT			Aegean
B	Y 253	RT			Aegean
N	Y 95	RT			Black Sea
A	Y 261	RT (250)	2016	March	Mediterranean Sea
A	Y 262	RT (250)	2016	March	Mediterranean Sea
A	Y 263	RT (250)	2016	March	Mediterranean Sea
A	Y 264	RT (250)	2016	March	Mediterranean Sea
A	Y 265	RT (250)	2016	March	Mediterranean Sea
A	NCTC 12266	RT	1978		USA
	NCTC 12267	Chinook salmon	1978		USA
	NCTC 12268	RT	1978		USA
	NCTC 12269	RT	1978		USA
B	NCTC 12270	Eel	1978		USA

all *Y. ruckeri* isolates clustered into the same group as the NCTC reference strains and some isolates from Iran, Russia, and Spain. The *Y. ruckeri* isolates were 87% similar to Asian, European, and American isolates, with the exception of those from France and Finland, which showed at least 80% similarity.

MICs, resistance genes, and sequencing

The MICs of *Y. ruckeri* are provided in Table 4. All isolates were found to be resistant to SUL; 21 isolates were resistant to FFC, 19 to TET, and 18 to SXT. The MICs (in mg l^{-1}) for FFC, SUL, TET, and SXT were 0.256–256, 256, 0.064–64, and 2–256 mg l^{-1}, respectively. The FFC and TET-resistant isolates were mainly observed in the Aegean and Central Anatolia.

The *sulIII, tetA, tetB, tetM*, and *tetS* genes were not detected amongst any of the *Y. ruckeri* isolates, whereas 8, 3, 4, 2, 1, and 1 isolates were positive for *sulII, sulI, tetC, tetE, tetD*, and *floR* genes, respectively (Table 5). Of the 19 *Y. ruckeri* isolates phenotypically resistant to TET, 1 isolate carried *tetE* and 1 carried both *tetC* and *tetD*. Among the phenotypically TET-susceptible isolates, 4 carried resistance genes. Among all of the isolates, 21 were phenotypically resistant to FFC, but only 1 carried a *floR* resistance gene. Three *sulI* and 8 *sulII* genes were detected in the SUL-resis-

Table 4. Minimum inhibitory concentrations (MIC) of *Yersinia ruckeri* isolates (n = 142). Numbers in the table are the numbers of isolates showing resistance at a given concentration. MIC reference values are taken from CLSI (2014): S, susceptible; R, resistant. SXT: sulfamethoxazole+trimethoprim

Antimicrobial	MIC (mg l^{-1})																MIC ref. value	No. of resistant strains (%)
	0.008	0.016	0.032	0.064	0.128	0.256	0.512	1	2	4	8	16	32	64	128	256		
Florfenicol						3	1	5	33	79	8	5	2	3	1	2	S < 8 > R	21/142 (14.8)
Tetracycline				1	2	5	24	61	22	8	7	1	7	4			S < 8 > R	19/142 (13.4)
Sulfamethoxazole																142	S < 128 > R	142/142 (100)
SXT									22	102	7	3	1		1	6	S < 8 > R	18/142 (12.7)

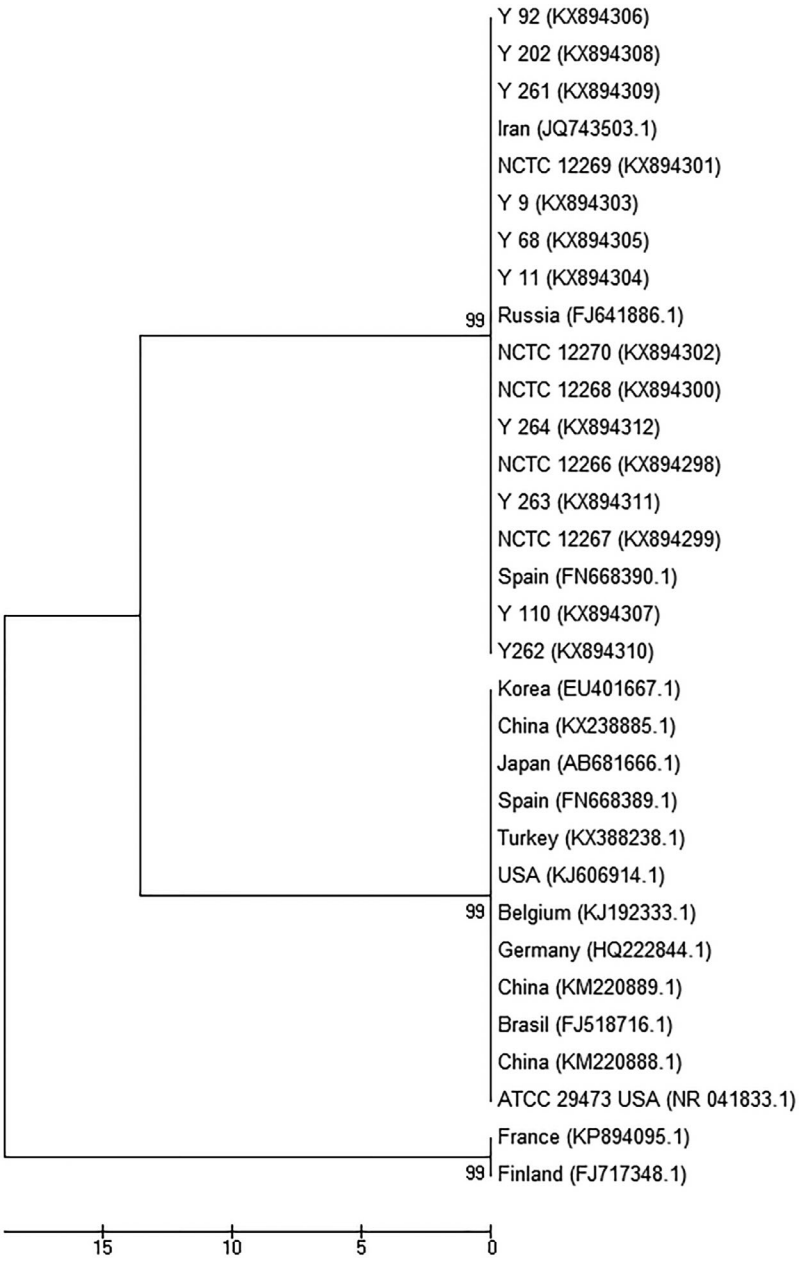

Fig. 4. Comparison of *Yersinia ruckeri* isolates in GenBank; accession numbers are given in brackets. In the tree, the percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are indicated next to the branches

tant isolates. All resistance genes were confirmed by sequence analysis, and the sequences have been deposited in GenBank under accession numbers KY001929 to KY001934. One isolate from Central Anatolia carried *sulI* and *tetE*, and 1 from the Eastern Anatolia region carried *tetC* and *tetD* (multiple antimicrobial resistance). Additionally, 2 reference strains were detected as phenotypically resistant to SXT.

DISCUSSION

Antimicrobial resistance surveys of *Yersinia ruckeri* are rare, and the characterization of isolates is important to acquire information about resistance. The regional and territorial similarities of 137 *Y. ruckeri* isolates isolated from 6 regions of Turkey in comparison to 5 reference strains were investigated in this study. The results showed that the isolates from Turkey and other countries present at least 80% similarity (the sequences have been deposited in GenBank). All of the isolates were resistant to SUL but were highly susceptible to SXT, which is the most commonly used antimicrobial agent in aquaculture for the control of ERM. To our knowledge, this is the first identification of *floR, sulI, tetC, tetD*, and *tetE* genes in *Y. ruckeri* isolates from aquaculture.

Although ERM causes acute infection, especially in younger fish (fry and fingerlings), disease can affect fish of all ages, resulting in high mortality. The disease appears as a more chronic condition in older/larger fish (Kumar et al. 2015), and in this study, *Y. ruckeri* was isolated from fish weighing between 0.1 and 3000 g. The risk of vertical transmission has been suggested based on the recovery of the bacterium from eggs and ovarian fluid (Kumar et al. 2015). However, this has yet to be demonstrated definitely. In the present study, the isolation of *Y. ruckeri* after the yolk sac stage from rainbow trout fry (0.1 g) that had been reared in full controlled hatcheries and had never been exposed to pathogens until this life stage supports the suspicion of vertical transmission. In addition to vertical transmission, *Y. ruckeri* has been isolated from animals other than fish, including muskrats *Ondatra zibethicus*, kestrels (*Falco* spp.), gulls (Laridae), turtles (Cheloniidae), and humans (Farmer et al. 1985, Willumsen 1989, Tobback et al. 2007).

In our study, *Y. ruckeri* was isolated from water samples in addition to rainbow trout, demonstrating that resistance genes could transfer from water to inland

Table 5. *Yersinia ruckeri* antimicrobial resistance genes. Only phenotypic and genotypic isolates are given; **bold** text indicates minimum inhibitory concentration (mg l^{-1}). FFC: florfenicol; SUL: sulfamethoxazole; TET: tetracycline; SXT: sulfamethoxazole+ trimethoprim. +: gene found; –: gene not found

Phylogenetic group	Isolate ID no.	FFC	*floR*	SUL	*sulI*	*sulII*	TET	*tetC*	*tetD*	*tetE*	SXT 19/1
B	Y 241	4	–	256<	–	–	1	–	–	–	**8**
B	Y 248	**32**	–	256<	–	–	2	–	–	–	4
M	Y 121	4	–	256<	+	–	0.512	–	–	+	4
G	Y 89	4	–	256<	+	–	0.512	–	–	–	4
A	Y 13	**64**	–	256<	–	–	4	–	–	–	2
A	Y 14	**128**	–	256<	–	–	1	–	–	–	4
D	Y 10	**16**	–	256<	–	–	0.512	–	–	–	**16**
D	Y 7	**16**	–	256<	–	–	4	–	–	–	**32**
A	Y 200	2	–	256<	–	–	**32**	–	–	–	4
J	Y 199	**8**	–	256<	–	–	1	–	–	–	**16**
A	Y 183	2	–	256<	–	–	**32**	–	–	–	4
A	Y 132	2	–	256<	–	–	**32**	–	–	–	4
L	Y 131	0.512	–	256<	–	–	**8**	–	–	+	**8**
A	Y 120	4	–	256<	–	–	**32**	–	–	–	4
A	Y 91	**8**	–	256<	–	–	1	+	–	–	4
A	Y 66	4	–	256<	–	–	**64**	–	–	–	4
O	Y 68	**16**	–	256<	–	–	4	–	–	–	**8**
A	Y 71	4	–	256<	–	–	**8**	–	–	–	4
A	Y 78	4	–	256<	–	–	**32**	–	–	–	4
A	Y 106	4	–	256<	–	–	1	+	–	–	4
A	Y 107	4	–	256<	–	+	1	–	–	–	4
C	Y 11	**256**	+	256<	–	–	2	+	–	–	**256**
E	Y 9	**256**	–	256<	–	–	**64**	+	+	–	**256**
A	Y 40	4	–	256<	–	–	1	–	–	–	**256**
A	Y 43	4	–	256<	–	–	0.512	–	–	–	**256**
A	Y 255	**8**	–	256<	–	–	4	–	–	–	4
A	Y 256	**8**	–	256<	–	–	**32**	–	–	–	4
A	Y 212	2	–	256<	–	–	2	–	–	–	**8**
A	Y 217	2	–	256<	–	+	**8**	–	–	–	4
A	Y 206	2	–	256<	–	–	**64**	–	–	–	4
A	Y 232	4	–	256<	–	–	**64**	–	–	–	4
A	Y 231	4	–	256<	–	–	1	–	–	–	**8**
A	Y 260	**8**	–	256<	–	–	1	–	–	–	4
A	Y 257	**8**	–	256<	–	–	2	–	–	–	4
A	Y 258	**8**	–	256<	–	–	2	–	–	–	4
A	Y 210	0.256	–	256<	–	+	1	–	–	–	4
A	Y 225	4	–	256<	–	–	**32**	–	–	–	4
A	Y 211	0.256	–	256<	–	+	1	–	–	–	4
A	Y 96	**8**	–	256<	–	–	1	–	–	–	4
A	Y 233	4	–	256<	–	–	**16**	–	–	–	**256**
A	Y 234	4	–	256<	–	–	**8**	–	–	–	2
A	Y 236	1	–	256<	–	–	**8**	–	–	–	4
A	Y 239	4	–	256<	–	–	**8**	–	–	–	2
A	Y 244	**16**	–	256<	–	–	1	–	–	–	4
A	Y 247	**32**	–	256<	–	–	2	–	–	–	2
A	Y 252	4	–	256<	–	–	**8**	–	–	–	4
H	Y 92	**64**	–	256<	–	–	2	–	–	–	**128**
I	Y 93	**16**	–	256<	–	–	1	–	–	–	**16**
N	Y 95	**64**	–	256<	+	–	1	–	–	–	**256**
A	Y 261	1	–	256<	–	+	0.128	–	–	–	2
A	Y 262	4	–	256<	–	+	0.256	–	–	–	4
A	Y 263	4	–	256<	–	+	0.128	–	–	–	2
A	Y 265	1	–	256<	–	+	0.512	–	–	–	2
NCTC	12268	4	–	256<	–	–	0.256	–	–	–	**8**
NCTC	12270	4	–	256<	–	–	0.256	–	–	–	**8**
E. coli	25922	2	–	256	–	–	0.128	–	–	–	**2**

ecosystems. Both PCR analyses and biochemical tests have been used to detect the bacterium in infected or carrier fish and water or sediment samples previously (Gibello et al. 1999). We sampled infected or seemingly healthy fish, water, and sediment from different rainbow trout farms and isolated *Y. ruckeri* from infected or seemingly healthy fish tissue or organs and one isolate from water samples.

Analyses of the pathogen's genetic diversity and population structure using genotyping techniques are required to improve our understanding of *Y. ruckeri* and subsequently farm management. Several investigations have genotyped *Y. ruckeri* using different RAPD-PCR methods (Coquet et al. 2002, Altun et al. 2013, Huang et al. 2013, Glenn et al. 2015, Altinok et al. 2016a). Huang et al. (2013) and Bastardo et al. (2011) used different PCR methods to determine the molecular characteristics of *Y. ruckeri* and found that ERIC-PCR exhibited the highest discriminatory power compared with other PCR-based methods. We determined 18 fingerprinting patterns in our RAPD analysis of 141 Turkish *Y. ruckeri* isolates characterized by ERIC-PCR and detected 4 different genogroups with 72% similarity coefficients. Most of the isolates (82.3%) showed 100% fingerprinting pattern similarities with the serotype O1 reference strain. Additionally, 4 isolates exhibited 100% similarity with the serotype O2 reference strain, and the other reference strains exhibited at least 90% similarity with these 2 groups. Using RAPD-PCR, previous investigators identified 5 or 6 genogroups of *Y. ruckeri* isolates from Turkey (Onuk et al. 2011, Altun et al. 2013). Although Altun et al. (2013) found that 15 Turkish *Y. ruckeri* isolates and a strain from Denmark were very similar, we found that all of the Turkish isolates from the present study were from a different genogroup than those from other countries, with the exception of the isolates from Iran (JQ743503.1), Russia (FJ641886.1), and Spain (FN668390.1). The isolates from France and Finland exhibited the lowest (at least 80%) similarity. These findings support the results of a study by Altinok et al. (2016a) in which 38 *Y. ruckeri* strains were characterized using other methods. All 137 Turkish isolates were classified into genogroups different from the isolates from other countries (except for the Iranian, Russian, and some Spanish isolates). These findings indicate that ERM must be controlled by different measures specific for territorial outbreaks.

Although intensive vaccination programs against yersiniosis are performed in Turkey, ERM causes serious mortality in both vaccinated fish and fish with weights unsuitable for vaccination in Turkey (Bastardo et al. 2011, Altinok et al. 2016b). We sampled and observed rainbow trout from the stage when they first started feeding through all vaccination periods (before and after vaccination). Isolation of the bacterium from most samples indicates that efficient immunization and control for yersiniosis are not occurring in Turkey. Therefore, antimicrobials (e.g. SXT, FFC, and TET) are used in rainbow trout farms. Previous studies demonstrated variation in *Y. ruckeri* antimicrobial resistance levels (Balta et al. 2010, Altun et al. 2013, Huang et al. 2013, Orozova et al. 2015, Ture & Alp 2016). We found that more than 80% of the *Y. ruckeri* isolates were susceptible to SXT, FFC, and TET; in contrast, no isolates were susceptible to SUL, similar to other investigations (Balta et al. 2010, Ture & Alp 2016). The most commonly used antimicrobials (SXT and FFC) may still be effective for the control of ERM because only 12.6 and 14.7%, respectively, were resistant. As shown in the present study, sulfonamide group antimicrobials cannot be successfully used alone to control ERM. Field observations suggest that TET resistance has increased compared with FFC and SXT resistance, likely because rainbow trout farmers prefer to use TET-group antimicrobials in uninfected fish during various handling procedures.

Genotypically, antimicrobial resistance surveys in *Y. ruckeri* are rare, whereas phenotypic antimicrobial resistance has been analyzed in previous investigations (Balta et al. 2010, Huang et al. 2013, Ture & Alp 2016). In *Y. ruckeri* isolates, Huang et al. (2013) and Ture & Alp (2016) detected only the *sulII* sulfonamide resistance gene, and Balta et al. (2010) detected only the *tetA* and *tetB* resistance genes. To our knowledge, our study is the first to characterize antimicrobial resistance genes, including *floR, sulI, tetC, tetD*, and *tetE*, in *Y. ruckeri* isolated from aquaculture. In this study, we detected the *sulII* gene, similar to other investigations, but we did not detect *tetA, tetB, tetM, tetS*, or *sulIII*, which was in contrast to previous investigations (Balta et al. 2010, Huang et al. 2013, Ture & Alp 2016). We confirmed all resistance genes by sequencing and deposited the information in GenBank under accession numbers KY001929 to KY001934. All *sulII* resistance genes were detected in common *Y. ruckeri* genogroup isolates (with 82.3% similarity); all other resistance genes (except for 1 *tetC* gene) were detected in the *Y. ruckeri* isolates, except for the most common genogroup. One isolate from 2014 carried both *tetC* and *tetD*. We detected 1 *floR* gene, and 21 *Y. ruckeri* isolates were phenotypically resistant. Interestingly, although 19 isolates were phenotypically resistant to TET, most (except 2) did not carry

TET-resistance genes. Our findings demonstrate that even phenotypically resistant isolates do not necessarily carry those specific resistance genes. We isolated a *Y. ruckeri* isolate from water samples that was resistant to TET and SXT; this isolate also carried the *tetE* gene, which was identified here for the first time in *Y. ruckeri*. Water samples carrying the bacteria harboring resistance genes show potential for resistance-gene transfer from water to inland ecosystems and humans.

CONCLUSION

Yersinia ruckeri isolates from 18 different genogroups were isolated from 6 different regions of Turkey; most of these isolates clustered into a genogroup similar to that of serotype O1. All of our isolates separated into genogroups that differed from most of the isolates from other countries, indicating that different control measures should be used. We detected some resistance genes in *Y. ruckeri* in our study, and detection of antimicrobial resistance genes in water samples indicated high gene transfer potential from aquaculture to other ecosystems. Thus, region- or farm-specific control measures should be implemented to control yersiniosis and the presence of resistance genes. This study is the first to describe some antibiotic resistance genes of *Y. ruckeri* isolates. This is the most comprehensive study between antimicrobial resistances and genetic relationships of *Y. ruckeri* isolated from 6 regions including the biggest rainbow trout farm in Turkey and shows that antibiotic-resistant *Y. ruckeri* isolates are genetically diverse. Fish and water samples may be an important reservoir for dissemination of antibiotic resistance, with implications for public health.

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