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Virulence of *Aeromonas hydrophila* and some other bacteria isolated from European eels *Anguilla anguilla* reared in fresh water

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ABSTRACT: Studies were conducted to determine the pathogenicity for European eels *Anguilla anguilla* and rainbow trout *Oncorhynchus mykiss* of several strains of the species *Aeromonas hydrophila*, *A. jandaei*, *A. sobria*, *A. caviae*, *Plesiomonas shigelloides*, *Vibrio anguillarum*, *Pseudomonas fluorescens* and *Shewanella putrefaciens* isolated from an eel farm. Virulence of 20 isolates obtained during epizootics from diseased eels, asymptomatic carriers and tank water and of 20 isolates obtained during the absence of disease from healthy eels and water was evaluated by 2 challenge methods: intraperitoneal injection (IP) and bath exposure. Of the isolates tested by IP, all strains of *A. hydrophila*, and those of *A. jandaei* mainly from epizootics, were pathogenic for eels (LD_{50} dose $10^{5.4}$ to $10^{7.5}$ cfu $fish^{-1}$). Strains of these species caused an ulcerous disease by bath exposure to 10^7 to 10^8 cfu ml^{-1}. In contrast, rainbow trout were less susceptible to infection caused by *A. hydrophila* and *A. jandaei* isolates, perhaps because of the lower water temperatures at which they were held during challenge. The epizootics in the eel populations occurred in spring and summer when water temperatures were 17 to 22 °C. The data indicate that *A. hydrophila* and *A. jandaei* are European eel pathogens and that infections may occur by waterborne transmission. Therefore, the presence of these bacteria in the farm environment probably constitutes a health hazard for eels.

INTRODUCTION

Epizootics caused by obligate pathogens such as *Pseudomonas anguilliseptica* and *Vibrio vulnificus* biotype 2 have been reported in cultured European eels *Anguilla anguilla* (Nakai & Muroga 1982, Biosca et al. 1991, Michel & Bernardet 1991). However, there are cases of disease in which other bacteria seem to be implicated (MacCarthy 1976, Jensen et al. 1983, Mellergaard & Dalsgaard 1987, Esteve & Garay 1991).

In a previous study, 3 disease outbreaks were recorded in an eel farm located in Valencia (Spain) during spring and summer 1987 and 1988 when water temperatures were 17 to 22 °C (Esteve & Garay 1991). The disease in these outbreaks resembled 'red fin disease' of Japanese eels (Rickards 1978) with regard to external lesions, mortality rates and seasonal pattern. Losses were high (80 %) when glass eels in the initial stages of feeding were involved. These losses were economically important because eel production depends on the availability of 'seed' from nature. The mortality occurring in silver eels (adults) was lower (approximately 30 %). Eels were treated with tetracycline (40 mg kg^{-1} of fish) for 7 d, and mortality decreased. Aetiology of the disease was complex. The following bacterial species were isolated on tryptic soy agar plus 1 % (w/v) NaCl (TSA-1) from freshly dead eels: (1) motile *Aeromonas* spp. (*A. hydrophila* and *A. jandaei*), (2) *Pseudomonas fluorescens* and (3) *Shewanella putrefaciens*. Although these species were also present in samples from routine surveys (tank water/healthy eels), their levels in the routine surveys were different from those observed during epizootics. On the other hand, the species *A. sobria*, *Vibrio anguillarum* and *Plesiomonas shigelloides*, which are described as potential fish pathogens (Austin & Austin 1987), were mainly isolated on TSA-1 from healthy eels and their surrounding water.

The present work was a continuation of the study started by Esteve & Garay (1991). Its objective was to determine the virulence of the bacterial species (*Aeromonas hydrophila*, *A. jandaei*, *A. sobria*, *A. caviae*,

Plesiomonas shigelloides, *Vibrio anguillarum*, *Pseudomonas fluorescens* and *Shewanella putrefaciens*) isolated from the affected European eel farm during epizootics as well as during routine surveys, so that their importance as European eel pathogens could be assessed.

MATERIAL AND METHODS

Experimental fish. The infectivity study was conducted using juvenile European eels *Anguilla anguilla* (elvers) (5 to 12 g) and rainbow trout *Oncorhynchus mykiss* (4 g). Groups of 6 fish (elvers or trout) were transferred to 24 l plastic tanks supplied with dechlorinated municipal water. Water temperatures were optimal for these fish, ranging from 20 to 22 °C for elvers and from 13 to 15 °C for trout. Effluent water from the tanks was treated with sodium hypochlorite in sufficient concentration to kill pathogens present. Tanks were treated with 0.1N HCL and irradiated with ultraviolet light when the experiment was over.

Challenge bacteria. A number of strains of the species *Aeromonas hydrophila* (10), *A. jandaei* (8), *A. sobria* (4), *A. caviae* (3), *Plesiomonas shigelloides* (2), *Vibrio anguillarum* (4), *Pseudomonas fluorescens* (7) and *Shewanella putrefaciens* (2) were evaluated for their virulence (see Tables 2 & 3). The number of isolates tested for each species approximately reflected the numerical relationship previously observed among these species (Esteve & Garay 1991). Oxidative bacilli (*P. fluorescens* and *S. putrefaciens*) were identified using the tests reported by Palleroni (1984) and Thampuran & Lyer (1989). Fermentative bacilli (*Aeromonas* spp., *V. anguillarum*, and *P. shigelloides*) were classified by a numerical taxonomy study (Esteve & Garay 1992). Some biochemical characteristics of the strains tested are given in Table 1. For challenging, previously lyophilized strains were used.

Challenge. ***IP challenge:*** Bacterial strains were grown overnight on TSA-1 at 25 °C. For each strain, 6 fish (elvers or trout) were injected intraperitoneally (IP) with 0.1 ml of a suspension containing 10^9, 10^8, 10^7, 10^6, 10^5 and 10^4 cells ml^{-1} in phosphate-buffered saline (PBS, pH 7.0). Six fish (controls) injected with 0.1 ml of sterile PBS were held under the same conditions as the challenged specimens. Mortalities were recorded daily for 7 d (Santos et al. 1988). Liver and kidney samples from freshly dead fish were cultured on TSA-1, and isolates were subsequently tested for their biochemical properties (see Table 1). Mortalities were only considered if the injected bacterium grew in pure culture from these samples. For each strain, the 50 % lethal dose (LD_{50}) was calculated by the method of Reed & Müench (1938). According to Santos et al. (1988), strains that exhibited $LD_{50} \geq 10^8$ cfu $fish^{-1}$ were considered avirulent.

Bath challenge: Only the most virulent strains in IP experiments were selected for bath challenges (see Table 4). Strains were grown for 24 h in tryptic soy broth (Oxoid) supplemented with 1 % NaCl (TSB-1). For each strain, duplicate groups of 6 elvers (average weight 12 g) were held for 60 min in an aerated bath (6 l) containing 2 and 20 ml broth culture l^{-1} and then returned to their tanks (Markward & Klontz 1989). Fish were exposed to 10^7 and to 10^8 cells ml^{-1} during each treatment. Mortalities were recorded daily for 14 d (Baxa et al. 1990). Samples from ulcers, livers, and kidneys of freshly dead elvers were examined for the presence of the challenge bacterium. At 14 d post-exposure, samples of gills, liver and kidney from all survivors were also analyzed.

Statistical analysis. The association between virulence for eels and origin of the isolates (epizootics/non-epizootics) was assessed using the Chi-square (χ^2) test.

Table 1. Biochemical characteristics of the Gram-negative, oxidase-positive and motile bacterial species that were tested for virulence. 1: O (oxidative)/F (fermative); 2: colony pigment; 3: pyoverdines production; 4: H_2S production from thiosulphate; 5: gelatinase; 6: DNase; 7: growth at 42 °C; 8: O/129 test (150 µg) (R: resistant; S: sensitive); 9: growth with 0 % NaCl; 10: growth with 6 % NaCl; 11: arginine dihydrolase (ADH); 12: ornithine decarboxylase (ODC); 13: Voges-Proskauer; 14: acid production from salicin; 15: esculin hydrolysis; 16: elastin hydrolysis; 17: sulphatase; 18: gas production from glucose. (+) All isolates were positive; (–) all isolates were negative; v: variable response (20 to 80 % of isolates were positive)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Aeromonas hydrophila	F	–	–	–	+	+	v	R	+	–	+	–	+	+	+	+	–	+
Aeromonas jandaei	F	–	–	–	+	+	+	R	+	–	+	–	+	–	–	v	v	+
Aeromonas sobria	F	–	–	–	+	+	v	R	+	–	+	–	v	–	–	–	–	+
Aeromonas caviae	F	–	–	–	+	+	+	R	+	–	+	–	–	+	+	–	–	–
Plesiomonas shigelloides	F	–	–	–	–	–	+	S	+	–	+	+	–	–	–	–	–	–
Vibrio anguillarum	F	–	–	–	+	+	–	S	+	+	+	–	v	–	+	+	–	–
Pseudomonas fluorescens	O	–	+	–	+	–	–	R	+	–	+	–	–	–	–	–	–	–
Shewanella putrefaciens	O	+	–	+	+	+	–	R	+	–	–	–	–	–	–	–	–	–

RESULTS

IP challenges

Of the isolates recovered during epizootics only those of the species *Aeromonas hydrophila* (100%) and *A. jandaei* (71%) were pathogenic for elvers: LD_{50} values were $10^{5.4}$ to $10^{7.5}$ cfu fish^{-1} (Table 2). Mortalities began at 18 h post-challenge, and 'red fin disease' as described by Rickards (1978) was produced in elvers that died during the experiment. Pure cultures of the inoculated *A. hydrophila* and *A. jandaei* strains were recovered from kidney and liver of all dead and moribund eels. In contrast, only 16% of these strains of motile *Aeromonas* spp. produced mortalities in the groups of challenged trout (LD_{50} values ca 10^7 cfu fish^{-1}) (data not shown).

Table 2. Virulence of strains of various bacteria isolated from eels and eel farm water during epizootics as determined in *Anguilla anguilla* elvers by IP challenge. E: diseased glass eel; EA: diseased silver eel (gills); EO: diseased silver eel (liver); A: asymptomatic glass eel; AO: asymptomatic silver eel (liver); TW: tank water. Virulence on a scale from 'not virulent' (–) to 'very virulent' (+++)

Species Strain	Virulence	LD_{50} (cfu fish^{-1})
Aeromonas hydrophila		
EO-63 (replicate 1)	+++	$10^{5.4}$
EO-63 (replicate 2)	+++	$10^{5.6}$
EA-59	++	$10^{6.3}$
EO-64	++	$10^{6.2}$
EA-68	++	$10^{6.4}$
E-37 (replicate 1)	+	$10^{7.0}$
E-37 (replicate 2)	+	$10^{7.1}$
A-19 (replicate 1)	++	$10^{6.5}$
A-19 (replicate 2)	+	$10^{7.1}$
TW-1	+	$10^{7.2}$
Aeromonas jandaei		
E-273	++	$10^{6.6}$
E-30 (replicate 1)	++	$10^{6.3}$
E-30 (replicate 2)	+	$10^{6.9}$
AO-60	++	$10^{6.4}$
AO-62	+	$10^{7.5}$
TW-9 (replicate 1)	+	$10^{7.4}$
TW-9 (replicate 2)	+	$10^{7.0}$
E-44	–	$\geq 10^{8.4}$
A-26 (replicate 1)	–	$\geq 10^{8.6}$
A-26 (replicate 2)	–	$\geq 10^{8.3}$
Aeromonas sobria		
A-13	–	$\geq 10^{9.0}$
A-21	–	$\geq 10^{8.4}$
TW-4 (replicate 1)	–	$\geq 10^{8.1}$
TW-4 (replicate 2)	–	$\geq 10^{7.9}$
Pseudomonas fluorescens		
E-294	–	$\geq 10^{8.2}$
E-280	–	$\geq 10^{8.3}$
Shewanella putrefaciens		
EO-89	–	$\geq 10^{8.3}$

Of the strains recovered from tank water and from eels in absence of disease, those of the species *Aeromonas hydrophila* (100%), one of *Vibrio anguillarum* (25%), and one of *Pseudomonas fluorescens* (20%) were virulent for challenged elvers. *A. hydrophila* strains displayed LD_{50} values of $10^{6.2}$ to $10^{7.4}$ cfu fish^{-1}, and the virulent strains of *V. anguillarum* and *P. fluorescens* showed LD_{50} values of $10^{7.3}$ cfu fish^{-1} (Table 3). Mortalities began at 2 d post-challenge. The bacteria were recovered from the kidneys and livers of all dead and moribund challenged elvers. External clinical signs in these fish included cutaneous petechiation on the ventral portions of the body and occasionally ulcerations. These strains were non-pathogenic for trout (LD_{50} values were $\geq 10^8$ cfu fish^{-1}) (data not shown).

Table 3. Virulence of strains of various bacteria isolated from eels and eel farm water during routine surveys as determined in *Anguilla anguilla* elvers by IP challenge. S: healthy glass eel; SA: healthy silver eel (gills); SO: healthy silver eel (liver); TW: tank water. Virulence scale as in Table 2

Species Strain	Virulence	LD_{50} (cfu fish^{-1})
Aeromonas hydrophila		
S-188 (replicate 1)	++	$10^{6.5}$
S-188 (replicate 2)	++	$10^{6.2}$
TW-170	++	$10^{6.8}$
SO-324 (replicate 1)	+	$10^{7.2}$
SO-324 (replicate 2)	+	$10^{7.4}$
Aeromonas jandaei		
SO-337 (replicate 1)	–	$10^{8.4}$
SO-337 (replicate 2)	+	$10^{7.6}$
Aeromonas sobria		
SA-253 (replicate 1)	–	$\geq 10^{8.6}$
SA-253 (replicate 2)	–	$10^{8.0}$
Aeromonas caviae		
S-184	–	$\geq 10^{8.7}$
SA-251	–	$\geq 10^{8.1}$
SO-314	–	$\geq 10^{8.7}$
Pseudomonas fluorescens		
SO-317	+	$10^{7.3}$
S-220 (replicate 1)	–	$10^{8.6}$
S-220 (replicate 2)	–	$10^{8.0}$
S-355	–	$10^{8.3}$
SA-306	–	$\geq 10^{8.2}$
SO-332	–	$\geq 10^{8.2}$
Shewanella putrefaciens		
TW-172 (replicate 1)	–	$\geq 10^{8.3}$
TW-172 (replicate 2)	–	$\geq 10^{7.7}$
Plesiomonas shigelloides		
SO-328	–	$\geq 10^{8.5}$
SO-334 (replicates 1 and 2)	–	$\geq 10^{8.7}$
Vibrio anguillarum		
TW-157	+	$10^{7.3}$
TW-137	–	$\geq 10^{8.3}$
TW-139	–	$\geq 10^{8.4}$
TW-151	–	$10^{8.0}$

Bacteria were not present in samples from survivors, and mortalities did not occur in the control groups injected with sterile PBS.

A positive relationship was found between virulence of the isolates for elvers and their origin (χ^2 test = 5.013, $p_{(n=1)} < 0.05$). This result indicates that strains isolated during epizootics tended in general to be more virulent than those recovered in the absence of disease. This finding could be explained by the higher prevalence of *Aeromonas hydrophila* and *A. jandaei* in samples during epizootics.

Bath challenges

Mortalities in the bath challenge experiments began at 9 to 13 d post-challenge, depending on challenge received. There was a 17 to 34 % mortality in elvers exposed to certain of the *Aeromonas hydrophila* strains and a 17 to 50 % mortality in elver groups challenged with one of the *A. jandaei* strains (Table 4). All moribund fish showed ulcerations on the tail, and purulent liquid in the gills. In one case, petechiation of the fins was also detected. Internally, signs of infection were lacking. Large numbers of the bacteria used for the challenge were obtained on TSA-1 from the ulcers of freshly dead fish. They were also isolated from liver as pure cultures but in low numbers. In most cases, bacteria were not recovered from liver or incipient cutaneous lesions from the survivors (Table 4). Mortalities were not recorded in the group of elvers challenged with sterile TSB-1.

Table 4. *Aeromonas hydrophila* and *A. jandaei*. Mortality of *Anguilla anguilla* elvers following immersion challenges with selected strains. Symbols as in Tables 2 & 3

Species Strain	Bacterial dose (cfu ml^{-1})	Mortality No.	%	Reisolation Dead	Survivor	Time to death (d)
A. hydrophila						
EO-63	4.8×10^8	2/6	34	2/2	0/4	10
	1.7×10^7	1/6	17	1/1	0/5	13
EA-68	2.7×10^8	1/6	17	1/1	0/5	9
	2.0×10^7	0/6	0	–	0/6	–
EA-59, S-188	1.3×10^8	0/6	0	–	0/6	–
	4.2×10^7	0/6	0	–	0/6	–
A. jandaei						
E-273	5.0×10^8	0/6	0	–	0/6	–
	4.4×10^7	0/6	0	–	0/6	–
AO-60	3.0×10^8	3/6	50	3/3	0/3	9
	1.2×10^7	1/6	17	1/1	5/5[a]	11

[a]Bacterium was isolated from gills, liver or kidney

DISCUSSION

The infectivity experiments reported here demonstrate that *Aeromonas hydrophila* and *A. jandaei* strains isolated from the European eels in 1987–1988 were the cause of the eel disease originally reported to be the result of a mixed septicaemia by Esteve & Garay (1991). These results are in agreement with those of Rickards (1978) and Kou (1981) who recognized the species *A. hydrophila* as an important pathogen for Japanese eels in Japan and Taiwan.

Challenged eels were resistant to strains of *Pseudomonas fluorescens* (2) and *Shewanella putrefaciens* (1) that had been isolated from diseased eels along with the mentioned *Aeromonas* species (Esteve & Garay 1991). At various times, the species *P. fluorescens* and *S. putrefaciens* have been considered as fish spoilage organisms (Gillespie 1981) or as trout (Roberts & Horne 1978) or rabbitfish (Saeed et al. 1987) pathogens. Our data indicate that they probably represented secondary invaders that took advantage of damaged eel tissues.

Isolation of *Aeromonas jandaei* from eels and water has not been reported previously. This species has recently been described by Carnahan et al. (1991) from human infections. Identification of *A. jandaei* isolates was a difficult problem because their phenotypic characteristics are very similar to those of the species *A. sobria* (Carnahan et al. 1991). Many *A. jandaei* strains from fish were elastase positive as well as pathogenic, whereas none of the tested *A. sobria* isolates was positive for these features. Thus, it should be kept in mind that virulent and elastase positive strains of *A. sobria* may actually correspond to *A. jandaei* strains. In fact, it is generally accepted that *A. sobria* is relatively non-pathogenic for fish, and there are very few reports of epizootics associated with *A. sobria* (Toranzo et al. 1989).

In the present study, disease produced by *Aeromonas hydrophila/A. jandaei* was induced by injection as well as by waterborne exposure. The disease was acute in eels infected by IP challenge, whereas it produced lower mortality rates and progressed more slowly when bath challenge was used. Although water transmission of the disease has been demonstrated with both of these species, we consider that water is not likely to be an important reservoir of these species because low numbers of *Aeromonas* spp. (approximately 18 cfu per 100 ml) were always found in tank water (Esteve & Garay 1991). In contrast, *Aeromonas* species were much more numerous in fish (Esteve & Garay 1991),

A. hydrophila and *A. jandaei* being especially abundant in eels during the epizootics. Some authors (Cahill 1990) have found that *Aeromonas* species occur in the gastrointestinal tract of salmonids and warm-water fish.

The eel disease showed a seasonal occurrence, appearing only in the warmest months when water temperatures increased from 17 to 21 °C. These temperatures are close to that described as optimal for European eels (Gault 1986). Mellergaard & Dalsgaard (1987) reported that outbreaks caused by *Aeromonas hydrophila* in European eel farms in Denmark were always associated with fish grading and poor water quality, but these conditions did not appear to explain the epizootics studied by us (Esteve & Garay 1991). On the other hand, it is well established that temperature influences the pathogenicity of *A. hydrophila* (Groberg et al. 1978, Schubert & Matzinou 1990) because its ability to produce cytotoxins, hemolysin and enterotoxins is much higher at temperatures of 22 °C than at 4 to 10 °C (Krovacek et al. 1991). In fact, most of our *Aeromonas* strains proved to be non-pathogenic for trout held at temperatures of only 13 to 15 °C.

In conclusion, the role of *Aeromonas hydrophila* and *A. jandaei* as primary pathogens for European eels is supported by their ability to reproduce disease in the eels under laboratory conditions using 2 challenge methods: IP injection and bath exposure. Moreover, the results obtained in these experiments suggest that *Pseudomonas fluorescens* and *Shewanella putrefaciens* act as opportunistic pathogens of European eels.

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