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Source: Journal of Wildlife Diseases, 8(3) : 207-214

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-8.3.207

Vibrio anguillarum ISOLATED FROM A NASAL ABSCESS OF THE COD FISH *(Gadus morhua)* [1]

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Abstract: An abscess in the nasal region of a cod fish, held for experimental work, resulted in a large swelling. Purulent exudate filled the nostrils. Among the signs of distress was an apparent lack of equilibrium. Histological examination of the nasal area showed extensive destruction of the muscle and connective tissue and leucocytic infiltration.

Six microorganisms were isolated from the infected region. The suspected pathogen was identified as *Vibrio anguillarum*. The other isolates, felt to be secondary invaders or bacteria normally present in sea water, were *Achromobacter, Flavobacterium* and *Pseudomonas* species. The vibrio grew well at salinities of 2-5% and at temperatures of 10-24 C. It exhibited strong proteolytic activity.

Intramuscular injection of the suspected pathogen into mummichogs *(Fundulus heteroclitus)* resulted in a lesion similar to that originally observed.

The results of electron microscopy are described.

INTRODUCTION

Infections of marine fish by *Vibrio* spp. have been described frequently and the importance of vibrio infections in fish has been documented often.[6,14,18]

During the summer of 1970 a large swelling in the nasal region of a cod fish being held in the aquarium[19] for experimental work, developed into an ulceration of the musculature. The nares were filled with a purulent exudate. The lesion was due apparently to an abscess in the region of the nares. A *Vibrio* sp., which appeared to be the causative agent, was isolated from the lesion. This report describes the degenerative changes in the tissue at the site of the lesion, the preliminary results of the pathogenicity test of the isolate, and the morphology and physiology of the organism.

MATERIAL AND METHODS

Microbiological Studies

The surface of the infected area was washed repeatedly with 70% ethanol and samples taken aseptically from the interior of the abscess were streaked on trypticase soy agar prepared with both distilled water and a basal salt solution.[11] The isolates were maintained at 15 C. The biochemical tests followed the procedures outlined in the Manual of Microbiological Methods.[17] The pattern of carbohydrate metabolism was determined by the method of Hugh and Leifson,[7] the presence of cytochrome oxidase and production of 2,3-butanediol were determined by the methods outlined by Bullock.[3] The taxonomic schemes of Shewan *et al.*[16] and Bain and Shewan[2] were followed in identifying the organisms.

Trypticase soy broth was the medium used to determine the growth characteristics of the suspected pathogen at various temperatures and salt concentrations. Growth was estimated by measuring the increase in optical density of the cultures at 12 hour intervals.

The sensitivity to various antibiotics was determined by inoculating trypticase soy agar plates with 0.1 ml of a 24 hour culture. Sensidiscs (BBL) were placed on the agar and the plates were incubated at 15 C for 24 hours.

[1] This paper was presented at the Conference of the International Association of Aquatic Animal Medicine, University of Guelph, Guelph, Ontario, Canada, April 29-30, 1971.

Histological Studies

Muscle tissue removed from the infected area and from a healthy fish was fixed in 10% formalin and embedded in paraffin. The tissue was sectioned and stained with hematoxylin and eosin before examination.

Smears from the abscess of the infected fish were stained with either the Wright or the Gram stain.

Pathogenicity Tests

Groups of 5 mature mummichogs *(Fundulus heteroclitus)* were injected intramuscularly with 0.1 ml of a 24 hr culture of the three salt requiring bacteria grown in trypticase soy broth. Controls were inoculated with 0.1 ml of sterile medium. The fish were kept isolated in separate tanks supplied with running sea water at 15 C, and examined frequently for pathological signs and mortality.

Assay of Proteolytic Activity

The organisms were grown in litmus milk and incubated at 15 C for 3-4 weeks. After centrifugation at 12,800 x g for 30 minutes in a refrigerated centrifuge the supernatant solution was used for the enzyme determination. The assay system contained from 0.1 to 0.5 ml of the enzyme solution, 5 ml of 0.1 M Tris buffer (pH 7.2) and 25 mg of Azocoll* in a 25 ml Erlenmeyer flask. The reaction mixtures were incubated in a shaking water bath at 38 C for 20 minutes. The mixture was filtered and the optical density of the filtrate was determined at 520 mμ in a Coleman Junior Spectrophotometer. Blanks for comparison were prepared in the same manner except that the enzyme solution was heated at 100 C for 10 minutes.

Electron Microscopy

Stock cultures were grown on trypticase soy agar at 15 C. For negative contrast staining, organisms were suspended in a 1% aqueous solution of potassium phosphotungstate at pH 7.0.15. The suspension was placed on Formvar coated grids and drained. For the examination of ultrathin sections the cells were fixed with osmium tetroxide.[8] The sections were stained with uranyl acetate followed by Millonig lead staining as previously described.[10] Sections were cut on an LKB microtome and the prepared specimens examined in a Hitachi HS7S electron microscope.

RESULTS

The severely swollen and abscessed nasal region is shown in Fig. 1. The infection developed into an ulcer surrounded by inflamed tissue. The fish swam with a lack of equilibrium and became lethargic, lying motionless at the surface.

There was extensive damage to the connective and muscle tissues surrounding the lesion, with pronounced leucocytic infiltration.

Smears from inside the inflamed area showed the presence of numerous gram negative microorganisms, many of which exhibited a slight curvature (Fig. 2).

A total of six bacteria were isolated from the abscess and classified as: two species of *Achromobacter,* two *Pseudomonas,* one *Flavobacterium* and one *Vibrio.* The biochemical characteristics of the *Vibrio* sp. are shown in Table 1. The organism was a motile gram negative rod. Elongated cells were often present in pure cultures depending upon the age of the culture. Older cultures appeared to be more variable in cell morphology. Only the *Vibrio* sp. liquified gelatin and hydrolized litmus milk.

Preliminary pathogenic tests with the three salt requiring bacteria injected intramuscularly into mummichogs showed that the *Vibrio* sp. caused a mortality of 60% compared to the 12% in the controls. The mortality of the fish injected with the *Pseudomonas* and *Achromobacter* spp. was 40% and 24%, respectively. Furthermore, only the fish injected

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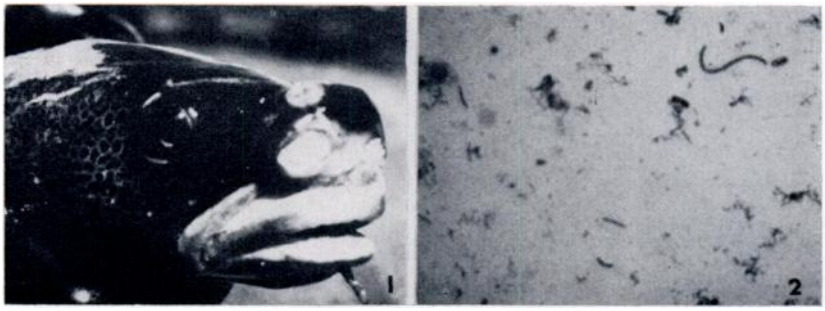

FIGURE 1. Nasal abscess of cod fish showing swelling and ulceration.

FIGURE 2. Smear from the interior of the abscess. Gram Stain, X 1340.

TABLE 1. **Characteristics of cod isolate.**

Test	Results	Test	Results
Nitrate reduced	+	Litmus milk	Hydrolysis
Indole produced	+	Cytochrome oxidase	+
H_2S produced	—	2,3-butanediol	—
Gelatin hydrolyzed	+	Glucose oxidation	Acid, no gas
Starch hydrolyzed	+	Glucose fermentation	Acid, no gas
Citrate utilized	—	Sucrose oxidation	Acid, no gas
Methyl red	—	Sucrose fermentation	Acid, no gas
Acetylmethylcarbinol	—	Mannitol oxidation	Acid, no gas
Catalase produced	+	Mannitol fermentation	Acid, no gas

+ = positive
— = negative

with the *Vibrio* sp. showed initial pathological signs of swelling and discoloration at the site of injection followed by the development of an open lesion. The organism was reisolated from the lesion and when reinoculated caused 60% mortality.

The organism grew well in salinities of 2-5% at 15 C (Fig. 3). The isolate grew as well in a medium prepared with 3% sodium chloride as with supplemented salt solution, indicating a requirement for sodium chloride. The organism failed to grow at 37 C but grew well from 10-24 C (Fig. 4).

The biochemical and physiological characteristics of the suspected pathogen identify it as *Vibrio anguillarum*. The sensitivity to various antibiotics is shown in Table 2. The organism exhibited strong proteolytic activity (Fig. 5) when grown in litmus milk at 15 C.

Electron micrographs of *V. anguillarum* (Fig. 6a) revealed polymorphic cells with a single polar flagellum. Thin sections showed an undulant cell wall parallel to, but separated from, the plasma membrane by an electron-translucent area (Fig. 6b). The apparent nuclear material was diffuse and poorly defined (Fig. 6c).

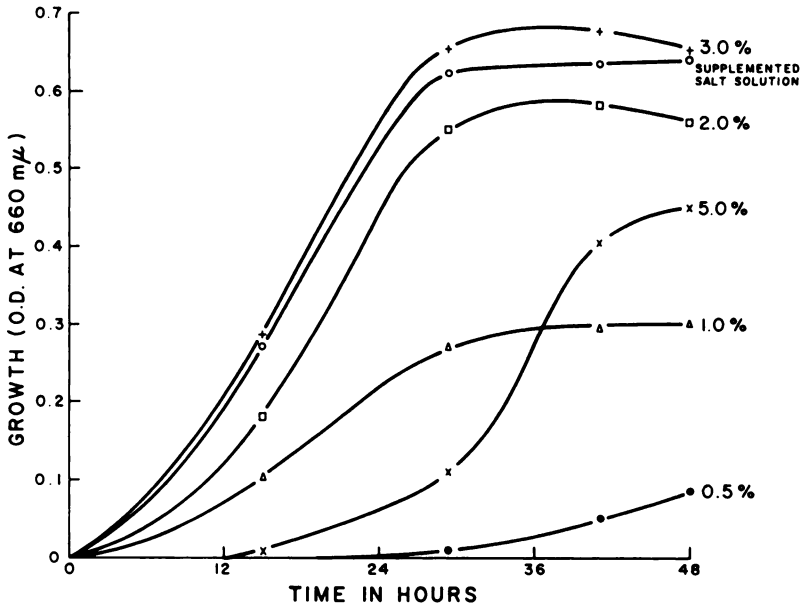

FIGURE 3. Growth of the vibrio at various salinities in trypticase soy broth medium.

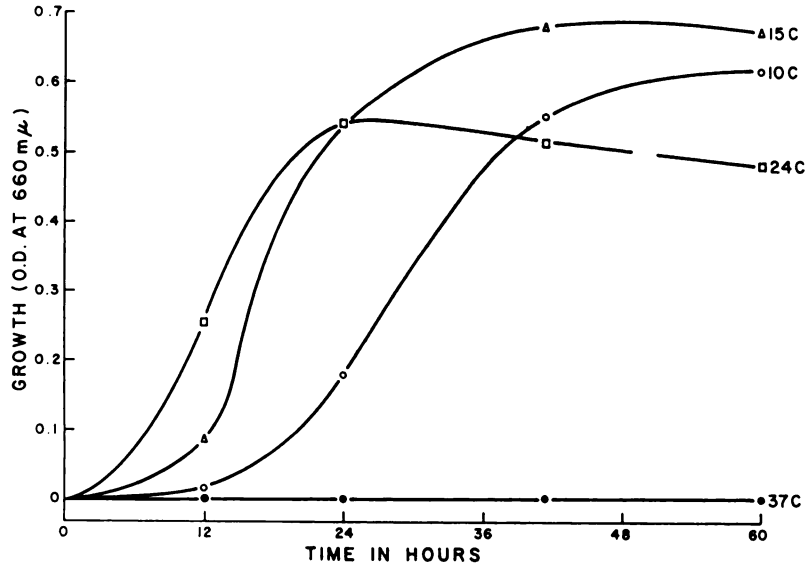

FIGURE 4. Growth of the vibrio at various temperatures in trypticase soy broth medium.

TABLE 2. **Sensitivities to various antibiotics of Vibrio anguillarum isolated from cod.**

Antibiotic	Concentration	Cod isolate	* Shewan
Chloramphenicol	30 μg	+	+
Dihydrostreptomycin	10 μg	—	+
Erythromycin	15 μg	+	+
Neomycin	30 μg	—	+
Novobiocin	30 μg	+	+
Penicillin	10 units	—	—
Polymyxin B	300 units	+	+
Tetracycline	30 μg	+	+

* reported by Pacha and Kiehn, 1969.
+ = sensitive
— = resistive

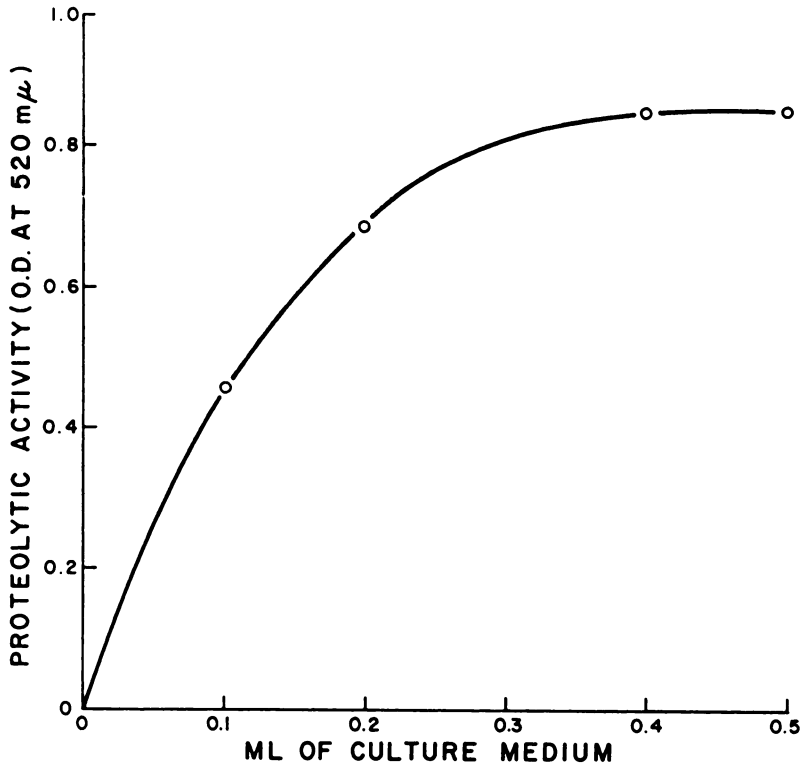

FIGURE 5. Proteolytic activity of culture medium.

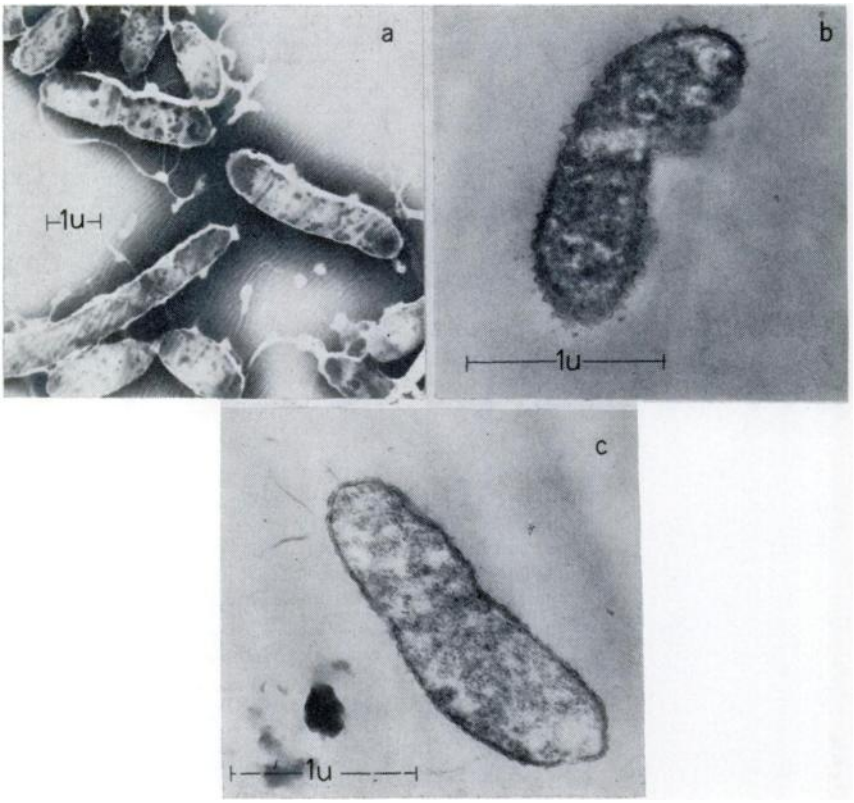

FIGURE 6. Fine structure of the vibrio: (a) negative stain showing polymorphic nature of the cells; (b) ultrathin section (note the irregular undulant cell wall); (c) ultrathin section (note the single polar flagellum and the diffuse nature of the nuclear material).

DISCUSSION

The signs of the infection in the nasal region of the cod are similar to the signs of vibrio infections outlined by Bullock and McLaughlin.[1]

The preliminary pathogenicity tests using mummichogs as the test animal indicate that *V. anguillarum* was the pathogen. The effects, however, of the *Pseudomonas* and *Achromobacter* spp. cannot be disregarded and will be the subject of further investigation.

Anderson and Conroy[1] outlined the biochemical characteristics used to differentiate the three biotypes of *V. anguillarum*. The ability of our isolate to produce indole and acid from sucrose and mannitol places it with type A. Slight biochemical and morphological differences are apparent between our isolate and a strain of *V. anguillarum* type A isolated from a cod by Shewan and reported by Pacha and Kiehn.[13] Our isolate lacked the ability to produce acetylmethylcarbinol, utilize citrate and grow at 37 C. In addition, our isolate was not sensitive to dihydrostreptomycin and neomycin.

Anderson and Conroy indicated that *V. anguillarum* showed optimum growth in media containing 1.5-3.5% NaCl.[1]

This is supported by the evidence that maximum growth at 15 C for our isolate occurred at salinities from 2-5%.

The electron micrographs of *V. anguillarum* revealed a structure typical of gram negative organisms.[20] The cell wall was irregularly undulant lying parallel to a well defined cytoplasmic membrane. A similar cell wall and plasma membrane structure was observed by Wiebe and Chapman[20] in marine pseudomonads but not achromobacters. The diffuse and poorly defined nuclear area of *V. anguillarum*, while similar to that of *V. parahaemolyticus*,[5] was in contrast to the well defined nuclear area of the marine pseudomonads.[20]

The mode of pathogenicity of vibrio infections is unknown. Bullock[4] mentioned the possible role of endotoxins. The proteolytic activity of our isolate and the histological evidence of extensive tissue damage indicate a possible role of the proteinase in the development of the lesions. The production of an extracellular proteinase by a pseudomonad, was important in the development of lesions in rainbow trout.[11] A compound, produced by *Pseudomonas aeruginosa*, toxic to insect larvae, was isolated and characterized as proteinase.[12] Although no special effort has been made in this study to characterize the proteinase, the use of a non-specific substrate to detect proteolytic activity cannot rule out the possible production of collagenase by our isolate. The characterization and determination of the role of the proteinase in the development of the lesions is an area which warrants and will receive further attention.

Acknowledgements

We thank Dr. J. E. Stewart for his interest and assistance in this work. We also thank Dr. R. A. Laycock for her helpful discussions in identifying the bacteria; and Mrs. B. M. Zwicker for her suggestions in performing the antibiotic sensitivity tests.

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Received for publication June 11, 1971