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ISSN 0792 - 156X

PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

Bacteria in Rainbow Trout (*Oncorhynchus mykiss*) in the Southern Black Sea Region of Turkey - A Survey

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(Received 24.9.08, Accepted 18.3.09)

Key words: antimicrobial susceptibility, bacterial fish disease, coldwater diseases, Black Sea fish farms

Abstract

Thirty-two freshwater rainbow trout (*Oncorhynchus mykiss*) farms, two rainbow trout marine cages, and one research station in the Black Sea region of Turkey were surveyed for bacterial pathogens and diseases in 2006-2008. Forty bacterial isolates were phenotypically identified in 558 fish. Yersiniosis, furunculosis, vibriosis, motile *Aeromonas* septicemia, bacterial cold water disease, and *Pseudomonas* infection were recorded. Infections caused by *Yersinia ruckeri*, *A. hydrophila*, and *A. salmonicida* occurred most frequently, but one or two outbreaks of *P. putida*, *Flavobacterium psychrophilum,* and *P. luteola* infection were also recorded. Most of the bacteria were isolated in spring and summer rather than fall and winter. Susceptibility to antibiotics was fairly consistent regardless of geographic area or year of isolation. Fifty percent or more of the bacteria were resistant to ampicillin, cephalothin, erythromycin, neomycin, sulfamethoxazole, tetracycline, and O/129. The most effective antibiotics were oxolinic acid and florefenicol.

Introduction

Disease is a primary constraint in aquaculture and can severely impact economic and socio-economic development in many countries (Subasinghe and Bernoth, 2000). The appearance and development of a fish disease is the result of pathogen-host interactions and the environment. A relatively small number of pathogenic bacteria are responsible for the most important economic losses in cultured fish (Toranzo et al., 2005).

Antibacterial treatments are most often administered to fish through medicated feed. Contamination of the surrounding environment by these drugs occurs principally through leaching of uneaten food and feces (Hirsch et al., 1999). In addition to the release of active molecules into the aquatic ecosystem, normal therapeutic aquaculture practices can lead to the selection of antimicrobial resistance in fish pathogens and environmental bacteria (Alderman and

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Hastings, 1998). Antibacterial resistance in the freshwater environment of fish farming activities is more recent and poorly documented (Schmidt et al., 2000). Nowadays, because of land-use management of freshwater and river ecosystems, it is necessary to enhance this knowledge (Gordon et al., 2007).

Many of the diseases that affect domestic fish populations are also a threat to wild fish populations (Thoesen, 1994). Any systematic study of fish populations will undoubtedly reveal the presence of novel disease conditions. Whether they are truly emerging or were merely ignored in the past is a subject of conjecture. New pathogens may appear to take the place of existing problems (Austin and Austin, 2007). Therefore, fish farms should be routinely surveyed for pathogens.

The objectives of this study were to isolate and identify pathogenic and nonpathogenic fish bacteria from 35 rainbow trout farms in the southern region of the Black Sea and examine the resistance of the isolates to antimicrobials. The resulting data will serve as a basis for future comparisons.

Materials and Methods

Sampling. Rainbow trout (*Oncorhynchus mykiss*) were sampled from 34 sites in northeast Turkey: two farms that culture trout in cages in the Black Sea and 32 farms located on different streams that are not interconnected. The annual production of each of the 34 farms ranged 5-100 tons. Fish were sampled from 13 farms in the winter, 31 in the spring, 29 in the summer, and 17 in the fall of 2006-2008. During the two years, 540 fish were sampled during 90 samplings; the samples usually comsisted of six fish per farm per season. In addition, 18 fish were sampled as a result of disease outbreaks. Fish were randomly sampled when diseased fish were not present in the farm. Water quality characteristics were measured in all samples (Table 1): total hardness and total alkalinity by the titration method (Boyd and Tucker, 1992), dissolved oxygen and water temperature with a WTW 330i polarographic oxygen meter and thermistor (WTW Wissenschaftlich-Technische Werkstatten GmbH, Weilheim, Germany), and pH with a WTW 330 glass electrode (WTW Wissenschaftlich-Technische Werkstatten).

Identification of bacteria. The 558 fish (5-250 g) were transported to the laboratory on ice and dissected for bacteriological examination. All were examined externally and internally. The gills and body surface were examined microscopically for the presence of *Flavobacterium* sp. Then the body surface was swabbed with 70% ethyl alcohol to prevent contamination of the culture by normal external bacterial flora. The liver, trunk kidney, and spleen were aseptically streaked on tryptic soy agar (TSA) or marine agar, depending on the origin of the fish. Samples from external lesions, if present, were streaked on cytophaga agar, Shu-Shotts agar, and TSA. After incubation at 20°C for 4 days or at 15°C for 10 days, isolated bacteria were subcultured on the same medium to check the purity of the isolate. Pure cultured colonies were biochemically characterized with API 20E, API 20NE (Biomerieux, Marcy l'Etoile, France), and the following biochemi-

Table 1. Water characteristics (mean±SD) during the sampling period.

	Season			
	Spring	*Summer*	*Fall*	*Winter*
Dissolved oxygen (mg/l)	8.78±1.36	8.99±1.45	10.52±1.78	10.71±0.82
Temperature (°C)	11.4±3.6	17.6±4.7	10.3±4.4	7.7±1.4
pH	7.07±0.36	7.63±0.25	7.25±0.35	7.53±0.14

cal tests: Gram staining, cytochrome-oxidase, catalase, ß-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophane deaminase, indole production, voges proskauer, gelatinase, fermentation of glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, amygdalin, methyl red, arabinose, lactose, esculine, xylose, motility, and oxidative/fermentative tests. Isolates were identified to the genus or species level by standard bacterial taxonomy procedures (Krieg and Holt, 1984; Holt et al., 1994; Austin and Austin, 2007). Isolates were stored in a broth culture supplemented with 15-20% glycerol at -70°C.

Susceptibility to antibiotics. Susceptibility of all isolated bacteria was determined by the agar diffusion method using 9 mm diameter commercial discs (Bioanalyse, Ankara, Turkey) that contained the following antibiotics (mg/disc): ampicillin 10, cephalothin 30, erythromycin 15, gentamycin 10, kanamycin 30, neomycin 5, oxolinic acid 2, oxytetracycline 30, streptomycin 10, sulphamethoxazole 100, tetracycline 10, and florfenikol 30. Antimicrobial tests were applied on Muller Hinton agar. Bacteria were incubated at 15 or 20°C overnight and the susceptibility of the isolates was determined according to NCCLS (2002). Isolates were considered susceptible if the inhibition zone equaled or exceeded 17 mm for ampicillin, 18 mm for cephalothin and kanamycin, 23 mm for erythromycin and neomycin, 15 mm for gentamycin, oxytetracycline, streptomycin, and florfenikol, 20 mm for oxolinic acid, and 16 mm for sulphamethoxazole.

Results

Forty kinds of bacteria were isolated from 115 fish (Table 2). Thirty-four were identified to the species level and six to the genus level. Eighteen were putative pathogens while the remainders were ubiquitous environmental organisms.

The most common pathogenic bacteria isolated from both freshwater and marine farms were sorbitol-negative *Y. ruckeri*. *Yersinia ruckeri* was isolated from the liver, spleen, and kidney in all seasons but was most common in summer. Of the pathogenic bacteria, *Aeromonas caviae, A. hydrophila,* and *Pseudomonas putida* were isolated from kidney, liver, spleen, and skin lesions while *A. sobria* and *Photobacteriom damsella* were isolated solely from the kidney. When fish had clinical signs of disease, the following bacteria were isolated; *A. caviae*, *A. hydrophila, Flavobacterium psychrophilum* (dark pigmentation, fried fin), *Pseudomonas aeroginasa* (dark pigmentation, ascites, and exophthalmia) *P. fluorescens, V. fluvialis* (pitechial hemorrhage on the abdomen), and *Y. ruckeri* (hemorrhage on liver, enlarged spleen, and exophthalmia).

The disk susceptibility pattern was fairly consistent regardless of geographic area or year of isolation. Fifty percent or more of the bacteria were resistant to ampicillin, cephalothin, erythromycin, neomycin, sulfamethoxazole, tetracycline, and O/129 (Table 3). The most effective antibiotics were oxolinic acid and florefenicol.

Discussion

The range of diseases found in aquaculture vary, some with low or unknown host specificities and many with non-specific symptoms. *Pseudomonas luteola* has been associated with human clinical infections but was not identified in Turkish aquaculture until 2006 (Altinok et al., 2007). *Pseudomonas luteola* infection in fish was reported by Altinok et al. (2007). Although *Burkholderia cepacia, Cedecea davisae, Chromobacterium violaceum, Enterobacter amnigenus, Ewingella americana, Pasteurella pneumotropica, Pseudomonas caryophylii, P. dilafialdi, P. diminuta, P. solanacearum, Shigella sonnei,* and *Weeksela virosa* were isolated in the present study, they are not cited as a fish pathogens. Koch's rules that determine whether a given organism can cause a given disease will be applied to these bacteria in our future research.

There were seasonal trends in fish diseases. When the water temperature was 11.4°C in the spring and 10.3°C in the fall, the numbers of isolated bacteria were 37 and 14, respectively. In spring, small young fish join the stock. Since their immune system is not entirely established they

Table 2. Bacteria found in 35 rainbow trout farms in Turkey (2006-2008).

Bacteria	Season					Organ			
	Spring	Summer	Fall	Winter	Total	Kidney	Spleen	Liver	Skin
*Aeromonas caviae**	4	3	-	-	7	5	3	4	1
*A. hydrophila**	3	3	1	4	11	5	6	8	1
*A. salmonicida**	-	1	2	1	4	1	2	2	-
*A. sobria**	1	-	-	-	1	1	-	-	-
Aeromonas spp.	-	1	-	1	2	1	2	1	-
Burkholderia cepacia	5	1	-	-	6	4	1	3	1
Cedecea davisae	1	1	-	-	2	2	2	2	-
Chromobacterium violaceum	-	1	-	-	1	1	-	-	-
*Citrobacter freundii**	-	-	-	1	1	-	1	-	-
Edwardsiella sp.	-	1	-	-	1	-	-	1	-
*Eikenella corrodens**	-	-	1	-	1	1	-	-	-
Enterobacter amnigenus	1	-	-	-	1	-	-	1	-
*E. cloacae**	1	-	-	-	1	1	1	1	-
Erwinia sp.	-	1	1	1	3	2	-	1	-
Ewingella americana	1	-	-	1	2	3	1	1	-
*Flavobacterium psychrophilum**	-	-		1	1	-	-	-	1
*Hafnia alvei**	1	1	-	-	2	2	1	1	-
Mannheimia haemolytica /Pasteurella trehalosi	-	1	-	-	1	-	-	1	-
Pantoea spp. 1	-	1	-	-	1	1	-	1	-
Pasteurella pneumotropica	2	4	1	1	8	3	5	5	-
*Photobacteriom damsella**	-	-	-	1	1	1	-	-	-
*Plesiomonas shigelloides**	2	-	-	-	2	1	1	1	-
*Pseudomonas aeroginasa**	2	1	-	-	3	2	2	2	-
P. caryophylii	-	-	1	-	1	-	-	1	-
P. dilafialdi	1	1	-	-	2	1	-	1	-
P. diminuta	-	-	2	-	2	-	2	-	-
*P. floresans**	1	-	-	-	1	1	-	1	-
*P. luteola**	-	1	-	1	2	1	1	-	-
P. solanacearum	-	-	1	-	1	1	-	-	-
*P. putida**	1	-		-	1	1	1	1	1
Pseudomonas spp.	-	1	1	1	3	2	2	3	-
Salmonella sp.	-	-	1	-	1	1	-	-	-
*Serratia liquefeciens**	1	-	-	-	1	2	-	-	-
Shewanella putrefaciens grp*	1	-	-	-	1	-	-	-	1
Shigella sonnei	-	1	-	-	1	-	-	1	-
*Stenotrophomonas maltophilia**	2	-	1	-	3	1	1	1	-
*Vibrio fluvialis**	2	-	-	-	2	2	2	2	-
Weeksela virosa	1	-	-	-	1	1	-	-	-
Yersinia pseudotuberculosis	-	-	-	2	2	-	-	2	-
*Y. ruckeri**	3	20	1	3	27	27	20	20	-
Total	37	45	14	19	115	78	57	69	6

* putative pathogens to fish

Table 3. Disc susceptibility of bacteria to antimicrobial agents.

Antimicrobial agent	*No. isolates tested*	*Resistance (%)*	*Intermediate (%)*	*Susceptible (%)*
Ampicillin (AM10)	89	65.17	1.12	33.71
Cephalothin (KF30)	97	71.13	3.09	25.77
Erythromycin (E15)	98	52.04	37.76	10.20
Gentamycin (CN10)	90	20.00	12.22	67.78
Kanamycin (K30)	52	26.00	14.00	60.00
Neomycin (N5)	30	86.67	6.67	6.67
Oxolinic acid (OA2)	79	22.78	3.80	73.42
Oxytetracycline (T30)	99	26.26	8.08	65.66
Streptomycin (S10)	29	31.03	17.24	51.72
Sulfamethoxazole (SMZ100)	73	54.79	5.48	39.73
Tetracycline (TE10)	27	66.67	3.70	29.63
O/129*	7	85.71	0.00	14.29
Florfenicol	20	10	5	85

* Vibriostatic agent O/129 is 2,4-diamino-6,7-diisopropylpteridine

are very susceptible to pathogenic diseases (Plumb, 1999). Therefore mortality in spring was higher than in fall. In summer, the combined effect of young fish and high water temperature resulted in higher mortality.

The most commonly reported bacterial fish diseases in Europe are vibriosis, pasteurellosis, enteric red mouth disease, furunculosis, marine flexibacteriosis, bacterial coldwater disease, rainbow trout fry syndrome, columnaris disease, motile *Aeromonas* septicemia, pseudomoniasis, streptococcocis, mycobacteriosis, and epitheliocystis (Toranzo, 2004). All these disease agents, except marine flexibacteriosis, columnaris disease, streptococcocis, mycobacteriosis, and epitheliocystis, were isolated in this survey.

According to regulation 91/67/EEC of the European Union Council, screening of fish farms reduces the risk that animals carrying opportunistic agents proliferate during shipping, handling, or change of environment and that resistant or tolerant animals transfer a significant pathogen to a population that may be susceptible to infection.

Tetracycline is the most commonly used antibiotic in fish farms in Turkey. Therefore, 67% of the bacteria were resistant to it. On the other hand, florfenicol has been used in aquaculture for only a couple years and most of the bacteria were not resistant to it. The use of both these antibiotics in aquaculture is authorized by the European Union by council regulation (EEC) No. 2377/90 (Costello et al., 2001). Antibiotics such as ampicillin, erythromycin, gentamycin, kanamycin, neomycin, oxolinic acid, oxytetracycline, streptomycin are also commonly used in Europe and Turkey (Costello et al., 2001). The greater incidence of resistant strains in fish farms in our study is of concern since intensive fish farming in Turkey has lead to the widespread use of antibiotics for treatment and prevention. The horizontal spread of resistance genes might have occurred, as several antibiotics resistance determinants associated with mobile genetic elements have been identified with regard to aquaculture (Rosser and Young, 1999).

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