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Authors: HACKING, M. A., and BUDD, JOAN

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VIBRIO INFECTION IN TROPICAL FISH IN A FRESHWATER AQUARIUM

M. A. HACKING and JOAN BUDD, Department of Pathology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

Abstract: Vibrio anguillarum was identified as the causative agent of an epizootic in tropical freshwater fishes. It was pathogenic for selected species of other freshwater fishes, and was isolated from inoculated gravid guinea pigs, and their fetuses and dead young. Gross and microscopic lesions are described.

INTRODUCTION

Vibrio anguillarum has been described as the cause of disease in numerous marine fishes from many areas of the world,[2,3,6,7,13] but there are fewer reports of vibriosis in fishes reared in fresh water. From North America there are two reports of such an outbreak. Rucker et al.[12] reported an outbreak in rainbow trout *(Salmo gairdneri)* in a freshwater hatchery in Washington. The infection may have been introduced with marine fish which were used in the diet. Ross et al.[10] described an epizootic caused by *V. anguillarum* at the Willow Beach National Fish Hatchery, Arizona, in which the affected rainbow trout were maintaintd in freshwater and fed on a pelleted diet. The source of the infection was not determined.

The case described in this paper is assumed to be the first report of a *Vibrio* sp. causing death in tropical fishes maintained in a freshwater aquarium.

HISTORY

On November 10, 1970 several small tropical fish frozen in blocks of ice were brought to the fish pathology laboratory at the Ontario Veterinary College. The owner had purchased a used 30 gallon glass tank from the local pet supply shop and had added field stone from his farm and water from a 200 foot drilled well. Water from this well was used in the household and was reported to be "very hard".

Plants in the tank were from two sources: the majority were imported, having been acquired with the fish, but some had come from the local pet supply shop. He had stocked the tank with 30 fish: a selection of barbs *(Cyprinidae)*, tetras *(Characidae)*, loaches *(Cobitidae)* and corydoras *(Callichthyidae)* which he had imported from Holland. About two weeks after setting up the tank he had added two guppies *(Lebistes reticulatus)* which he had purchased from the local pet supply shop.

Temperature in the tank was maintained at 74 to 76 F (23 to 24 C), and aeration was achieved with charcoal filters and a small electric pump. Fish were fed on a flaked commercial dry diet (TetraMin) and live white worms *(Enchytraeus albidus)*. A pinch of salt was added to the tank water at irregular intervals.

No losses occurred for the first two months of operation but pale thickened areas on the tips of the fins of some fish had been noted. These had disappeared after the daily addition to the tank of a few drops of a solution containing malachite green. Water was added to the tank to replace loss by evaporation, but no water had been siphoned from the tank since the aquarium was established.

One of the guppies was the first to die. Two weeks later losses occurred in other fish and continued for the following two weeks. Affected fish had hyperemic areas at the base of the fins and on the ventral surface of the body. Death occurred soon after clinical signs of illness were noted. By the time specimens were brought to

the laboratory, at least 50% of the fish had died. A tiger barb *(Puntius* sp.) and loach *(Acanthrophthalmus* sp.) were selected for examination.

MATERIALS AND METHODS

Bacteriology

Blood agar plates (5% citrated calf blood) were used for primary isolation from the liver. These were incubated at room temperature (approximately 22 C).

Standard bacteriological methods were used for the following tests: ' oxidation-fermentation, motility, hydrogen sulfide, indole, urease, citrate, nitrate, methyl red, Voges-Proskauer, phenylalanine deaminase, starch hydrolysis, gelatin liquefaction, and carbohydrate utilization. The latter was determined in phenol red broth base (Difco) with 0.5% NaCl. Pathotec test papers (Warner-Chilcott, N.J.) were used for the cytochrome oxidase and lysine decarboxylase tests. Flagellation was demonstrated by the use of electron microscopy.

The ability to grow at 4, 15, and 37 C was determined by incubating trypticase soy agar plates (B.B.L.) for one week. The requirement for salt was determined by using nutrient broth (Difco) and tubes of the same medium containing 0.5 and 7.0% NaCl, and incubating them at room temperature for one week. Sensitivity to antibiotics and to the vibriostatic agent 0/129 (2, 4-diamino- 6,7 - di - isopropyl pteridine) was determined by placing discs of the following concentrations on heavily inoculated trypticase soy agar plates: chloramphenicol (5 μg), neomycin (5 μg), nitrofurazone (100 μg), novobiocin (5 μg), oxytetracycline (5 μg), penicillin (2 u), polymyxin B (50 u), triple sulfa (0.25 mg), and 0/129 (20 μg).

Strains

For comparison with our isolate (the OVC strain), three known isolates of *V. anguillarum* were obtained as follows:

1. American Type Culture Collection No. 14181 (12301 Parklawn Dr., Rockville, Maryland 20852) (ATCC 14181).
2. National Collection of Marine Bacteria No. 6 (A. J. Ross, Western Fish Disease Laboratory, Sand Point Naval Support Activity, Seattle, Washington 98115) (NCMB #6).
3. Willow Beach Strain (A. J. Ross).

Histopathology

Tissues were fixed in either 10% buffered formalin or Bouin's fixative, processed by routine methods, and stained with hematoxylin and eosin.

Infectivity

The owner submitted two live fish, a tiger barb and a corydoras, with hyperemia of the ventral surface and areas adjacent to the fins. These were placed in a small tank and three guppies were introduced to test transmissibility.

The following species of fish were injected intraperitoneally with either 1.0×10^{10} or 1.0×10^{11} organisms: pumpkinseeds *(Lepomis gibbosus L.),* brook trout *(Salvelinus fontinalis M.),* and goldfish *(Carassius auratus L.).* The doses of bacteria were determined by plating serial saline dilutions of the cells in plate-count agar supplemented with 0.5% NaCl.

To determine pathogenicity for warm-blooded animals, two each of mice, rabbits, and guinea pigs were inoculated intraperitoneally with 1 ml of a slightly turbid bacterial suspension in saline. Five pregnant guinea pigs were inoculated intraperitoneally with 1.0×10^{7} organisms. One pregnant guinea pig received an equal volume of saline, and served as a control.

Serology

Antiserum was produced in rabbits with two intramuscular injections 14 days apart each containing 0.5 ml of saline-washed cells of the OVC strain (adjusted to #2 McFarland nephelometer) to which was added 0.5 ml of Freund's complete adjuvant (Difco). The rabbits were exsanguinated and serum was collected 21 days after the second injection. A rapid slide agglutination test using undiluted antiserum was used to determine serological relationships between the strains,

and between cultures isolated from exposed and inoculated experimental fish and guinea pigs.

RESULTS

Bacteriology

Pure cultures of a small (1.1 - 2.8μ x 0.4 - 0.7μ), gram - negative, curved rod were isolated from both fish. On blood agar there were small (1 mm), convex, translucent, greyish colonies after 24 hours. These became green in heavy growth after 72 hours. There was no hemolysis and no diffusible pigment. Colonies became yellowish after 72 hours on trypticase soy agar. Most cells possessed a single polar flagellum (Figure 1); however, occasional bipolar flagallation was seen. Growth occurred in 10% CO_2.

Comparisons were made of the cultural reactions of the four isolates (Table 1). Variations occurred in the production of indole and in citrate utilization. No two strains were alike in these reactions. Variations occurred in acid production from arabinose, galactose, lactose, mannose, and sorbitol; again, no two strains were identical. The OVC strain appeared to have less tolerance for low temperatures, and a higher tolerance for low sodium chloride content than the other three strains. Reactions to antibiotics and to the vibriostatic agent 0/129 were identical with the exception of polymyxin B.

Pathogenicity

Two days after the admission of the live fish and the addition of guppies to the tank, the tiger barb died. Four days after this death one of the guppies was found dead, and a pure culture of *Vibrio*

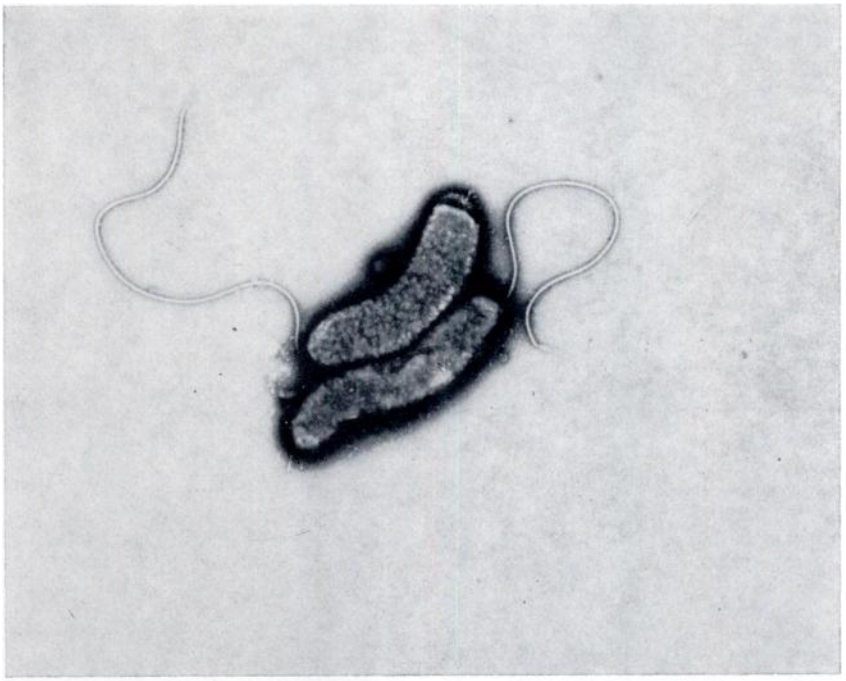

FIGURE 1. Electron micrograph of **Vibrio** sp. (OVC strain) showing single polar flagella and curved morphology. X14,280.

TABLE 1. Comparison of strains of **Vibrio anguillarum**

Test	OVC	ATCC 14181	Willow Beach	NCMB #6
Cytochrome oxidase	+	+	+	+
Fermentative	+	+	+	+
Motility	+	+	+	+
Indole	+	—	—	+
H_2S	—	—	—	—
Urease	—	—	—	—
Simmon's citrate	—	+	—	+
Nitrate reduction	+	+	+	+
Methyl red	—	—	—	—
Voges-Proskauer	+	+	+	+
Phenylalanine deaminase	—	—	—	—
Lysine decarboxylase	—	—	—	—
Starch hydrolysis	+	+	+	+
Gelatin liquefaction	+	+	+	+
Acid production in:				
arabinose	—	—	+	+
dextrose	+	+	+	+
dulcitol	—	—	—	—
galactose	+	—	+	—
inositol	—	—	—	—
inulin	—	—	—	—
lactose	+ *	—	+ *	—
levulose	+	+	+	+
maltose	+	+	+	+
mannitol	+	+	+	+
mannose	—	+	+	+
raffinose	—	—	—	—
rhamnose	—	—	—	—
salicin	—	—	—	—
sorbitol	—	+	+	+
sucrose	+	+	+	+
trehalose	+	+	+	+
xylose	—	—	—	—
Growth at: 4 C	—	(+)	(+)	(+)
15 C	(+)	+	+	+
37 C	+	+	+	+
Growth with NaCl 0%	(+)	—	—	—
0.5%	+	+	+	+
7.0%	—	—	(+)	(+)
Susceptibility to:				
chloramphenicol	+	+	+	+
neomycin	+	+	+	+
nitrofurazone	+	+	+	+
novobiocin	+	+	+	+
oxytetracycline	+	+	+	+
penicillin	—	—	—	—
polymyxin B	—	+	—	+
triple sulfa	+ **	+ **	+ **	+ **
0/129	+	+	+	+

* = positive after 14 days
** = weakly resistant after 48 hours
(+) = much reduced growth

sp. was isolated from the liver. The remaining guppies and the corydoras survived. The details of this experiment, and of others in which pumpkinseeds, brook trout, and goldfish were inoculated with the OVC strain, are summarized in Table 2. The injection of $1.0x10^{10}$ organisms failed to kill goldfish with either the OVC or the ATCC strains. In all cases in which mortality occurred, pure cultures of *Vibrio* sp. were isolated.

Our isolate was not pathogenic for non-gravid mice, rabbits, or guinea pigs. The results using gravid guinea pigs are given in Table 3.

Pathology

Hemorrhage was the most consistent lesion. It was evident to some degree in all fish but the affected areas varied as did the severity of the lesions.

Neither the tiger barb nor the loach from the original submission had gross lesions. Microscopically the loach had localized accumulations of slightly curved organisms within the blood vessels of the dorsal muscles. In the tiger barb there were small granulomas containing acid-fast organisms in the mesentery adjacent to the liver.

TABLE 2. Vibrio Infectivity Studies with Fish

Species	No. of Specimens	Approx. Size (cm)	Water Temp. (°C)	Vibrio Strain	Dose/Route	Time to Die (hrs.)
Guppy	2	2.5	24	OVC	Exposed	
Guppy	1	2.5	24	OVC	Exposed	144
Pumpkinseed	3	10.0	21	OVC	$1.0x10^{11}$/IP	18
Brook trout	2	18.0	10	OVC	$1.0x10^{11}$/IP	
Goldfish	1	17.5	21	OVC	$1.0x10^{11}$/IP	44
Goldfish	2 *	11.0	21	OVC	$1.0x10^{10}$/IP	
Goldfish	2 **	11.0	21	ATCC	$1.0x10^{10}$/IP	

* One fish was killed 1 month and the other 3½ months postinoculation. No vibrio was isolated.

** One fish was killed 1 month and the other 2 months postinoculation. No vibrio was isolated.

TABLE 3. Vibrio Infectivity Studies with Gravid Guinea Pigs

Guinea Pig No.	Dose/Route	Time to Give Birth (days)	No. of Dead Young/Total No. of Young	Results of Culture
1	$1.0x10^{7}$/IP	2	2/2	Vibrio isolated from one
2	$1.0x10^{7}$/IP	4 (adult died)	3/3 (fetuses)	No vibrio isolated
3	$1.0x10^{7}$/IP	4 (adult died)	4/4 (fetuses)	Vibrio isolated from adult and fetuses
4	$1.0x10^{7}$/IP	4	0/1	
5	$1.0x10^{7}$/IP	5	2/6 *	Pure culture of vibrio
6	Control	21	0/2	

* One of the live young died two days later. Vibrio was isolated in pure culture from the liver.

The guppy exposed in the laboratory aquarium had a pale liver and spleen. Histologically there was hemorrhage in the gills, and hemorrhage and small granulomas containing acid-fast organisms in adjacent tissues.

The goldfish had reddish discolorations on the body surface and the fins had a pinkish tinge from congested blood vessels. The abdomen was distended, and a gelatinous material was present in the abdominal cavity. The serosal surface of the abdominal organs was hyperemic, and the spleen was cherry red and somewhat swollen. The cut surface of the dorsal muscles was dark red, as were the muscles at the base of the fins and around the vent. Microscopically there was lymphocytic infiltration and increased fibrous tissue in the epicardium and an edematous appearance to the myocardium. In the liver there was marked cytoplasmic degeneration of hepatocytes, and sinusoids were distended with blood. In the head kidney abundant macrophages had replaced normal tissue. In the intestine focal areas of increased lymphocytes were present in the submucosa. There were hemorrhages on the serosal surface of the ovary and the ova were degenerate.

In each of the pumpkinseeds the liver was pale and blood vessels were prominent. The spleen was mottled and slightly enlarged. Histologically blood vessels in most organs were congested and there were many bacteria free in the lumen. There was massive hemorrhage obliterating the normal leucocytic elements in the kidney and spleen with large numbers of organisms present, many within macrophages. The epithelial cells of many kidney tubules were in various stages of degeneration.

Serology

The three stains used for comparison were not agglutinated by antiserum prepared against the OVC strain. However, cultures isolated from exposed and injected fish, and from adult and young guinea pigs were all agglutinated by this antiserum.

DISCUSSION

The bacterium isolated in this outbreak was a gram-negative, curved rod which was cytochrome oxidase positive, fermented glucose with the production of acid but no gas, was motile by a single polar flagellum, and was sensitive to the vibriostatic agent, 0/129. These criteria were used to place it in the genus *Vibrio*. Vibrios reported by Anderson and Conroy[1] to be pathogenic to fish include *V. piscium*, *V. ichthyodermis*, and *V. anguillarum*. Confusion is evident in the classification of these organisms. *V. piscium* is considered by some authors to be *V. anguillarum*, while others doubt that it is a true vibrio since it fails to attack carbohydrates. It is uncertain whether *V. ichthyodermis* is a valid species or whether it should be considered a variant of *V. anguillarum*. Our isolate showed a strong resemblance to the three cultures used as comparisons and to other *V. anguillarum* strains described.[2,3,5,6,7,9,13]

This isolate is a late lactose fermentor. The Willow Beach strain also was found to produce slight acid in lactose after 14 days. Strains fermenting lactose were also described by Nybelin[8] and Pacha and Kiehn.[9] Based on the work of Nybelin and Smith,[13] *V. anguillarum* has been subdivided into Types A, B, and C. Using this method of typing, the present isolate could be considered *V. anguillarum* Type A.

According to Anderson and Conroy, several workers have attempted to study the antigenic properties of the vibrios pathogenic to fish, and have concluded that cross reactions even between strains of *V. anguillarum* are rare when the isolates have been obtained from different epizootics. Pacha and Kiehn, on the other hand, found that 12 vibrio strains from the U.S. Northwest and three strains of European vibrios all cross reacted, and they further distinguished three serotypes among them. No cross reaction was found in the present study between the OVC strain, and the three strains of *V. anguillarum* used for comparison.

The OVC strain proved capable of killing experimental fish which were exposed to the original source, and those

injected intraperitoneally with $1.0x10^{11}$ organisms. The exception of the brook trout was attributed to the low water temperature. Rucker[11] stated in 1959 that fish vibrios are not pathogenic for warm-blooded animals, and under the usual testing methods, the present results are in agreement. However, LaGarde and Chakroun[9] reported in 1965 that their isolate was highly pathogenic for mice. The present studies indicate that this organism is capable of producing a septicemia in gravid guinea pigs, but the significance of the fetal deaths is difficult to interpret since the stage of gestation was not known. Further studies are necessary.

Although acid-fast organisms also were present in the barb and in the exposed guppy, it was evident from the infectivity studies that the vibrio alone, when present in sufficient numbers, caused death.

Clinical signs of disease were absent in some of the experimentally infected fish, and there was variation in the signs observed. This agrees with the experience of Smith who noted that there was no single set of signs of disease common to the finnock she examined. The hyperemia of body and fins and the lesions of hemorrhage and congestion of various organs have been described in vibrio infections by various authors.[1,10] However, in none of the fish we examined did we notice the blister-like lesions, muscle necrosis, or exophthalmus described in rainbow trout.[10,11] Possibly death intervened before these lesions developed in our experimentally infected fish. Intraperitoneal injection of the organisms is not the portal of entry in a natural infection.

The source of the vibrio in this case was not determined. Whether the imported fish were carriers, or whether the locally acquired guppy was the source, is unknown. One wonders why it was nearly three months before the outbreak occurred, and what was the triggering mechanism.

The disease was self limiting. No further losses occurred. The only changes made were the gradual replacement of the water in the aquarium and the owner stopped adding the malachite green solution and the pinch of salt.

The salt content of a water sample from his tank was 80 ppm (0.008%), so low that it seems insignificant as a factor in this outbreak of vibriosis. This occurrence emphasizes that the possibility exists of introducing fish diseases with imported tropical fish. Perhaps disease regulations concerning salmonid fishes should be expanded and control should be established to regulate the importation of all species of fishes.

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