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Occurrence of Drug-Resistant Bacteria in Two European Eel Farms

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The occurrence of strains that are resistant to oxolinic acid, oxytetracycline, sulfamethoxazole-trimethoprim, and nitrofurantoin among heterotrophic bacteria, including human and fish pathogens, in two freshwater eel farms was investigated. High levels of individual- and multiple-drug-resistant bacteria were detected, although sampling events were not correlated with clinical outbreaks and drug therapy.

The intensive use of chemotherapeutic agents to treat infectious diseases in reared fish has led to an increased frequency of drug-resistant microorganisms, as well as bacteria showing multiple-antibiotic resistance (1, 2, 9, 14). Several studies have reported the development of bacterial resistance to antimicrobials in different types of aquacultural production (7, 13, 14, 16–20). However, there are few available data about the incidence of drug-resistant bacteria in intensive freshwater culture of eels (3).

The aim of the present study was to determine the occurrence of drug-resistant heterotrophic bacteria associated with eel culture in two freshwater farms located in Valencia, Spain. Moreover, we focused on human pathogens, such as *Salmonella* spp. and *Vibrio cholerae*, as well as the fish pathogen *Vibrio vulnificus* serovar E, responsible for severe outbreaks among eels (4). The antimicrobials selected were oxolinic acid (OXA), oxytetracycline (OTC), and sulfamethoxazole-trimethoprim (SXT), all of which were used for the treatment of bacterial infections in both eel farms and allowed by the European Community regulations (no. 2377/90, annex I), and nitrofurantoin (NIT), which was sporadically used several years ago.

Two sampling events, which took place in February and June 2002, were carried out in two intensive eel hatcheries located in Valencia, Spain. Farm 1 is densely stocked (300 kg fish m^{-2}), obtaining glass eels from different Spanish and European sources. Farm 2 obtains glass eels from a lake nearby and grows them to adulthood at densities of 4 kg fish m^{-2} in order to repopulate natural habitats. The farms are located approximately 60 km apart, and each uses freshwater from springs nearby. Eels in both farms are reared with dried food. Water samples were collected from inlet, tank, and outlet waters as previously described (16). Two silver eels and around 50 g of glass eels (average weight, 0.3 g per fish) were caught in each sampling. Glass eels were homogenized in 200 ml of phosphate-buffered saline (pH 7) by use of an IUL masticator. Silver eel intestines were processed as described before (9). Serial 10-fold dilutions of the samples in phosphate-buffered saline were prepared, and 0.1-ml aliquots were streaked in duplicate on tryptone soy agar (TSA) (Pronadisa, Madrid, Spain) and TSA plates supplemented with OXA (10 μg ml^{-1}), OTC (15 μg ml^{-1}), SXT (30 μg ml^{-1}), and NIT (15 μg ml^{-1}) (Sigma-Aldrich Co., St. Louis, Missouri). Stock solutions of the drugs were prepared as previously described (12). TSA plates were incubated at 25°C for 24 h. Ten colonies were randomly picked from TSA and TSA-drug-supplemented plates. Isolation and identification were conducted as previously described (11), and further characterization was carried out by using API 20E and API 20 NE strips (BioMérieux, Marcy-L'Etoile, France). The selected strains from TSA-drug-supplemented plates were tested for additional resistances to the other drugs by the disk diffusion method in Mueller-Hinton agar (Pronadisa, Madrid, Spain) (8). Disks containing SXT (25 μg), OTC (30 μg), NIT (50 μg), and OXA (2 μg) (Oxoid, Madrid, Spain) were used, and sensitivity/resistance was determined according to the sizes of the inhibition zones (12).

On the other hand, 10 ml of each sample was inoculated on 100 ml of alkaline-peptone-water (pH 8.5) enrichment, and 0.1 ml was subcultured 6 h later in thiosulfate citrate bile salt (TCBS) agar (Difco) and cellobiose-polymixin B-colistin agar (5). All the samples were also streaked on Hektoen enteric (HE) agar plates (Pronadisa). Plates were incubated at 37°C for 24 h. Presumptive black and green colonies on hemagglutinin and yellow colonies on TCBS agar were identified as mentioned above and tested for sensitivity to the four drugs used as previously mentioned (8).

The results of heterotrophic bacterial counts are shown in Table 1. The percentages of drug-resistant isolates were computed for each eel farm and for all kinds of samples. Two analyses of variance (for water and fish samples) were performed using the SPSS program, version 10, to detect differences between the rates of resistance to the drugs used, the farms, and the types of samples. Resistance to SXT was significantly higher ($P = 0.001$) than resistance to any other individual drug, and this highest resistance was followed by resistances to OTC, NIT, and OXA. The levels of resistance to individual and multiple antibiotics for bacteria in farm 1 were significantly higher ($P = 0.024$) than those for bacteria in farm 2. Comparison of water samples showed that inlets contained significantly ($P = 0.0001$) smaller fractions of resistant bacteria in both farms. Samples from the eel intestines and outlet water had the highest numbers of strains showing multiple resistance, whereas no multiple resistance was detected for samples from inlet water (Table 1). These results show that levels of individual- and multiple-drug resistance increase for bacteria in pond

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TABLE 1. Percentages of antimicrobial resistance among the heterotrophic bacteria recovered from water and fish samples in two eel farms and isolated on TSA, HE, and TCBS agar

Sample	Count[a]	% Resistant to[b]:				
		OTC	NIT	OXA	SXT	≥2 R
Total heterotrophic bacteria						
Farm 1						
Water						
Inlet	1.5×10^2	7.6	14.1	13.2	26.8	0
Pond	1.7×10^7	44	32	41	33.3	60
Outlet	4.1×10^7	41.3	53.4	49.5	80	70
Glass eel	4.1×10^6	17.6	28.6	15.2	75.7	50
Eel intestine	7.6×10^7	18.8	38	14.9	86.7	80
Farm 2						
Water						
Inlet	3.3×10^3	19.4	23.4	20.8	30.4	0
Pond	4×10^4	20.1	33.2	21.6	50	25
Outlet	7.8×10^5	19.3	34.8	26	87.2	60
Glass eel	3.6×10^5	25.5	39.6	11.8	70.3	45
Eel intestine	4.2×10^7	18.2	27.2	22.5	75.4	70
Motile *Aeromonas*						
Farms 1 and 2						
Water						
Inlet	7	25	0	0	50	20
Pond	9	20	30	10	70	40
Outlet	10	33.3	41.6	16.6	75	58.3
Glass eel	9	44.3	33.3	11.1	41.6	66.6
Eel intestine	12	58.3	50	16.6	58.3	58.3
Salmonella						
Farm 1 (outlet water)	3	33.3	100	0	100	100
Vibrio furnissii						
Farms 1 and 2						
Glass eel	5	40	60	20	60	60
Eel intestine	9	33.3	77.5	22.2	33.3	77.5
Vibrio mimicus						
Farm 1 (water)						
Pond	3	0	100	0	0	0
Outlet	3	0	100	0	66.6	66.6

[a] Counts of total heterotrophic bacteria are expressed as CFU ml^{-1}, and counts of identified anaerobic facultative bacteria are expressed as number of strains isolated.

[b] Percentages of total heterotrophic bacteria are derived from differential plating on TSA medium with and without the respective drugs. Percentages of resistance of motile *Aeromonas, Salmonella*, *V. furnissii*, and *V. mimicus* were obtained after testing isolates by the agar diffusion method. ≥2 R, percentages of isolates resistant to two or more antibiotics.

and outlet waters, probably due to the impact of farming, as stated before (16). Previous studies of aquaculture systems suggested a relationship between the levels of resistance and the drugs used in farms (9). However, in our study, a correlation between antibiotic usage and antibiotic resistance could not be established, since the sampling events were not correlated with clinical outbreaks and antibiotic therapy. Thus, our data support the hypothesis that resistant bacteria are present in eel farm environments during periods in which no antibacterial therapy is administered. In this sense, silver eels could be a reservoir for resistant bacteria, as the highest percentages of multiresistant strains were recovered from eel intestines as well as from outlet water, in which eel feces are present (Table 1).

The predominant microbiota recovered from TSA plates with water samples were gram-negative nonfermentative bacteria, whereas gram-negative fermentative bacteria were the most frequently recovered from fish samples, as reported before (9, 11, 14, 15, 16). In contrast, motile *Aeromonas* strains were preponderant in all types of samples when selective media (HE agar and TCBS agar) were used. These aeromonads were mainly resistant to SXT and OTC (Table 1), in accordance with previous reports (14, 15). No *V. cholerae* strains were isolated on TCBS agar; instead, *Vibrio mimicus* and *Vibrio furnissii* were recovered from water and fish, respectively, on this medium (Table 1). Previous studies reported similar resistance patterns for *V. furnissii* strains recovered from a water pond in a brackish-water eel farm (10). Three *Salmonella* strains from HE agar plates were identified, coming from different sampling events in outlet water in farm 1 (Table 1). Both farms studied are located some way from human habitation and are not integrated with any other animal exploitations, which probably accounts for the low incidence of human pathogens. Interestingly, no growth on the plates used for the isolation of the eel pathogen *V. vulnificus* was detected. Previous studies reported that *V. vulnificus* could not be isolated from eel farm water, even when the environmental conditions were optimal for its recovery, suggesting that it was probably present below the level of plate detection (6).

Our results showed a high frequency of individual- and multiple-drug-resistant microorganisms occurring in the freshwater eel farm environment. These heterotrophic bacteria associated with eel culture included potential pathogens for humans and fish, such as *Salmonella* spp., *Aeromonas* spp., *V. mimicus*, and *V. furnissii*.

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