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Characterization of *Vibrio anguillarum* Strains Isolated from Diseased Fish in Finland

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Tiainen, T., J. L. Larsen and S. Pelkonen: Characterization of *Vibrio aguillarum* strains isolated from diseased fish in Finland. Acta vet. scand. 1994, 35, 355-362. – Characterization of *V. anguillarum strains* (n=109) isolated from diseased salmonids was performed. Eight O serovars were found among the strains. Serovar O1 was predominant (90 %), while serovars O2, O3, O5, O8, O9, and a new serovar Va NT2, were represented by 1 or 2 strains. Two strains remained non-typeable. One of these was cross-reactive with several antisera, but had a LPS profile identical to that of serovar O8. All serovars showed specific LPS profiles. All but 1 of the O1 strains had a plasmid comparable in size to the pJM1 virulence plasmid, while plasmids of different sizes were found in O2, Va NT2 and the non-typeable strains. Apart from a single strain resistant to tetracycline, all the strains were sensitive to oxolinic acid, tetracycline, and trimethoprim-sulfonamides. By their biochemical and antigenic properties strains causing vibriosis among salmonids in Finland closely resemble Scandinavian strains. Predominance of the serovars O1 and O2 suggests that commercial vaccines containing these serovars should afford sufficient protection against vibriosis in Finland.

salmonids; serovar; plasmid; lipopolysaccharide; biochemistry; susceptibility to antibiotics.

Introduction

Vibriosis is one of the most important bacterial diseases in aquaculture (*Austin & Austin* 1987). Mortalities, together with retarded growth, bad slaughter quality and treatment with antibiotics influence the economy of fish farming. In Europe vibriosis is mainly caused by *Vibrio anguillarum*, while in North America and Japan *Vibrio ordalii* is also associated with the disease. Other *Vibrio* species and recently *V. anguillarum* related (VAR) organisms have also been found as fish pathogens (*Colwell & Grimes* 1984, *Myhr et al.* 1991, *Pazos et al.* 1993).

V. anguillarum strains have traditionally been divided into 10 serovars according to their O-antigen structure (*Sørensen & Larsen* 1986), but recently additional serovars have been proposed (*Pedersen et al.* 1993). Strains belonging to serovars O1 and O2, and to a lesser extent O3 are commonly associated with vibriosis, while strains in the other serovars or non-typeable strains are opportunistic pathogens, i.e they are present in the aquatic environment but only rarely cause disease outbreaks (*Muroga et al.* 1986, *Sørensen & Larsen* 1986, *Larsen et al.* 1988, *Larsen et al.* 1994).

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Vaccines against the most common causative agents of vibriosis, *V. anguillarum* O1 and O2 and *V. ordalii*, are available, and are widely used in Finnish fish farming. However, outbreaks of vibriosis in vaccinated fish are reported in Finland as well as in Canada and Norway (*Paterson et al.* 1989, *Myhr et al.* 1991). The failures in vaccination have raised the question whether commercial vaccines are unsuitable against vibriosis in Finland or whether the vaccination methods are improper. To address this point, and to understand more about the epidemiology of *V. anguillarum* infection in Finland, we have characterized strains isolated from cases of vibriosis in salmonid fish.

Materials and methods

Bacterial strains

A total of 109 *V. anguillarum* strains isolated from diseased fish were included in the study. The strains were isolated during 1988-91 from 3 different fish species by The National Veterinary and Food Research Institute, and The University of Åbo Academy (Table 1). The strains were stored at -80 °C until analysis. The isolates were initially identified as *V. anguillarum* from their colony morphology, hemolysis, oxidase and catalase reactions, Gram-staining, motility, and utilization of glucose. The identity of the strains was confirmed by susceptibility to the vibriostatic agent 2,4-diamino-6,7-di-isopropyl pteridine, 0/129 (Neo-sensitabs 10 µg, A/S Rosco, Taastrup, Denmark) and by the symptoms in the fish.

Serogrouping

All strains were serogrouped by the slide agglutination test using polyclonal O-antisera produced in rabbits against *V. anguillarum* serovars O1-O10 (*Sørensen & Larsen* 1986), as well as 5 previously non-typeable strains (Va NT1-5) (*Pedersen et al.* 1993). Serovar O2 strains were devided into subgroups O2a and O2b with absorbed antisera according to *Larsen et al.* 1994. The preparation of antigens, production of antisera, preparation of samples for agglutination and slide agglutination test were performed as described by *Sørensen & Larsen* (1986).

Lipopolysaccharide (LPS) profiles

Proteinase-K treated samples were prepared using a method modified from *Hitchcock & Brown* (1983). Overnight bacterial cultures were harvested with 1 ml phosphate-buffered saline (pH 7.3), incubated for 20 min at 60 °C and centrifuged at 12 000 rpm for 10 min. Equal amounts of supernatant and sample buffer (2 % SDS, 4 % 2-mercaptoethanol, 10 % glycerol, 1 M Tris [pH 6.8], bromophenol blue) were mixed and heated at 100 °C for 10 min. Ten µl proteinase-K (2.5 g/l) solution per 50 µl sample solution were added. Samples were incubated at 60 °C for 1 h, and then subjected to electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (12 % [wt/vol]) at 100 V for 3-4 h according to *Laemmli* (1970). LPS was silver stained by the method of *Tsai & Frasch* (1982) using a silver stain kit (Bio-Rad Silver Stain, Bio-Rad Laboratories, Ca.). Danish type strains (O1-O10, Va NT1-5) were used as reference strains (*Sørensen & Larsen* 1986, *Pedersen et al.* 1993).

Biochemical and physiological tests

Strains were grown on tryptone soy broth (TSB, Difco) or tryptone soy agar (TSA, Difco) containing 1 % NaCl. All other substrates were supplemented with 1 % NaCl. All strains were examined for production of acid from arabinose, galactose, sorbitol and trehalose, production of indole (Kovac's), growth on TSA at 37 °C (*Cowan* 1974) and hydrolysis of Tween 80 on Shotts-Waltman plates (*Waltman & Shotts* 1984).

Table 1. Distribution of *V. anguillarum* serovars among fish species in Finland.

	Serovar[a]									
Fish species	O1	O2	O3	O5	O8	O9	NT2[b]	CR[c]	NT[d]	TOTAL
Rainbow trout	91		2	1	1	1	2	1		99
Atlantic salmon		1								1
Aea trout	1	1							1	3
Not known	6									6
Total	98	2	2	1	1	1	2	1	1	109

[a] Number of bacterial strains; [b] Typeable with new antisera Va NT1-5; [c] Several cross reactions; [d] Non-typeable with *V. anguillarum* 01-010 or Va NT1-5 antisera.

Table 2. Biochemical and physiological characteristics of Finnish *V. anguillarum* strains.

Serovar	O1	O2	O3	O5	O8	O9	NT2[a]	CR[b]	NT[c]
No. of strains	98	2	2	1	1	1	2	1	1
Acid from:									
Arabinose	67	+	+	+	-	+	+	-	+
Sorbitol	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+
Trehalose	-	+	+	+	+	+	+	+	+
Indole	97/2[d]	+/2	+/2	+/1	+/1	+/1	+/2	+/1	+/0
Tween 80[e]	+	+	+	+	+	+	+	+	+
Growth at 37 °C	+	+	+	+	+	+	+	+	+

+: all strains positive; -: all strains negative; numbers indicate number of positive strains; [a] Typeable with new antisera Va NT1-5; [b] Cross reactive; [c] Non-typeable with *V. anguillarum* O1-O10 or Va NT1-5 antisera; [d] Number of strains with strong color reaction with Kovac's indole; [e] Tween 80 hydrolysis.

Plasmid screening

Plasmid DNA was prepared according to *Kado & Liu* (1981). Samples were run by electrophoresis on 0.8 % agarose gels (100mm x 100mm) in TAE-buffer (40 mM Tris-acetate, 1 mM EDTA). Gels were stained with ethidium bromide (0.5 g/l), and photographed in UV light on Polaroid film. *Escherichia coli* V517 (*Macrina et al.* 1978) and *E. coli* 39R861 (*Threlfall et al.* 1986) were used as plasmid size reference strains while *V. anguillarum* NCMB 2129 was used as a reference for a strain having the pJM1 plasmid (*Conchas et al.* 1991). Plasmid sizes were calculated according to the method of *Rochelle et al.* (1985).

Susceptibility to antibiotics

Strains were examined for susceptibility to tetracycline, oxolinic acid and trimethoprim-sulfamethizol by the disc diffusion method (*Casals & Pringler* 1991) using Neo-Sensitabs (Rosco A/S Diagnostica, Taastrup, Denmark).

Results

Serogrouping

Seven different serovars were found amongst the *V. anguillarum* strains (Table 1). Most of the strains belonged to serovar O1 (90 %) while serovars O2, O3, O5, O8, O9 and Va NT2 were represented by 1 or 2 strains. One

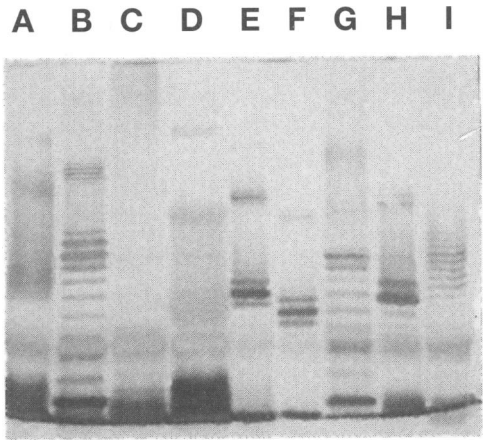

Figure 1. SDS-PAGE electrophoresis of proteinase-K prepared LPS-samples stained with silver stain from Finnish *Vibrio anguillarum* strains. A: Serovar O1; B: Serovar O2; C: serovar O3; D: serovar O5; E: serovar O8; F: serovar O9; G: serovar Va NT2; H: cross-reactive strain; I: non-typeable strain.

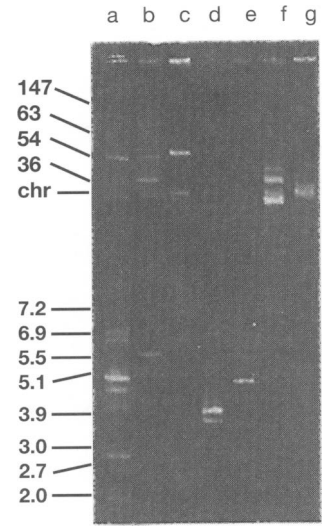

Figure 2. Different plasmid profiles of Finnish *V. anguillarum* strains isolated from diseased fish on ethidium bromide-stained 0.8 % agarose gels. Lane a: E. coli V517; b: E. coli 39R861; c: serovar O1; d and e: serovar O2; f: serovar Va NT2; g: non-typeable. Numbers correspond to molecular weights in kilobases. chr=chromosomal-DNA.

strain did not react with any of the used antisera, while another strain showed cross-reactions by reacting equally with O8, O10, Va NT1 and Va NT2. Both serovar O2 strains belonged to O2a subgroup.

LPS profiles

All the strains shared some common structures of LPS, but 8 distinct LPS profiles were recovered. Strains belonging to the same serovar had identical or very similar LPS profiles. The cross reacting strain had a LPS profile identical to serovar O8 (Fig. 1).

Biochemical and physiological tests

All strains produced acid from sorbitol and galactose (Table 2). Acid production from arabinose varied: 32 % of the O1 strains and both the O8 and the cross reactive strains were negative. All the strains belonging to non-O1 serovars produced acid from trehalose, while none of the O1 strains did. All strains, except one, were indole positive. However, there was a clear difference in the intensity of the indole reaction: 12 strains were strongly positive while the others were less intense. Almost all (10/12) of the strongly reacting strains were non-O1 strains. All strains hydrolyzed Tween 80 and grew at 37 °C.

Plasmids

All O1 strains except one, harboured one large plasmid (65-67 kilobase [kb]) comparable in size to the pJM1 virulence plasmid. One serovar O2 strain had a small plasmid (5.2 kb) while the other had 2 small plasmids (3.9 and 4.2 kb). One of the Va NT2 strains

had 3 large plasmids (48, 36 and 30 kb) and the non-typeable strain possessed 1 large plasmid approximately 160 kb (Fig. 2). No plasmids were detected in 8 of the strains.

Susceptibility to antibiotics

All strains were susceptible to oxolinic acid and trimethoprim-sulfamethizol. Except for the non-typeable strain, all strains were susceptible to tetracycline.

Discussion

The present study shows that *V. anguillarum* serovar O1 is predominant as a causative agent of vibriosis in Finland, as 90 % of the strains belonged to serovar O1. Eleven strains belonging to non-O1 serovars included O2, O3, O5, O8, O9 and NT Va2 strains. Two strains remained non-typeable. LPS profiles mainly supported the sero-grouping results. The cross-reactive strain resembled the serovar O8 strain in its LPS profile and biochemical reactions. Thus the strain might belong to serovar O8.

All strains were isolated from salmonid fish, primarily rainbow trout. This may explain the predominance of serovar O1 in our strain collection. Common occurrence of serovar O1 in rainbow trout has previously been reported by *Sørensen & Larsen* (1986). Several authors have reported that *V. anguillarum* O1, O2, and O3 are common fish pathogens; serovar O1 being associated with salmonids, sea bream, sea bass, and turbot, O2 causing disease especially in cod and eel, and O3 commonly occurring in eel (*Pacha & Kiehn* 1969, *Sørensen & Larsen* 1986, *Toranzo et al.* 1990, *Myhr et al.* 1991, *Larsen et al.* 1994). However, only few reports exist of the pathogenicity of the other serovars. Recently, serovars O4, O5, O6, and O8 have been isolated from diseased fish or fish larvae (*Myhr et al.* 1991, *Pazos et al.* 1992, *Buchmann et al.* 1993, *Pazos et al.* 1993), but only O4 strains have been proved to be pathogenic by experimental infection (*Pazos et al.* 1993). In this study the isolates belonging to serovars O5, O8, O9, and Va NT2, as well as the non-typeable and cross reactive strains, originated from different pathogenic processes. Both Va NT2 strains and the O5 strain were isolated from the kidney, which is indicative of invasive and pathogenic capacity of these bacteria. The pathogenicity of the serovar O8 and O9 strains, however, is questionable as the strains were isolated from intestine and a surface wound, respectively. The non-typeable strains were both isolated in a double infection, thus it is difficult to estimate the role of *V. anguillarum* in these cases.

Biochemically and physiologically, Finnish *V. anguillarum* strains resembled the Scandinavian control strains. All produced acid from sorbitol and galactose, hydrolysed Tween 80 and grew at 37 °C. Acid from arabinose was produced by most of them. It has previously been reported (*Larsen* 1990) that *V. anguillarum* serovar O1 strains with pJM1 plasmid do not produce acid from trehalose, while those without this plasmid do. None of the O1 strains in this study produced acid from trehalose, including the only strain without plasmid. This indicates that there is no connection between pJM1 plasmid and fermentation of trehalose. Current studies in our laboratory seem to support this observation (*Pedersen & Larsen*, unpublished). However, all non-O1 strains did produce acid from trehalose.

Grisez et al. (1991) proposed 6 different phena for *V. anguillarum*, including a separate phenon for Scandinavian strains. Scandinavian strains were negative for indole and acid production from arabinose. Here the indole reaction was positive for all except 1 of the Finnish strains, while the arabinose reaction varied. The vast majority of the Finnish strains would therefore remain outside the

proposed Scandinavian phenon. *Grisez et al.* (1991) has not included differences in the reactions between the various serovars. However, in this study there was a clear difference in the colour intensity in the indole reaction, whereas most of the O1 strains were weakly positive while non-O1 strains had a strong colour reaction. This, and a use of self prepared biochemical reagents instead of API 20 E, may in part explain the difference in indole reactions between our results and those of *Grisez et al.* (1991).

Plasmid content of *V. anguillarum* has been widely examined (*Wiik et al.* 1989, *Bolinches et al.* 1990, *Conchas et al.* 1991, *Larsen & Olsen* 1991, *Olsen & Larsen* 1993). The existence of the pJM1 virulence plasmid coding for iron uptake in serovar O1 strains was recovered by *Crosa et al.* (1980). In the present study all the serovar O1 strains except 1 had a plasmid comparable in size to the pJM1. Four strains of the other serovars possessed plasmids all differing considerably from the virulence plasmid by their size. The O2 strains had small plasmids (5.2, 4.2, and 3.9 kb), while a nontypeable strain had a one large plasmid (160 kb). One of the Va NT2 strains had three plasmids (48 kb, 36 kb and 30 kb) comparable in size to those reported by *Pazos et al.* (1993) in some VAR strains. Connection between those VAR strains and newly created Va NT2 group is possible. The function of the plasmids, other than pJM1, is not known.

Vibriosis is one of the most common bacterial fish diseases occurring in the sea water farms in Finland (*Anonymous* 1991). Although most of the cultured fish populations are vaccinated before transfer to the sea, disease outbreaks still occur and fish farmers have to use medication to control the disease. According to this study, Finnish *V. anguillarum* strains are sensitive to the most commonly used antibiotics: oxolinic acid, tetracycline, and trimethoprim-sulfonamides. However, medication poses an economical problem for fish farmers and it also influences the surrounding ecosystem. Therefore prophylactic treatment of vibriosis by vaccination is highly recommended.

Commercial vaccines used in Finland include *V. anguillarum* O1 and O2. Here 92 % of the strains represented these serovars, indicating that the vaccines used should confer protection in most cases. Furthermore, with regard to the biochemical and growth properties, the Finnish strains seem to be closely related to strains isolated elsewhere in Scandinavia (*Larsen* 1990, *Larsen & Olsen* 1991, *Myhr et al.* 1991, *Wiik et al.* 1989). Thus, it may be indicated that insufficient protection given by commercial vaccines in some fish farms results from improper vaccination rather than from vaccines themselves. In Finland, salmonid fry is usually produced in fresh water and transferred to the sea in late spring or early summer during the first year of life. There are recommendations for the minimum fish size and water temperature at the time of vaccination which is conducive for protective immunity. In practise, however, it may be difficult to adhere to these recommendations. Furthermore, the transfer to sea may be initiated prematurely after vaccination. It is difficult to receive reliable information from fish farmers regarding vaccination of fish populations, obstructing explanations for the failure in vaccination of vibriosis. Hence, it is crucial to analyze the development of immunity in the Finnish fish farming conditions and immunogenic and pathogenic properties of *V. anguillarum* strains isolated from outbreaks occurring in properly vaccinated fish populations.

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Sammendrag

Karakterisering af Vibrio anguillarum isoleret fra syge fisk i Finland.

I alt 109 V. anguillarum stammer isoleret fra salmonider blev karakteriseret. Stammerne var delt i 8 serovar, hvoraf serovar O1 var dominerende (90 %). Serovar O2, O3, O5, O8, O9 og en ny serovar Va NT2 var repræsenteret med enkelte stammer. To stammer kunne ikke types, en af dem krydsreagerede med flere antisera. Krydsreagerende stamme havde LPS-profil som lignede serovar O8 LPS-profil, ellers havde hver serovar en specifik LPS-profil. Alle O1 stammerne, undtagen én, havde et stort pJM1 virulens-plasmid lignende plasmid. Plasmider i forskellige størrelser blev fundet i O2, Va NT2 og i ikke typbare stammer. Bortset fra en enkelt stamme, som var resistent overfor tetracyclin, var alle stammerne sensitive for oxolinsyre, trimetoprim-sulfamethizol og tetracyklin. I deres biokemiske reaktioner lignede finske stammer skandinaviske stammer. At flertallet af de finske stammer tilhørte serovar O1 og O2 tyder på, at kommercielle vacciner anvendt i finsk akvakultur burde være effektive mod vibriose i Finland.

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