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Virulence, Cytotoxic and Inflammatory Activities of Vibrio Anguillarum and Aeromonas Salmonicida Isolated from Cultivated Salmonid Fish in Sweden

By *Karel Krovacek, Ahmed Faris, Laila Eriksson, Eva Jansson, Olle Ljungberg* and *Ingmar Månsson*

Krovacek, K., A. Faris, L. Eriksson, E. Jansson, O. Ljungberg and I. Månsson: Virulence, cytotoxic and inflammatory activities of Vibrio anguillarum and Aeromas salmonicida isolated from cultivated salmonid fish in Sweden. Acta vet. scand. 1987, 28, 47–54. – Extracellular products in culture filtrates of Aeromonas salmonicida subsp. achromogenes and Vibrio anguillarum isolated from infected fish have been shown to possess skin inflammatory factor. The extracellular products from Vibrio anguillarum were cytotoxic in HeLa and CHO cells. In addition to the skin lesions, the culture filtrates of V. anguillarum caused necrotic reaction on the rabbit skin. Five of 6 strains of V. anguillarum were lethal to mice after intraperitoneal administration of 3×10^7 CFU. Only 1 strain of 5 A. salmonicida subsp. achromogenes produced extracellular products which elicited cytotoxic effects in the CHO cells. None of the A. salmonicida subsp. achromogenes strains were lethal to mice. The cytotoxins were inactivated when heated at 65°C for 30 min. The results indicate that the thermolabile exotoxins are non-enterotoxic since they failed to stimulate fluid accumulation in the rabbit ileal loop and did not cause elongation of the CHO cells. The rounding off of CHO cells, as well as of HeLa cells indicate that the exotoxins may play an important role in fish diseases.

Aeromonas salmonicida; Vibrio anguillarum; bacterial toxins; fish diseases; virulence factors.

Introduction

In a recent review by *Austin & Allen-Austin* (1985), bacterial pathogens of fish, belonging to 25 genera, have been described as being associated with disease of fresh water as well as marine fish. The authors point out that fish diseases due to bacterial infections are major problems in aquaculture. There is therefore, a need for basic water hygiene, which when neglected may lead to the start of a disease cycle (*McCarthy* 1977). Research on fish diseases has so far been mainly orientated towards pathology of the diseases, vaccination and profylactic treatment (*Austin & Allen-Austin* 1985).

Infections by Aeromonas and Vibrio spp. are known to result to furunculosis and vibriosis (*Austin & Allen-Austin* 1985). Both species produce a range of extracellular products (ECP) such as haemolysins, enterotoxins, proteases and endotoxins. (*Bernheimer et al.* 1974, *Sanyal et al.* 1975, *Donta et al.* 1978, *Eurell et al.* 1978, *Huizinga et al.* 1979, *Jiwa* 1983). Experimentally, it was de-

monstrated that the ECP from some of Aeromonas and Vibrio organisms are lethal to salmoid fish (*Cipriano et al.* 1981, *Ellis et al.* 1981, *Shieh et al.* 1981). Thus the aim of this investigation was to biologically characterize the virulent properties of Vibrio anguillarum and Aeromonas salmonicida subsp. achromogenes and to study their extracellular products with special reference to toxin production.

Materials and methods

Strains

The clinical history of 6 strains of Vibrio anguillarum and 5 strains of Aeromonas salmonicida subsp. achromogenes, isolated from rainbow trout, grayling, sea trout and char is summarized in Table 1. All strains were isolated from fish with clinical symptoms in fish farms with disease outbreaks.

Main source of isolation was the kidney; but strain B 98 (A. salmonicida subsp. achromogenes) was isolated from grayling skin sore. Pathological changes of the diseased fish ranged from muscular and fin bleeding to skin sore, and sloughing off of scales. In general, the fish (Table 1) underwent tissue damage of one form or another, apart from 1 sea and 1 rainbow trouts which lacked post mortem findings of interests.

Growth condition

Strains were streaked onto blood (5 % horse blood) agar with 2 % NaCl and incubated 48 h at 20°C. Thereafter the bacteria were harvested and inoculated into 50 ml Trypticase yeast broth in shaker flasks for 48 h at 20°C. The cultures were centrifuged at 16.000 xg at 4°C for 30 min and the supernatants were then millipore filtered (Millipore filter 0.22 µm). Sterile cell-free culture filtrates were then immediately tested for cytotoxic and hemolytic activities or aliquoted and frozen at –20°C for later studies in the rabbit skin test and the rabbit ileal loop test.

Assay of haemolytic activity

Haemolytic activity was detected by washed 1 % sheep erythrocyte suspension in phosphate buffered saline (PBS) (pH 7.0). 100 µl culture filtrates were 2-fold diluted with PBS in microtitre plates with U-shaped wells. Sheep erythrocytes (100 µl) were added into each microtitre well. The plate was incubated for 1 h at 37°C and afterwards for 24 h at 4°C. The haemolysin activity-titer was measured visually.

Assay of cytotoxic activity

Aeromonas and Vibrio isolates were examined for their ability to produce cytotoxin with activity to Chinese hamster ovary cells (CHO) and HeLa cells. Cells (2×10^6 cells/ml) were seeded in each well of a 96-well microtiter plate containing 200 µl of Hams-F12 medium supplemented with 10 % foetal calf serum, 1 % L-glutamine (200 mM), penicillin-G (100 units/ml) and streptomycin (100 µl/ml). The plates were incubated at 37°C for 2–3 days for formation of monolayers in a humidified atmosphere of 95 % air and 5 % CO_2. Fifty microliters (50 µl) of two-fold diluted culture filtrates were added into each well of monolayer containing fresh complete Hams-F12 medium. After 18 h of incubation as above, the cells were microscopically examined for any form of destruction or morphological alterations (*Janda et al.* 1985).

Rabbit skin assay

48 h culture filtrates were tested in the rabbit skin test in 2 to 2.5 kg New Zealand white rabbits. Evans blue (5 % w/v) was injected intravenously 18 h after 0.1 ml intradermal injection of test samples and the test was read 1 h later (*Sandefur & Peterson* 1976).

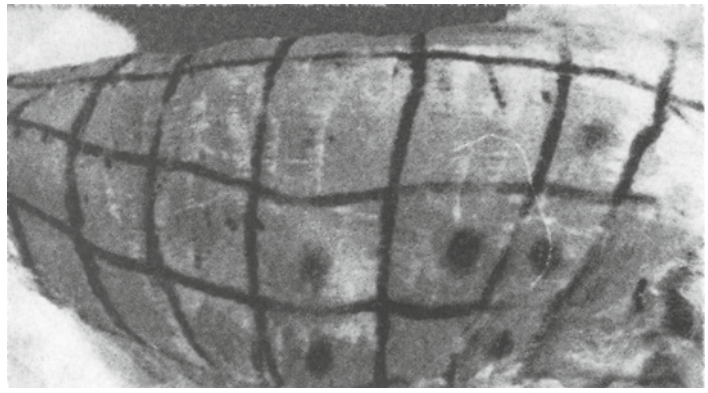


Figure 1. Rabbit skin showing induration and necrosis caused by Vibrio anguillarum culture filtrate.

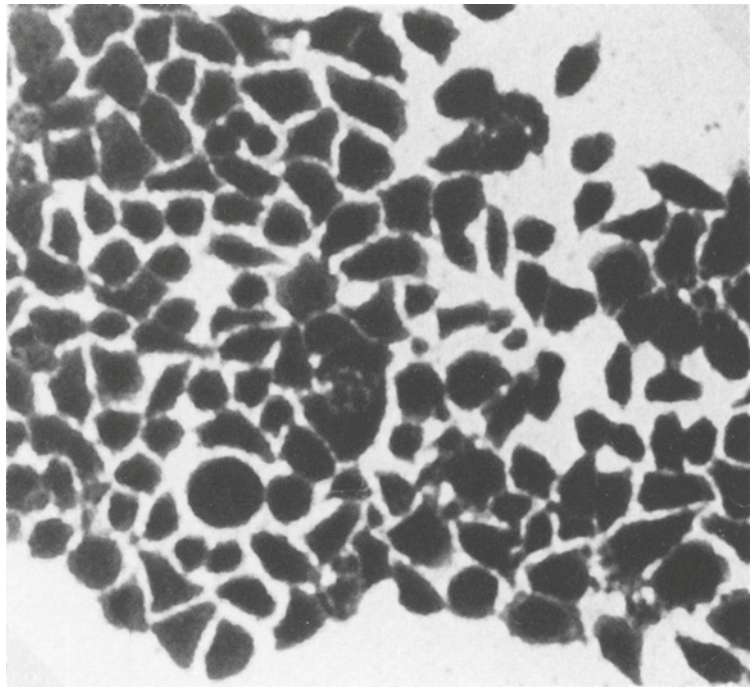


Figure 2. Untreated normal Chinese hamster ovary cells.

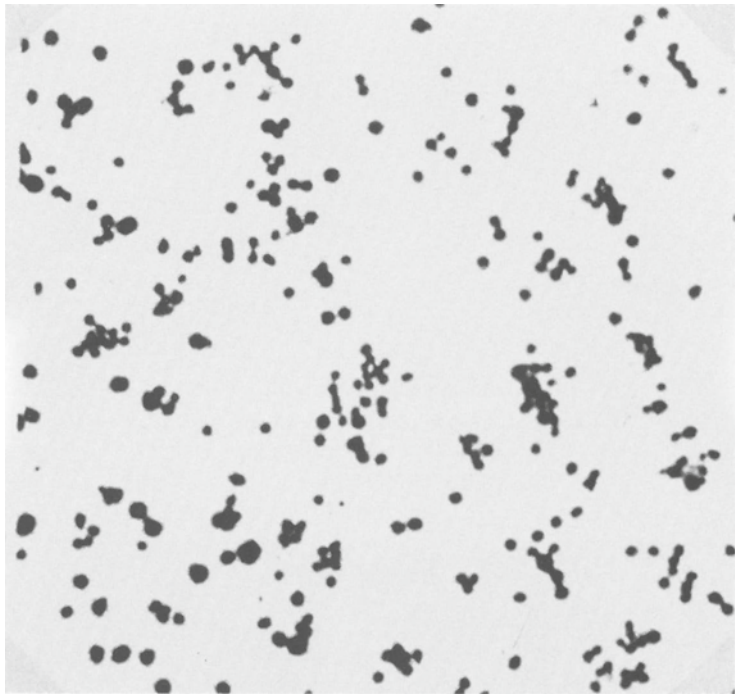
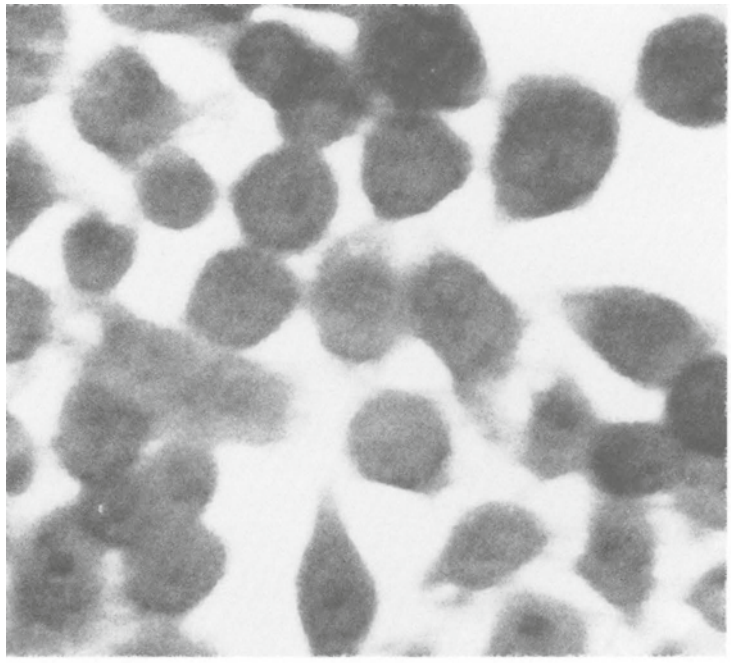
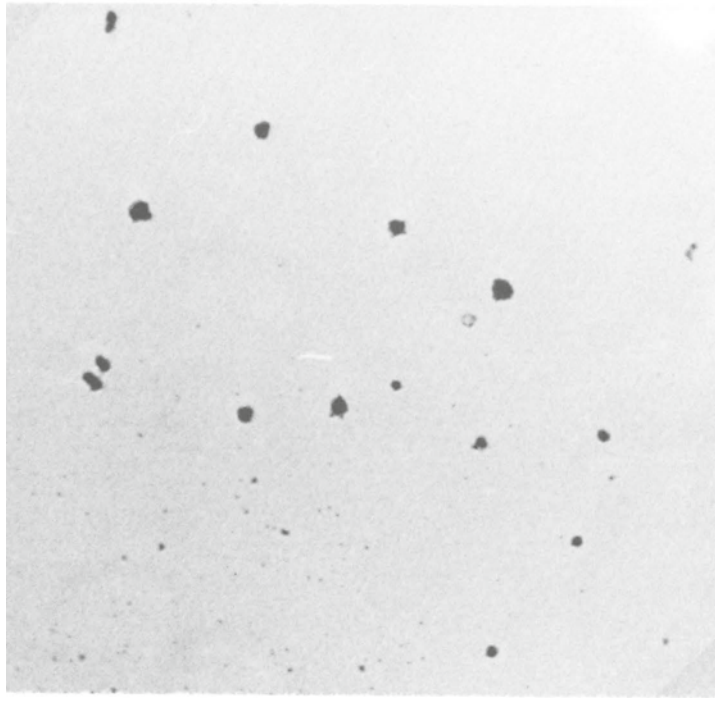


Figure 3. Cytotoxic effect in Chinesć hamster ovary cells caused by culture filtrate of Vibrio anguillarum.

Figure 4. Effect in HeLa cells 24 h after exposure to Vibrio anguillarum culture filtrate.
(A) untreated normal HeLa cells, (B) rounding effect and loss of adherence, (C) destruction of monolayer, (D) lysis and death of cells.



A



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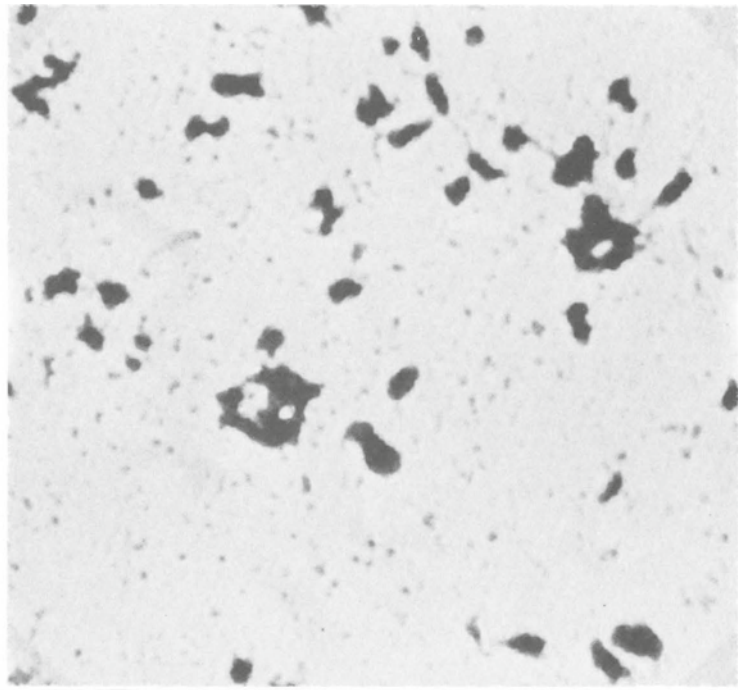

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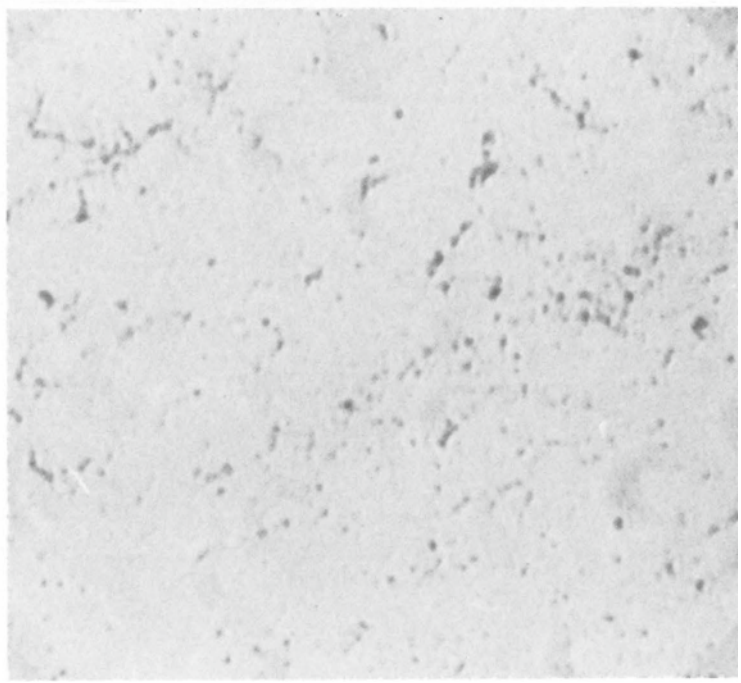

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Table 1. Clinical history of fish from which Vibrio anguillarum and Aeromonas salmonicida subsp. achromogenes were isolated.

Strain no.	Species and type	Host	Bacteria isolated from	Date for primary isolation	Gross pathology
B 2158	Va 1	Rainbow trout	Kidney	840923	Not investigated
B 2362	Va 1	Rainbow trout	Kidney	841012	Muscular bleeding
B 1924	Va 1	Rainbow trout	Kidney	850709	Muscular bleeding, skin lesion
B 2294	Va 1	Rainbow trout	Kidney	850806	Skin lesion
B 2299	Va 1	Rainbow trout	Kidney	850806	Muscular bleeding
B 2362	Va 2	Rainbow trout	Kidney	841012	Muscular bleeding
B 98	ASA	Grayling	Skin lesion	850114	Sloughing off of scales, skin lesion
B 1675	ASA	Sea trout	Kidney	850607	Bleeding at the fin basal region
B 1957	ASA	Sea trout	Kidney	850710	Pectoral fin damaged,
B 2296	ASA	Char	Kidney	850806	Bleeding at the basal region of pectoral fin skin lesion
B 2502	ASA	Sea trout	Kidney	850823	Negative

Va 1: Vibrio anguillarum, serotype 1.
Va 2: Vibrio anguillarum, serotype 2.
ASA: Aeromonas salmonicida subsp. achromogenes.

Rabbit ileal loop test

1 ml 48 h culture filtrates were tested in rabbit ileal loop test. Intestinal fluid accumulation were measured after 18 h for detection of enterotoxin effects (*Sanyal et al.* 1975).

Mouse lethality test

The strains were incubated on horse blood agar for 48 h at 20°C. The cultures were suspended in physiological saline, pelleted and washed in saline prior to photometric standardization of the suspension to 3×10^8 CFU/ml (OD 0.4 at $A_{430\ nm}^{10\ mm}$). For each strain 5 albino mice weighing 20–25 g each were injected intraperitoneally with 200 µl of the bacterial inoculum (6×10^7 CFU). The mice were observed daily for 1 week for determination of the pathogenicity of the strains (*Janda et al.* 1985).

Results

Cultural conditions on agar

Cultures on 5 % horse blood agar with and without 2 % NaCl at 20°C showed that Vibrio strains grew as medium size colonies as compared to the small Aeromonas colonies, after 16–24 h. Colonies of Aeromonas salmonicida subsp. achromogenes on blood agar, in contrast to those of Vibrio, were compact and on harvest, they dislocated from the agar surface as separate entities.

Biochemical tests

Biochemical profiles of and differences between Vibrio and Aeromonas strains showed that Vibrios fermented maltose, were indole, arginine, Voges-Proskauer and citrate positive. They produced amylase (starch hydrolysis positive). The Aeromonas strains were

Table 2. Biochemical and other characteristics of Vibrio anguillarum and Aeromonas salmonicida subsp. achromogenes isolated from different fish.

	Vibrio anguillarum						A. salmonicida subsp. achromogenes				
Biochemical tests	B 2158	B 2362 (Va 2)	B2362 (Va 1)	B 1924	B 2294	B 2299	B 98	B 1675	B 2296	B 2502	B 1957
Va 1 typing antisera	+	–	+	+	+	+	NT	NT	NT	NT	NT
Va 2 typing antisera	–	+	–	–	–	–					
Ramnose	–	–	–	–	–	–	–	–	–	–	–
Glucose	a	a	a	a	a	a	a	a	a	a	a
Lactose	–	–	–	–	–	–	–	–	–	–	–
Maltose	a	a	a	a	a	a	(–)	–	–	–	–
Saccharose	a	a	a	a	a	a	a	a	a	a	a
Indole	+	+	+	+	+	+	–	–	–	–	–
OF	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
Motility	+	+	+	+	+	+	–	–	–	–	–
15°C growth at	+	+	+	+	+	+	+	+	+	+	+
24°C growth at	+	+	+	+	+	+	+	+	+	+	+
37°C growth at	–	(+)	–	–	–	–	–	–	–	–	–
Arginine	+	+	+	+	+	+	–	+	–	–	–
Ornithine	–	–	–	–	–	–	–	–	–	–	–
Lysine	–	–	–	–	–	–	–	–	–	–	–
MR	–	–	–	–	–	–	+	–	+	–	+
VP	+	–	+	+	+	+	–	–	–	–	–
Gelatine	+	+	+	+	+	+	+	+	+	+	+
Aesculin	–	–	–	–	–	–	–	–	–	–	–
Starch	+	+	+	+	+	+	–	(–)	–	–	–
Vibriostat 0129 150 µg, 10 µg	++	++	++	++	++	++	NT	NT	NT	NT	NT

a = acid production.
NT = not tested.

negative on these tests, and were non-motile. Two Aeromonas strains were negative on the MR test, and only 1 strain (B 1675) was arginine dihydrolase positive. However, Vibrio anguillarum, strain B 2362 (serotype 2), differed from the other Vibrios by its negative VP reaction (Table 2).

Skin test

Intradermal inoculation of 20°C-grown culture filtrates of Vibrio and Aeromonas strains (Table 1), in the rabbit skin test gave induration of the skin in the average size of 14 mm (Table 3). Culture filtrates heated at 65°C for 30 min were inactivated. As can be seen in Table 3, the induration factor was best produced extracellularly into the medium at 20°C. Bacterial sonicates did not give any form of skin reaction. The culture filtrates on rabbit skin were also found to give necrotic reactions which were eliminated by heating the samples at 65°C for 30 min (Table 3). Necrosis was not associated with haemolysin production, since all the

Table 3. Effect of growth temperature on induration activity of culture filtrate from Aeromonas salmonicida subsp. achromogenes and Vibrio anguillarum in rabbit skin test.

Strain	4°C	10°C	20°C		30°C	
			induration (mm)	necrosis	induration (mm)	necrosis
B 98 ASA	ND[a]	0	16	–	0	–
B 1675 ASA	ND	0	16	–	0	–
B 2296 ASA	ND	0	9	–	8	–
B 1957 ASA	ND	0	8	–	0	–
B 2502 ASA	ND	0	8	–	0	–
B 2158 Va 1	0	0	24	+	0	–
B 2362 Va 1	0	0	25	+	0	–
B 2362 Va 2	0	0	23	+	0	–
B 2294 Va 1	0	0	23	+	0	–
B 2299 Va 1	0	0	24	+	0	–
B 1924 Va 1	0	0	24	+	15	+

[a] not done.

strains, with the exception of strain B 2362, were non-haemolytic. Strain B 2362 (serotype 2) was haemolytic at the 1:2 dilution (Table 4), and haemolytic on horse agar plates.

Cytotoxic and mouse lethality properties

None of the strains listed in Table 1 were enterotoxin positive in the rabbit ileal loop test. However, when Chinese hamster ovary (CHO) cells were used, for detection of ex-

Table 4. Haemolytic, cytotoxic and mouse lethality properties of Aeromonas salmonicida subsp. achromogenes and Vibrio anguillarum.

Strain	Haemolytic activity[a]	Cytotoxin[b]		Mouse lethality
		CHO	HeLa	
B 98 ASA	–	–	–	0/5
B 1676 ASA	–	+	–	0/5
B 2296 ASA	–	–	–	0/5
B 1957 ASA	–	–	–	0/5
B 2502 ASA	–	–	–	0/5
B 2158 Va 1	–	+	+	2/5
B 2362 Va 1	–	+	+	0/5
B 2362 Va 2	+[c]	+	+	5/5
B 2294 Va 1	–	+	+	5/5
B 2299 Va 1	–	+	+	5/5
B 1924 Va 1	–	+	+	3/5

[a] against sheep erythrocytes.
[b] against Chinese hamster ovary (CHO) cells and HeLa cells.
[c] haemolysin positive at the 1:2 dilution.

tra-cellular toxic products in the cell-free filtrates, morphological changes were demonstrated by all the Vibrio strains and 1 Aeromonas strain B 1675 ASA (Table 4). The other Aeromonas strains were negative. All toxic properties of the Vibrio and 1 Aeromonas cell-free filtrates were inactivated at 65°C for 30 min. The unheated filtrates caused rounding off (Fig. 3) of the CHO cells, a phenomenon comparatively different from the typical CHO elongation caused by E. coli LT enterotoxin. None of the cytotoxins from the individual strains under these experimental conditions were cytoinvasive to the CHO cells. When HeLa cells were used, they underwent shrinkage, loss of adherence to the plastic surface of tissue culture plates and cell death.

Mouse lethality test

Mouse lethality tests for virulence of the individual strains suggested that intraperitoneal inoculation of 6×10^7 bacteria harvested from blood agar cultures of Vibrio anguillarum was effective in determining the virulence status of the Vibrio strains. As can be seen in Table 4 mice infected by B 2362 Va2, B 2294 Va1, and B 2299 Va1 followed by B 1924 Va1 and B 2158 Va1 died in great numbers. Although strain B 2362 Va1 was cytotoxic in the CHO and HeLa cell tissue culture test systems, none of the mice infected by this strain died during the experimental period (Table 4). Mice infected by Aeromonas salmonicida subsp. achromogenes survived during the experiment.

Discussion

Our studies show that HeLa and CHO cells detect measureable amounts of cytotoxic acticity in culture filtrates of Aeromonas salmonicida subsp. achromogenes and Vibrio anguillarum. As indicated in Fig. 4a-d HeLa cell line is most sensitive for detection of the cytotoxin(s). Such products, from V. anguillarum cause detachment, lysis, and destruction of HeLa cells from the tissue culture plates. Damage of HeLa cells by V. anguillarum extracellular products corresponds to the necrotic activity observed on the rabbit skin.

Using CHO cells, it was possible to show differences between the morphological change caused by E.coli enterotoxins, and the extracellular products from the fish pathogens herein investigated. E. coli and V. cholerae enterotoxins cause elongation of CHO cells (*Guerrant et al.* 1974) whereas the susceptibility of CHO cells to the culture filtrates of the strains is expressed as rounding off of such cells (Table 4). In addition it is known as well that E. coli heat-labile enterotoxins have no effect on HeLa cells (*Janda et al.* 1983). These observations and the negative rabbit ileal loop test, thus confirm the non-enterotoxigenicity of V. anguillarum and A. salmonicida subsp. achromogenes fish pathogens listed in Table 1.

On the basis of the findings shown in Fig. 1, the skin test may be used as one of the biological indicators of virulence of the investigated strains or other fish pathogens. However, infection studies in fish will be relevant to perform in future to testify the relevance of these tests in a fish model. Still the skin reactions in Table 3 resemble the fish skin lesions from which the organism were isolated (Table 1). Other investigators have used the rabbit skin test to detect tissue-damaging heat-labile enterotoxin from A. hydrophila (*Dubey & Sanyal* 1978). Similar skin damage has been observed in fish after spontaneous infection with A. hydrophila (*Huizinga et al.* 1979). These authors reported small surface lesions, sloughing of scales, local haemorrhage and septicaemia. In this connection it seems that the findings herein reported, on the extracellular products of

Vibrio anguillarum and Aeromonas salmonicida subsp. achromogenes are interesting and further characterization is necessary since reports by *Sakai* (1977) and *Hsu et al.* (1981) showed that extracellular protease produced by A. salmonicida and A. hydrophila are responsible for their virulence. We still do not now to what extent the rabbit skin reactions described here are due to proteolytic products. Furthermore, the negative mouse lethality test should not be negatively interpreted as the strains may still be virulent to fish. We would like to emphasize as well that the high virulence of V. anguillarum and the avirulent properties of A. salmonicida subsp. achromogenes in the mouse lethality test is correlated to the products produced by these organisms. It was further found that the main differences between V. anguillarum and A. salmonicida subsp. achromogenes in their biochemical profiles were mainly on fermentation of maltose, indole reaction and production of arginine dihydrolase. V. anguillarum were positive in these tests. We found no link between the biochemical profiles, and the organisms' cytotoxicity.

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Sammanfattning

Virulens, cytoxisk och inflammatorisk aktivitet hos Vibrio anguillarum och Aeromonas salmonicida isolerad från odlad laxfisk i Sverige.

Genom att använda olika biologiska tester kan man demonstrera hur Aeromonas salmonicida subsp. achromogenes och Vibrio anguillarum d.v.s. fiskpatogena bakterier producerar extracellulära produkter. Dessa extracellulära produkter i bakteriella kulturfiltrat är cytotoxiska för HeLa och CHO cell linjer och orsakar inflammatoriska och nekrotiska reaktioner i kanin hud. Genom upphettning av kulturfiltrat till 65°C under 30 min inaktiveras dessa effekter. Fem av 6 Vibrio anguillarum stammar var lethala för möss efter intraperitoneal administration av 3×10^7 CFU. Ingen stam av Aeromonas salmonicida subsp. achromogenes var lethal för möss.

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