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Vibrio species as agents of elasmobranch disease

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ABSTRACT: Two *Vibrio* species identified as *V. damsela* and a new sucrose-positive *Vibrio* sp., *V. carchariae* sp. nov., were simultaneously isolated from a brown shark which died while being held in captivity at a large aquarium. Pathogenicity studies were subsequently conducted using a variety of elasmobranchs, including smooth dogfish and lemon sharks. Both bacterial strains proved pathogenic, causing death in nearly all of the elasmobranch hosts challenged. Virulence studies revealed that both bacterial strains were cytotoxic for Y-1 mouse adrenal cells. The *V. damsela* strain was highly cytotoxic, causing Y-1 cellular damage at culture supernatant dilutions up to 1 : 128. Both strains were hemolytic, but neither exhibited the Kanagawa phenomenon. They were both capable of urea hydrolysis, an interesting trait, considering that elasmobranchs retain large (ca 300 milliosmolal) urea concentration in their tissue.

INTRODUCTION

Interactions among *Vibrio* species and aquatic animals, both vertebrate and invertebrate, have been of interest to marine biologists for many years. Of the studies published to date, the microbial interactions include crustaceans (Krantz et al., 1969), shellfish (Colwell & Liston, 1960, 1962; Tubiash et al., 1970; Hada et al., 1984), copepods (Huq et al., 1983; Kaneko & Colwell, 1978), sea urchins (Guerinot et al., 1982), and fin fish (Toranzo et al., 1983). In many cases, a mutualism has been hypothesized, e.g. *V. diazotrophicus* and sea urchins (Guerinot et al., 1982), and *Vibrio* spp. and shellfish (Colwell & Liston, 1960, 1962). In other cases, the interactions are clearly pathogenic, e.g. *V. anguillarum* and salmonids (Colwell & Grimes, 1984). We have recently documented a new *Vibrio*-marine animal interaction involving sharks (Grimes et al., 1984) and a description of vibrios involved and the relationship is provided here.

EXPERIMENTAL PROCEDURES

Two bacteria were isolated during necropsy of a brown shark *(Carcharhinus plumbeus)* that died in captivity at the National Aquarium in Baltimore, Baltimore, MD.

Isolate 1116a was cultured from the liver and 1116b from the kidney. Detailed descriptions of the isolation procedure and disease characteristics have been published elsewhere (May et al., 1983; Grimes et al., 1984). Briefly, the disease was characterized by lethargy, inappetence, disorientation and subdermal cysts with necrosis, prior to death of the shark. At necropsy, notable features were vasculitis, meningitis, encephalitis, marked kidney necrosis, and involvement of the spleen, liver, and kidney. Both vibrios were isolated from thioglycollate broths into which necropsied specimens had been placed.

Tests conducted on the two isolates included cellular and cultural morphology observations, biochemical tests, G + C ratios, DNA-DNA hybridization, virulence tests, urea metabolism studies, and plasmid analysis. Details of the procedures are provided by Grimes et al. (1984).

DESCRIPTION OF ISOLATES

Isolate 1116a is a gram-negative coccobacillus (ave. size 1.0 × 0.7 μm), motile by means of a polar flagellum (1 to 2 per cell). Isolate 1116b is also gram-negative, but is longer (ave. size 1.6 × 0.5 μm) and forms both polar and lateral flagella. Cultural and morphological features for both isolates are shown in Table 1.

Biochemical attributes of isolates 1116a and 1116b are shown in Table 2. Notable traits for 1116a are: oxidase positive, sensitive to 0/129 (both 10- and 150-μg concentrations); deamination and decarboxylation of arginine; production of gas from glucose; hydrolysis of urea, gelatin and chitin; and positive methyl red and Voges-Proskauer reactions. Isolate 1116b is oxidase positive; sensitive to 150 μg of 0/129; decarboxylates lysine and ornithine; reduces nitrate to a non-gaseous product more reduced than nitrate; produces indole (plates typically have a strong indole odor); hydrolyzes urea, gelatin and chitin; produces acid from sucrose and salicin; and grows in 8 %, but not 10 % NaCl. These reactions, along with others listed in Table 2, place both isolates in the genus *Vibrio* (Pacini).

Table 1. Morphological and cultural features of *Vibrio damsela* 1116a and *Vibrio carchariae* 1116b

Test	*V. damsela*	*V. carchariae*
Form	coccobacillus	bacillus
Arrangement	single, pairs, chains	single, pairs, chains, clusters
Gram reaction	negative	negative
Flagellation		
liquid media	polar	polar
solid media	polar	polar and lateral
PHB granules*	+	+
Anaerobic growth	+	+
Growth range	10–41 °C	11–40 °C

* PHB = poly-β-hydroxybutyrate; visualized as phase-bright intracellular granules and as black granules when stained with Sudan black B

Table 2. Biochemical characteristics* of *Vibrio damsela* 1116a and *Vibrio carchariae* 1116b

Test	*V. damsela*	*V. carchariae*
Oxidase	+	+
0/129 sensitivity**		
10 µg	S	R
150 µg	S	S
Decarboxylase (Møller):		
arginine	+	−
lysine	−	+
ornithine	−	+
tyrosine	−	−
Arginine dihydrolase	+	−
Phenylalanine deaminase	−	−
Nitrate reduction	+	+
Denitrification	−	−
Growth in 0 % NaCl	−	−
3 %	+	+
6 %	−	+
8 %	−	+
10 %	−	−
Indole	−	+
Methyl red	+ (pH 4.8)	+ (pH 5.0)
Voges-Proskauer	+	−
Citrate	−	−
Enzyme production:		
alginase	−	+
amylase	+	+
caseinase	−	+
catalase	+	+
cellulase	−	−
chitinase	+	+
gelatinase	+	+
lecithinase	−	+
lipase	−	+
pectinase	−	−
urease	+	+
Fermentation of:		
arabinose	−	−
glucose	AG	A
inositol	−	−
lactose	−	−
mannose	AG	A
salicin	A	A
sucrose	−	A
O/F reaction:		
arabinose	NC/NC	K/NC
arbutin	K/NC	K/NC
cellobiose	A/A	K/NC
glucose	A/A	A/A
inositol	K/NC	K/NC
lactose	K/NC	K/NC
mannitol	K/NC	A/A

Table 2 (continued)

Test	*V. damsela*	*V. carchariae*
O/F reaction:		
mannose	A/A	A/A
salicin	K/NC	A/A
sucrose	K/NC	A/A
trehalose	A/A	A/A
ONPG**	−	−
Malonate	−	−
H_2S production	−	−
Utilization of:		
arabinose	−	+
γ-aminobutyrate	−	+
ethanol	−	+
glycine	−	+
α-ketoglutarate	−	+
l-propanol	−	+
propionate	−	−
urea	−	+
xanthine	+	+
yeast extract	+	+
Swarming	−	+
Bioluminescence	−	−
Hemolysis	β	α
Kanagawa	NG	−
VFA in glucose broth**	a, f, s	a, l, s
Moles % G + C (T_m)***	42.4	46.3

* S = sensitive; R = resistant; A = acid; AG = acid + gas; K = alkaline; NC = no change; NG = no growth; a = acetic acid; f = formic acid; l = lactic acid; s = succinic acid

** 0/129 = 2,4-diamino-6, 7 diisopropyl-pteridine; ONPG = O-nitrophenyl-β-D-galactopyranoside; O/F = oxidative reaction/fermentative reaction; VFA = volatile fatty acid

*** Average of 6 different determinations each with a different DNA preparation

MOLECULAR GENETIC STUDIES

Vibrio 1116a and *Vibrio* 1116b were observed to possess guanine + cytosine (G + C) base ratios of 42.4 % (standard error = 0.5 %) and 46.3 % (s.e. = 0.8 %), respectively. These also are consistent with attributes previously determined for members of the genus *Vibrio* (Minutes, Subcommittee on Vibrionaceae, International Judicial Commission, International Association of Microbiological Societies, 1972). When DNA prepared from vibrios 1116a and 1116b was compared with DNA from other, similar *Vibrio* spp., by means of DNA hybridization, isolate 1116a was found to have a high degree of similarity to *V. damsela* CDC-2588-80 (Table 3). Therefore, based on biochemical attributes and DNA homology, isolate 1116a was determined to be *V. damsela* and, subsequently, was accessioned by the American Type Culture Collection, ATCC 35083. Isolate 1116b is biochemically similar, in many respects, to *V. parahaemolyticus, V. alginolyticus,* and *V. tubiashii.* However, DNA prepared from 1116b

Table 3. DNA hybridization

Test organism	Hybridization with ^{32}P-DNA from *V. damsella* 1116a	Hybridization with ^{32}P-DNA from *V. carchariae* 1116b
Vibrio damsela 1116a	100.0	nd
V. carchariae 1116b	4.7	100.0
V. alginolyticus ATCC 17749	nd*	28.6
V. anguillarum ATCC 19264	3.8	8.2
V. damsela CDC-2588-80	88.0	9.5
V. fischeri ATCC 25918	20.7	20.6
V. furnissii VL 2386**	5.7	nd
V. gazogenes ATCC 29988	1.5	nd
V. parahaemolyticus ATCC 17802	nd	42.6
V. tubiashii ATCC 19109	3.8	10.9
Escherichia coli ATCC 11775	3.9	4.2
Control	0.0	2.0

* nd = not done
** formerly *V. fluvialis* biotype II

was not sufficiently homologous with DNA prepared from these, or any other *Vibrio* sp. tested, to warrant classification as a previously recognized species (Table 3). It was, therefore, concluded that isolate 1116b represents a new species of the genus *Vibrio, V. carchariae,* "vibrio of sharks" (Grimes et al., 1984), and accessioned by the American Type Culture Collection as ATCC 35084.

Both *V. damsela* 1116a and *V. carchariae* 1116b were examined for plasmids, using the alkaline extraction method of Kado & Liu (1981), modified as previously described (Grimes et al., 1984). *V. damsela* 1116a was found to contain four distinct plasmid bands with masses of 3.3, 4.3, 10.5 and ca 30 megadaltons (Mdal). *V. carchariae* did not contain detectable plasmids. Interestingly, *V. damsela* did contain one plasmid band, the 30 Mdal band, that was identical to that detected in the type strain of *V. damsela* (strain CDC-2588-80, Love et al., 1981). We are currently examining other *V. damsela* strains for plasmid content, using both whole plasmid analysis and restriction endonuclease analysis.

VIRULENCE STUDIES

Vibrio damsela and *Vibrio carchariae* were injected intraperitoneally (IP), into spiny dogfish *(Squalus acanthias)* and lemon sharks *(Negaprion brevirostris).* The infected dogfish died within 18 hours, post injection. Both *V. damsela* and *V. carchariae* were recovered from the dead dogfish sharks. Lemon sharks appeared to be more resistant to infection. That healthy lemon sharks could not be infected with *V. damsela* was concluded, based on the observation that 46 h after IP injection with 5×10^7 total cells, *V. damsela* could not be recovered from the tissue of a sacrificed shark. *V. carchariae,* on the other hand, could be recovered from a lemon shark injected with *V. carchariae* at a concentration of 5×10^7 cells. While *V. carchariae* did not kill the lemon shark, it did cause abnormal histopathology of the kidney, liver, and spleen (Grimes et al., unpubl.

data). In progress is a survey of healthy sharks to determine whether *Vibrio* spp. comprise a component of the normal flora of sharks. To date, *V. damsela* has been isolated from the stomach of a healthy lemon shark, *V. carchariae* from a white-colored skin lesion on a captive lemon shark, and *V. carchariae* from trematodes (*Dermophthirius* sp.) infesting the skin of lemon sharks (Grimes et al., unpubl. data).

Previously, *V. damsela* was reported by Love et al. (1981) to have a narrow host range, infecting only the blacksmith *(Chromis punctipinnis)* and the garibaldi *(Hypsypops rubicunda)*. Thus, this report extends the host range for *V. damsela* to include the spiny dogfish and brown shark, and lemon shark. The fact that *V. damsela* is a potential human pathogen (Love et al., 1981) extends its host range to include both warm and cold-blooded animals.

Virulence of *V. carchariae* and *V. damsela* was investigated using the Y-1 adrenal cell assay (Maneval et al., 1980). *V. carchariae* was found to be slightly cytotoxic, with less than 25 % of the monolayer cells being affected by undiluted culture supernatant. *V. damsela,* on the other hand, was highly cytotoxic. While no toxicity was noted during the first 4 h of incubation, 1 : 16 dilutions of culture supernatant caused complete destruction of the monolayer after 18 h. A toxic effect for *V. damsela* was also obtained at a dilution of 1 : 128. Kreger et al. (1984) have observed *V. damsela* to be strongly cytotoxic. Using a mouse red blood cell system, crude toxin from five strains of *V. damsela* was found to be more cytotoxic than similar preparations from *V. vulnificus* (Kreger et al., 1984). These studies, combined with Y-1 assay data, support the hypothesis that *V. damsela* produces a toxin(s).

UREA METABOLISM

Both *Vibrio carchariae* and *Vibrio damsela* were found to be capable of hydrolyzing urea (Table 2), a trait not common to *Vibrio* spp. (West et al., in prep.). *V. carchariae* utilized urea as a sole source of carbon and nitrogen, although such use was stimulated by addition of 0.01 to 0.1 % yeast extract. *V. damsela* was also able to utilize urea as a carbon and nitrogen source, but only in the presence of yeast extract (0.01 %–0.1 %). Both vibrios, upon growth in 2 % urea broth, raised the pH of the medium from the initial 7.0 to a final pH 9.2 (Grimes et al., 1984), a significant observation in view of the fact that sharks maintain a high urea concentration in their plasma and tissue fluids (approx. 330 milliosmolal or 2 %).

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