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Vibrios Associated with Mortality in Cultured Plaice *Pleuronectes platessa* Fry

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Pedersen K, Austin B, Austin DA, Larsen JL: Vibrios associated with mortality in cultured plaice *Pleuronectes platessa* fry. Acta vet. scand. 1999, 40, 263-270. – Fifty two bacterial strains, identified as *Vibrio spp.*, were isolated from diseased plaice fry. The most numerous group comprised *V. anguillarum* (26/52), of which 3 isolates belonged to serogroup O2a, 16 corresponded to serogroup O18, and 7 isolates were nontypeable. All serogroup O18 isolates had identical ribotype patterns. Fourteen isolates were identified as *V. splendidus* biotype I (n = 11) or *V. splendidus*-like (n = 3). Seven isolates were *V. fluvialis*, representing the first isolation of this species in Denmark and the first description of *V. fluvialis* associated with diseased fish. All *V. fluvialis* isolates had identical ribotype patterns, indicating the presence of a single clone. The last 5 isolates belonged to 2 different, unidentified *Vibrio* species (n=2 and 3, respectively). Although all isolates were recovered from diseased plaice fry, their exact role as pathogens for the fry is as yet uncertain. Selected isolates were tested for virulence to salmon and turbot. When injected into juvenile salmonid fish, the recorded LD_{50} values were higher than 10^6, indicating that their virulence was relatively low. However, virulence seemed to deteriorate upon subculturing, and therefore, the strains may have been more virulent upon primary isolation from the plaice fry.

Vibriosis; Vibrio anguillarum; Vibrio splendidus; Vibrio fluvialis.

Introduction

Attempts are being made to grow an increasing number of fish species in aquaculture facilities. Many species breed readily in captivity, e.g. salmonids and turbot, whereas others, e.g. the eel, do not. Instead some fish have to be caught from wild stocks as juveniles for ongrowing in captivity. The plaice, *Pleuronectes platessa*, is one of the species that has not yet been bred in captivity. Instead the roe and milt is collected from wild fish and the resultant eggs hatched. In Denmark, it has been attempted to grow plaice fry using this technique, mainly destined for restocking of wild populations. Unfortunately, when a new species is introduced in aquaculture, new diseases will usually appear as well, and this has happened with plaice fry.

There is a dearth of information about diseases of plaice. However, infections caused by *Vibrio anguillarum* have been reported in plaice (*Anderson & Conroy* 1970) as well as in several other flatfishes, such as turbot *(Scophthalmus maximus) (Horne et al.* 1977, *Larsen & Olsen* 1993), winter flounder *(Pseudopleuronectes americanus) (Levin et al.* 1972), flounder *(Platichthys flesus)*, sole *(Solea solea)*, dab *(Limanda limanda)*, and halibut *(Hippoglossus hippoglossus) (Anderson & Conroy* 1970). Other *Vibrio* species causing diseases in flat-

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fishes include *Photobacterium (=V.) damselae (Fouz et al.* 1992) and *V. splendidus (Lupiani et al.* 1989), but infections with these bacteria have so far not been described in plaice. Likewise, infections caused by typical *(Hjeltnes et al.* 1995, *Pedersen & Larsen* 1996) and atypical *Aeromonas salmonicida* have been described in several flatfish species, including the plaice (for comprehensive review, see *Wiklund & Dalsgaard* 1998). Other diseases described in plaice include epidermal papillomas *(Sinderman* 1970), tuberculosis (in one fish landed in Hull), and infections with *Ichthyophonus hoferi,* ciliates, and various other parasites (*Kinne* 1984). In the present paper we describe some of the vibrios that were isolated from moribund and diseased plaice fry in Denmark. The purpose of the study was to improve our knowledge about the speciation of the vibrios associated with plaice and to investigate their potential pathogenic properties.

Materials and methods

Bacteriological examination of fish

Juvenile plaice, from hatching up to a size of approximately 10 cm in length, were kept in sea water, salinity approximately 20‰, in tanks of approximately 120 l. Water temperatures varied from 5 °C up to 22 °C. Roe and milt was collected during January–April 1994 and 1995, whereafter the eggs hatched approximately 90 degree-days (about 3 weeks) later. During the larval stage, the fish were fed live feed consisting of *Artemia* and *Brachionus,* and later, the live feed was substituted by a commercial pelleted feed. Metamorphosis occurred in May –June. During certain periods from June until September, when water temperatures were highest, the farmed stock experienced onset of disease with high mortalities, of up to 60%-70%, and some diseased fish displayed darkening of the skin, skin haemorrhages, and fin- and tail rot with erosion and decay of soft tissue between fin rays. During the summer and autumn of 1995 and 1996, diseased fish were examined bacteriologically. The fish were surface disinfected with ethanol whereafter, the abdominal cavity was opened, and material from internal organs was sampled with a sterile loop and streaked onto marine agar (Difco) supplemented with 5% (v/v) calf blood (MA). Some fish were too small to allow proper sampling from the kidney or spleen, whereas from others, inoculation was made from the kidney. From fish displaying skin ulcerations, ulcers were disinfected with ethanol on the surface, whereafter material from deep layers of the ulcers was collected with a sterile platinum loop and streaked onto MA. The plates were incubated at 20 °C for 2 days. Pure cultures and dominant colonies were subcultured for identification and further investigation. A total of 52 isolates were collected from 36 fish.

Identification of bacteria

The bacteria were identified using combinations of 42 biochemical and physiological criteria (*Larsen & Pedersen* 1995). The tests were carried out as described by *Barrow & Feltham* (1993). The criteria used were: Haemolysis, Gram staining, motility, catalase, oxidase, pellicle formation in broth culture, pigment production, arginine dihydrolase (Thornley [Barrow & Feltham 1993] and Møller [Barrow & Feltham 1993]), lysine decarboxylase, ornithine decarboxylase, growth in 6%, 7%, and 10% (w/v) NaCl, investigation of fermentative and oxidative pathway in Hugh and Leifson's medium, urease production, growth on thiosulphate-citrate-bile salts-sucrose (TCBS) agar, sensitivity to 10 and 150 μg of the vibriostatic agent, O/129, luminescence, citrate (Simmon's), indole, Voges-Proskauer reaction, gas from glucose, growth at 20 °C on MacConkey agar, nitrate reduction, production of alginase, amylase, and gelatinase, degradation of Tween

Table 1. Identification of 52 *Vibrio* isolates from diseased plaice fry.

	Serotype/biotype	No. isolates	Ribotype pattern
V. anguillarum (n = 26)	O2	3	
	O18	16	All identical
	Non-typeable	6	All identical
	Non-typeable	1	Unique
V. splendidus (n = 14)	Biotype I	11	3 different closely related ribotypes
	V. splendidus-like according to biochemical properties	3	All identical, but clearly distinct from the biotype I isolates
V. fluvialis (n = 7)			All identical, and almost identical to the type culture of the species
Vibrio sp. 1 (n = 3)			All identical
Vibrio sp. 2 (n = 2)			2 slightly different ribotypes

80, and acid from arabinose, cellobiose, galactose, glucose, glycerol, mannitol, salicin, saccharose, trehalose, lactose, and xylose. Isolates identified as *V. anguillarum* were subjected to O-serotyping as described by *Larsen et al.* (1994) using the antisera listed by *Austin et al.* (1995), *Grisez* (1997) and *Pedersen et al.* (1999a). Verification of identification was performed by ribotyping as described by *Pedersen & Larsen* (1993), *Koblavi* (1996), and *Austin et al.* (1995, 1997) by comparison with type cultures of *V. splendidus* I ATCC 33125, *V. anguillarum* NCMB 6, and *V. fluvialis* ATCC 33809.

Fish pathogenicity experiments

Six of the isolates, representing different species or groups, were tested for their virulence to fish. Bacterial cultures were grown overnight at 22 °C in tryptone soya broth (Oxoid) supplemented with 1% (w/v) sodium chloride, centrifuged at 5,000 × g for 10 min and resuspended in 10 ml volumes of 0.9% (w/v) saline to approximately 10^8 cells ml^{-1}. Ten fold dilutions were prepared to 10^3 cells ml^{-1}. A salmonid fish model (Atlantic salmon [*Salmo salar*], rainbow trout [*Oncorhynchus mykiss*] and turbot [*Scophthalmus maximus*]) was used to assess pathogenicity, as follows: Groups of 10 fish were infected by intraperitoneal injection with 0.1 ml volumes of the washed bacterial suspensions to achieve doses of 10^2-10^6 cells $fish^{-1}$. The infected animals were maintained for up to 14 days in covered polypropylene tanks supplied with dechlorinated, aerated static freshwater (the water was changed daily) or recirculating seawater at a temperature of ~15 °C, as appropriate. The LD_{50} dose was calculated by the Probit Method (*Wardlaw* 1985). Dead and moribund fish were removed, and subjected to standard bacteriological and pathological examination (*Austin & Austin* 1989). Any survivors at the end of the experiment were sacrificed and examined, as above. The disease signs were recorded, and attempts made to recover and confirm the identity of the pathogen by the methods described by *Austin & Austin* (1993).

Results and discussion

The results of the bacteriological examinations are given in Table 1. The bacterial isolates were equated with the genus *Vibrio*. This finding is in agreement with previous observations (*Muroga et al.* 1987, *Grisez* 1997) on larvae from various fish species. As observations on the microflora

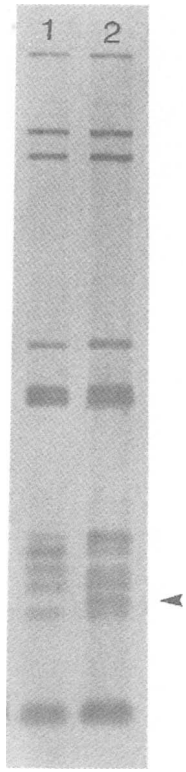

Figure 1. Ribotype patterns of plaice isolates of *V. fluvialis* compared to the *V. fluvialis* ATCC 33809 type culture. Notice that they deviate in only a single band (arrowhead). Lane 1, *V. fluvialis* ATCC 33809; lane 2, *V. fluvialis* plaice isolate.

of *Artemia* and *Brachionus* have revealed that these are also dominated by *Vibrio* species, it is believed that the fish larvae mainly acquire their *Vibrio* flora from the live feed (*Verdonck et al.* 1997).

Grisez (1997) described that for sea bass and sea bream larvae, disease outbreak could occur when *V. anguillarum* was dominant in the intestinal tract. Likewise, *Bolinches & Egidius* (1987) found that *V. anguillarum* could cause mortality in halibut larvae. In the present study, 26 out of the 52 isolates were identified as *V. anguillarum*. Three of these isolates belonged to serogroup O2, whereas the remaining 23 did not belong to any of the traditional pathogenic serogroups. However, 16 of them belonged to the new serogroup, O18 (*Pedersen et al.* 1999a), previously named VaNT4 (*Austin et al.* 1995). This serogroup has not previously been associated with disease, although isolates strongly cross-reacting between O18 and another new serogroup, preliminarily designated VaNT2, have been described from diseased salmonids in Finland (*Pedersen et al.* 1999b). All these O18 isolates displayed the same ribotype pattern, indicating that they belonged to the same clone, and the fact that the strains were isolated over an extended period indicates that they belonged to a clone capable of persisting in the aquatic environment or associated with healthy carrier fish. It has previously been described that *V. anguillarum* clones were able to persist in a hatchery for extended periods (*Grisez* 1997, *Pedersen et al.* 1999a). The remaining 7 *V. anguillarum* isolates did not react with any of the known antisera. Six of these isolates had identical ribotype patterns indicating that this was also a clone circulating in the fish farm, whereas the last *V. anguillarum* had a unique ribotype pattern. These results also demonstrate that additional *V. anguillarum* O-serogroups exist, and that some of these may have some significance as pathogens in fish larvae or juvenile fish.

Eleven isolates in the present study were identified as *V. splendidus* biotype I. These isolates displayed 3 almost identical ribotype patterns. *V. splendidus* I is not reputed as an important fish pathogen but it has been associated with disease in a number of fish species, among them turbot, sea bass, and sole (*Lupiani et al.* 1989, *Myhr et al.* 1991). An additional 3 isolates had biochemical and physiological reactions identical to the *V. splendidus* I isolates, and were identified as *V. splendidus* on the basis of these criteria (Table 2). However, they had a

Table 2. Biochemical and physiological properties of plaice isolates of *V. splendidus* and *V. fluvialis*.

	V. splendidus plaice isolates (n = 14)	*V. splendidus* I ATCC 33125	*V. fluvialis* plaice isolates (n = 7)	*V. fluvialis* ATCC 33809
Haemolysis	+1	+	+	+
Gram staining reaction	–2	–	–	–
Motility	+	+	+	+
Catalase	+	+	+	+
Oxidase	+	+	+	+
Pellicle	+		–	
Colony pigmentation	–	–	–	–
Arginine dihydrolase	+	+	+	+
Lysine decarboxylase	–	–	–	–
Ornithine decarboxylase	–	–	–	–
Growth in 6% (w/v) NaCl	+		+	
7% (w/v) NaCl	–		+	
10% (w/v) NaCl	–	–	–	–
O/F[3]	F	F	F	F
Urease	–	–	–	–
Yellow colonies on TCBS[4]	+	+	+	+
Sensitivity to 10 μg O/129	+	+	–	
Sensivity to 150 μg O/129	+	+	+	+
Luminescence	–	+	–	–
Citrate (Simmons)	+	+	+	+
Indole	+	+	+	+
VP	–	–	–	–
Glucose, gas	+	–	–	–
Growth at 37°C	–			
Growth on MacConkey agar	–			
Nitrate reduction	+	+		+
Alginase	+	+	–	–
Amylase	–	+	+	+
Gelatinase	+	+	+	+
Tween 80	+	+	+	+
Arabinose	–	–	+	+
Cellobiose	+	+	–	+
Galactose	+		+	
Glycerol	–		+	
Mannitol	+	+	+	+
Salicin	–		+	
Saccharose	+	+	+	+
Trehalose	+		+	
Lactose	–	–	–	(+)[5]
Xylose	–	–	–	–

[1] + indicates a positive reaction.

[2] – indicates a negative reaction.

[3] O/F means degradation of glucose by the oxidative or fermentative pathway in Hugh and Leifson's medium.

[4] TCBS is the vibrio selective medium thiosulphate-citrate-bile salts-sucrose agar.

[5] O/129 is the vibriostatic agent, 2,4-diamino-6,7-di-isopropyl pteridine.

[5] (+) indicates a week positive reaction.

clearly deviating ribotype pattern, which indicated that they were possibly not *V. splendidus* "sensu stricto", and we have consequently nominated them *V. splendidus*-like.

A very interesting observation was that 7 isolates were *V. fluvialis*. This species has been associated with human infections but, not so far, with infections in fish (*Balows et al.* 1991). Additionally, *V. fluvialis* has never previously been described from Denmark. The isolates were identified on the basis of their biochemical and physiological properties (Table 2), but the diagnosis was verified by ribotyping: the 7 isolates had the same ribotype pattern, that was almost identical to that of the *V. fluvialis* type culture, deviating in only one single band (Fig 1). This is in our experience highly unusual for epidemiologically unrelated clones.

The remaining 5 isolates belonged to 2 different *Vibrio* species, 2 and 3 isolates, respectively, that could not be identified to species level.

An evaluation of pathogenic properties of the bacteria was undertaken using 6 isolates representing each of the major groups for virulence testing in salmonids and turbot. In an initial experiment, groups of 10 salmon were injected i.p. with doses of 10^6 c.f.u. per fish of each of the strains *V. anguillarum* O18 95-6-161, *V. anguillarum* O2 95-6-285, *V. anguillarum* non-typeable 95-8-231, *V. splendidus* I 94-6-83, *V. splendidus*-like 94-6-81, and *V. fluvialis* 96-7-103. In general, virulence was low for all strains. *V. anguillarum* non-typeable 95-8-231 seemed to be the most virulent causing 100% mortality using this dose, but also *V. anguillarum* O18 95-6-161 caused some mortality. The strains *V. anguillarum* O2 95-6-285, *V. fluvialis* 96-7-103, and *V. splendidus*-like 94-6-81 caused low mortality, whereas *V. splendidus* I 94-6-83 caused neither mortality nor any pathological manifestations. On the basis of these results, LD_{50} experiments were carried out for the 2 strains, *V. anguillarum* non-typeable 95-8-231 and *V. anguillarum* O18 95-6-161, on salmonids. LD_{50} for *V. anguillarum* 95-8-231 was calculated to 1.5×10^6 and LD_{50} for *V. anguillarum* O18 95-6-161 to 1.6×10^6 c.f.u. For both isolates, it was characteristic that virulence deteriorated quickly upon subculturing. Therefore, the isolates may have been more virulent upon isolation than during the pathogenicity tests. The inoculated organisms were recovered from all dead and moribund fish, and the pathological findings recorded in the fish were all characteristics of a bacterial haemorrhagic septicaemia. Doses of 1.0×10^6 and 5.2×10^6 of strain 95-6-161 and 95-8-231, respectively, were injected into turbot. Strain 95-6-161 caused no mortality or pathological changes, whereas 95-8-231 caused 100% mortality. Like in salmon, the pathological findings were those characteristic for a bacterial haemorrhagic septicaemia. After i.p. injection, the most prominent findings were haemorrhages in the mouth, eye, skin - especially at the tail, fins, and flank -, abdominal organs, and in the gut, whereas after i.m. injection, these haemorrhages were supplemented with haemorrhages and necrosis of the muscle. It is interesting that the non-typeable *V. anguillarum* isolate displayed higher virulence to both salmonids and turbot than the recognized pathogenic serogroup O2 isolate.

Although all 52 isolates were recovered from diseased plaice, their pathogenic properties are somewhat uncertain. On the basis of the pathogenicity experiments it may be suggested that some of the isolates may have been involved in the mortality of the plaice fry, but it is likely that other factors have played a primary or predisposing role, as well.The larval and juvenile stages of fish are very vulnerable to disease, and several undetermined factors, such as temperature and nutrition, may also have been involved as causative factors in the high mortalities recorded on the farm. Mortalities in

juvenile fish has been attributed to several factors (*Buchmann et al.* 1993; *Grisez* 1997), but there is no doubt that various *Vibrio* species, in particular *V. anguillarum* play an important part as pathogens for larvae and juveniles of a number of fish species. In turbot, a mixed bacterial and viral etiology has been proposed as the cause of disease and mortality (*Lupiani et al.* 1989) and it should be mentioned that in the present study, no virological investigation was carried out.

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Sammendrag

Vibrioer associeret med dødelighed hos yngel af rødspætter (Pleuronectes platessa) i akvakultur.

I alt 52 bakteriestammer blev isoleret fra syge rødspætte-yngel og identificeret som *Vibrio spp. Vibrio anguillarum* var den mest talrige gruppe omfattende 26 ud af de 52 isolater. Af disse 26 *V. anguillarum* tilhørte de 3 serogruppe O2a, mens 16 tilhørte serogruppe O18, og 7 isolater var ikke-typbare. Alle serogruppe O18 isolater havde identisk ribotype mønster. Fjorten isolater blev identificeret som *V. splendidus* biotype I (n=11) eller *V. splendidus*-lignende (n=3). Syv isolater tilhørte *V. fluvialis,* og er således de første isolater af denne art i Danmark og tillige den første beskrivelse af *V. fluvialis* associeret med syge fisk. Alle *V. fluvialis* isolates havde identisk ribotype profil, hvilket indikerede tilstedeværelsen af en enkelt klon. De sidste 5 isolater tilhørte 2 forskellige, hidtil uidentificerede *Vibrio* arter (n=2 henholdsvis 3). Selvom alle bakterier blev isoleret fra syge rødspætte-yngel, er deres præcise rolle som patogener stadig noget usikker. Ved challengeforsøg blev juvenile laks og pighvar injiceret med forskellige doser af udvalgte isolater, men de fundne LD_{50} værdier fandtes at være højere end 10^6, hvilket antyder, at de er relativt lidt virulente. Imidlertid aftog virulensen tilsyneladende ved subkultivering, og isolaterne kan således have været mere virulente ved isoleringen fra rødspætteyngelen.

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