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The Role of Mechanical Injury in an Experimental Transmission of *Flexibacter columnaris* to Fingerling Walleye[1]

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The role of mechanical injury in transmission of *Flexibacter columnaris* to walleye fingerlings *(Stizostedion vitreum vitreum)* was investigated. Two groups (1 and 2) of 10 fish each, 7 to 9 cm total length, were exposed to a suspension of *Flexibacter columnaris* containing 3×10^5 cfu/mL; fish in group 1 were injured with a scratch along their flank; fish in group 2 were not injured. Two control groups (3 and 4) of 5 fish each were exposed to a sterile broth; group 3 was injured with a scratch and group 4 was not. In 72 h, morbidity was obvious in 7 of 10 fish in group 1, 1 of 10 in group 2, but none of the controls (groups 3 and 4). *F. columnaris* was isolated on cytophaga agar from swabs taken from moribund fish in groups 1 and 2, but not from healthy fish in any groups. In moribund fish, of group 1, *F. columnaris* was isolated from skin lesions of all 7 fish, from the gills of 2 fish, and from the kidney of 1 fish. Tissue damage in moribund fish in group 1 included extensive necrosis of the skin and underlying musculature; gill damage included epithelial separation, infarcts, and, in the most severe case, a secondary fungal infection. The inflammatory response included a substantial increase in relative abundance of lymphocytes in infected fish compared with healthy fish. In a plate culture, antibiotic sensitivity test, oxytetracycline was the most effective among the seven antibacterials examined.
INDEX DESCRIPTORS: *Flexibacter columnaris,* walleye, *Stizostedion vitreum vitreum,* bacterial diseases.

Columnaris disease, caused by the aquatic myxobacterium *Flexibacter columnaris,* was first described among warm-water fish of the Mississippi River Valley by Davis in 1922. The bacterium is not host specific, occurring in both warm and cold water fish in various regions of the United States (Nigrelli and Hunter, 1945; Davis, 1949; Johnson and Brice, 1952). It is now assumed to be ubiquitous to most freshwaters of the world (Post, 1987). Columnaris disease is recognized by the appearance of greyish-white or yellow areas of erosion, usually surrounded by a reddish hyperemic zone, on the body surfaces or the gills of fish. It has long been observed, although not experimentally evaluated, that an outbreak of columnaris disease is not spontaneous, but is influenced by combination of environmental (temperature) and other factors stressful to the host (Chen et al., 1982), such as nutritional deficiencies or injury (Post, 1987).

The disease is problematic in the warmer periods of the year. In salmonids, epizootics occur when the water temperature is in the range of 18-22°C, and the disease is rarely troublesome at <15°C (Amend, 1970). In some strains affecting warmwater and tropical fishes, optimal growth of the columnaris bacteria is at 28 to 35°C (Post, 1987). Although mechanical injury of the host is considered important for invasion of the bacterium, this has not been experimentally demonstrated.

Fingerling walleye *(Stizostedion vitreum vitreum)* reared in ponds on live feed to 35-50 mm can be transferred to intensive, tank-culture environments, trained to consume formulated feeds and reared to 150 mm (Nickum, 1978, 1986). The most serious constraint to the success of this procedure is the mortality caused by an epizootic of *Flexibacter columnaris* (Nickum, 1978) immediately after the pond-reared fish are transferred to the indoor, intensive culture environment. Since the tank environment has a continuous flow of clean, well-oxygenated water, it has been assumed, but not experimentally demonstrated, that the stress of capture by seining, handling and transporting the fish is the major factor responsible for incidences of columnaris disease in these fish and not the rearing environment of the intensive culture facility. Once the fish overcome the initial trauma, the disease is much less of a problem unless the environmental quality of the rearing environment deteriorates. Experimental observations on the role of trauma and mechanical injury in transmission of columnaris disease are lacking. Our present report: (1) demonstrates the role of mechanical injury in the transmission of *F. columnaris,* (2) describes the histopathological changes and hematological response of walleye fingerlings with a clinical infection of *F. columnaris* and (3) presents microbiological features to characterize the organism and evaluate its sensitivity to several antibacterial agents.

MATERIALS AND METHODS

Bacterial Strain

The culture used in this study was obtained from Mark Eimers of the National Veterinary Services Laboratories, USDA, Ames, Iowa. He obtained cultures from isolations made during epizootics of *F. columnaris* occurring in channel catfish *Ictalurus punctatus* at the Rathbun Hatchery, Iowa Department of Natural Resources, Moravia, Iowa (Moore et al. 1990). The primary isolate was from a kidney puncture streaked on Ordal's aga (Anacker and Ordal 1955), then subcultured in 10% trypticase soy broth (TSB). A sample of the TSB culture was frozen in a 50:50 ratio of TSB: glycerol at −70°C. We started a culture on TSB from a sample of the frozen sample.

Cytophaga agar (CA) (Anacker and Ordal, 1959) was used for bacterial isolation during this study. The plates were incubated at room temperature (≈22°C) for 2 to 5 days; colonies of *F. columnaris* were isolated and checked for purity and identity.

Preparation of Bacterial Culture

We used the method of Pacha and Ordal (1967) for the preparation of a live bacterial suspension. A single screw cap tube of 5 mL of 10% TSB was inoculated with a loopful of bacterial growth taken from a

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large colony grown on a CA plate; the TSB inoculum was incubated on a reciprocal shaker at room temperature for 24 h. Five mL of this bacterial suspension was used to inoculate 50 mL of broth in a 250 mL flask. After 16 to 18 h of incubation at 20°C, the flask culture was placed on a reciprocal shaker for 1 to 2 h, then the contents diluted 1:20 with water from the aquaria holding the experimental fish. This suspension was used as the contact medium to experimentally infect the fish.

Experimental Fish

The fish were 6-week-old, 7- to 9-cm fingerling walleye. Two groups of 10 fish each (groups 1 and 2) were exposed to the bacteria for 30 min by direct contact in a solution having 1:20 dilution of bacterial suspension. The solution contained 3×10^5 colony-forming units (cfu)/mL. Fish in group 1 were scratched on their flank above the lateral line under the dorsal fin with a sterile needle. Fish in group 2 were exposed to the solution without injury.

Two groups (groups 3 and 4) of 5 fish each were used as controls. They were exposed to a dilution of sterile broth for the same length of time as the experimental groups. After exposure to bacteria and broth, all groups of fish were placed in separate 37-L aquaria provided with a recirculating pump and a carbon filter.

Fish were inspected at 12- and 24-h intervals after exposure. Moribund fish, i.e., those exhibiting a severe infection, were removed before death for tissue and blood samples and for bacterial isolation.

Bacterial Isolation from Experimental Fish

Swabs from the skin, gills and kidneys of fish from each group were streaked onto CA for bacterial isolation. The plates were incubated at room temperature (≈22°C). Morphological and cultural characteristics of the bacterium were recorded. A bacterial suspension was made from the colonies in a small amount of TSB. This suspension was used as the inoculum for biochemical tests and sugar fermentation reactions to identify the isolates.

Antibacterial Sensitivity

Seven antibacterial chemotherapeutics (Becton Dickinson and Co., MD) were used to determine in-vitro sensitivity of *F. columnaris:*

Ampicillin	(10 mcg)	Streptomycin	(10 mcg)
Chloramphenicol	(30 mcg)	Tetracycline	(30 mcg)
Erythromycin	(30 mcg)	Triple sulpha	(1.0 mg)
Neomycin	(30 mcg)		

CA plates, one for each antibacterial compound, were streaked with bacterial suspension and antibiotic-discs were added. Plates were incubated at room temperature for 24-72 h to measure inhibition.

Blood Cell Response to F. Columnaris *Infection*

Blood was collected from 10 healthy (5 each, groups 3 and 4) and 13 infected (6 from group 1 and 7 from group 2) fish by cutting the caudal vein. Smears were stained with Giemsa, and differential leukocytic counts were made.

Preparation of Tissue for Histopathology

The body wall (skin and muscle), gills and kidneys of 3 normal (1 from group 3 and 2 from group 4) and 6 infected fish (5 from group 1 and 1 from group 2) were fixed in neutral, buffered formalin (10%). Parafiin sections were stained with haematoxylin and eosin.

RESULTS

Virulence

Direct exposure of walleye fingerlings to high concentrations of *F. columnaris* produced patent infections in group 1 fish by 72 h postexposure. The highest incidence of infection was recorded in group 1 where 7 out of 10 fish were infected after 72 h. Only 1 fish was affected in group 2 during the first 72 h, and none by 96 h when observation was terminated to obtain blood smears and tissue samples. None of the control fish (groups 3 and 4) had signs of infection.

Bacterial Isolation from Infected Fish

The organism isolated from infected skin, gill and kidney was *F. columnaris*. Morphological and biochemical tests and fermentation reactions gave similar results as described by Austin and Austin (1987). Colonies of *F. columnaris* developed after 48 h of incubation at room temperature. In 5 days, the colonies on CA were rhizoid and yellow-green, and varied in size from a pinpoint to large (≈4 mm). The colonies stuck tenaciously to the agar; they were so embedded in the medium that usually a small portion of agar had to be removed along with the colony to ensure growth in subcultures. Tissue sections revealed long, slender bocilli (Fig. 5). Morphological and biochemical tests and sugar fermentation reactions confirmed that the isolate was *F. columnaris* (Table 1). The organism was isolated from skin hemorrhagic lesions of all 7 fish that were near death in group 1, but only 2 fish had bacteria isolated from their gills, and only 1 from kidney tissue. Skin, gill and kidney of infected fish in group 2 also were streaked onto CA, but the organism was isolated from gill tissue form only 1 fish. *F. columnaris* was not isolated from fish in the control groups.

In-vitro Antibiotic Sensitivity Assay

Antibiotic sensitivity tests showed that the *F. columnaris* isolate was resistant to triple sulpha (i.e., no measurable inhibition), but sensitive to tretracycline (32 mm), chloramphenicol (24 mm), neomycin (20 mm), erythromycin (18 mm), streptomycin (16 mm) and ampicillin (10 mm).

Gross Pathology

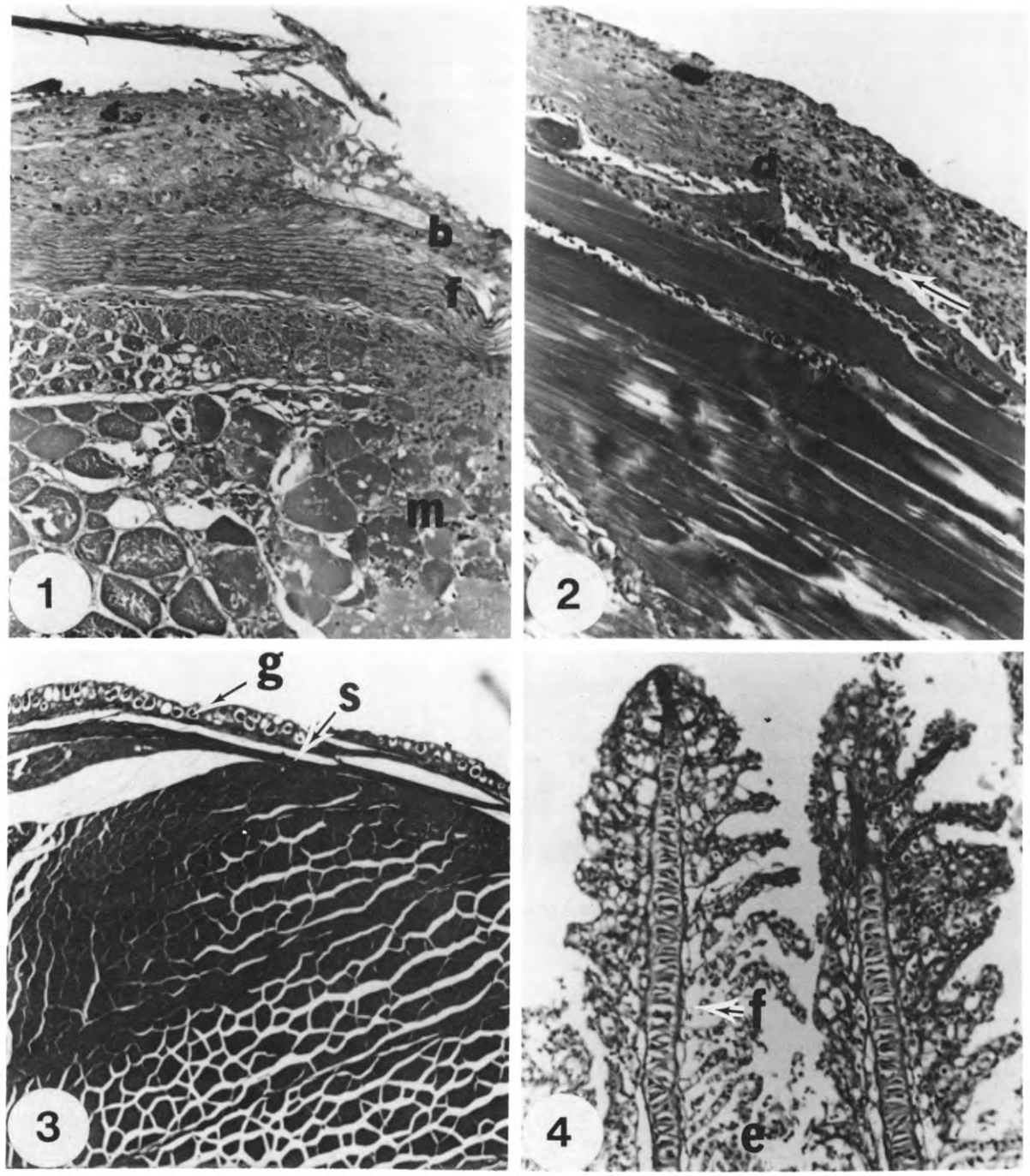
All fish were observed for gross pathology. Fish infected with *F. columnaris* (group 1) had 2- to 5-mm hemorrhagic patches along the lateral line and grey discoloration of the affected area. Of fish in group 1, only 2 of 7 developed discolored areas on the dorsal fin. Grossly, the internal organs of these fish did not show any lesions, nor were gross lesions observed in the control fish (groups 3 and 4).

Table 1. Morphological, biochemical tests and sugar fermentation reactions carried out for the confirmation of *F. Columnaris*.

Response (+/−) to morphological and biochemical tests								
Yellow-green colonies on CA	Motility	Gliding	Catalase	H_2S	Indole	Oxidase	Nitrate reduction	Voges Proskaver reaction
+	−	+	+	+	−	+	+	−
Production of acid in								
arabinose	glucose	lactose	mannitol	raffinose	sucrose	xylose		
−	−	−	−	−	−	−		

Microscopic Pathology

Skin and muscle from an area of hemorrhage in a group 1 fish showed an acute necrotizing ulceration with partial loss of the epidermis (Fig. 1). The epidermis surrounding the ulcer was hypertrophied (Fig. 1 and 2), and lacking in globlet cells (Fig. 2), which were abundant in the epidermis of healthy fish (Fig. 3). Degeneration occurred in the loose, hypodermis (Fig. 2) as a percursor to a sloughing of the skin.



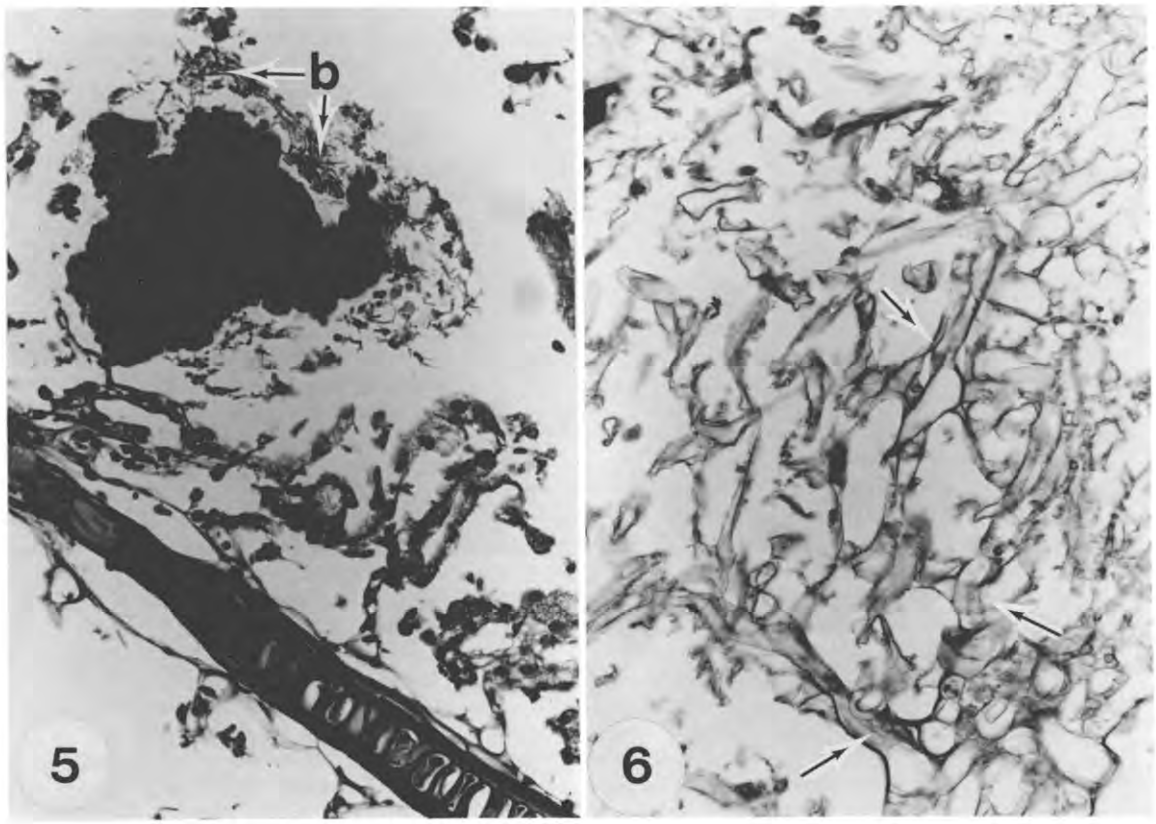
Figs. 1-4. Fig. 1. Transverse section through scratched skin showing epidermal hyperplasia, intracellular edema, and a lifting of the epidermis from the basal layer (b); this section also shows necrosis of underlying fibrous connective tissue (f) and muscle (m). Fig. 2. Epidermal tissue from scratched skin lacks goblet cells (contrast with Fig. 3); this section shows an extensive separation of the hypodermis (arrow) and degeneration (d) of collagen fibers. Fig. 3. Normal skin tissue showing numerous epidermal goblet cells (g) and thin dermis overlying the scales (s). Fig. 4. Gill filament of a fingerling walleye with an active columnaris infection. The epithelium (e) is separated from capillary bed, and edematous fluid (f) is present in both intracellular and extracellular spaces.

Bacteremia in infected fish in groups 1 and 2 produced conspicuous lesions on the gills (Fig. 4). Gill lesions were characterized by hyperplasia of the lamellar epithelium and dissociaiton of the epithelium from the capillary bed, which seems to be the result of accumulation of intra- and extracellular fluid. The gills had scattered areas of hemorrhage and foci of bacterial colonies (Fig. 5). A secondary mycotic invasion (presumably, *Saprolegnia*) also occurred in necrotic areas (Fig. 6). In normal gill tissue (Fig. 7), the epithelium of the lamellae adheres closely to the capillary bed.

There were no differences in the histopathology of kidney tissues from infected and healthy fish, and *F. columnaris* was isolated from the kidney of only one fish in group 1.

Differential Blood Cell Count

Blood cell response to *F. columnaris* infection was determined by differential leukocytic counts. Analysis of variance of the counts of lymphocytes, granulocytes and thrombocytes indicated a significant difference among the 4 groups (Table 2). There was a significant increase in lymphocytes, but a decrease in granulocytes and thrombocytes after infection as compared with control fish. Although a substantial increase in lymphocytes is evident, one cannot infer the granulocytes and thrombocytes actually decreased because differential counts from blood smears, in contrast to counts in a hemocytometer, only reflect relative proportions not absolute numbers. For lymphocytes, Duncan's multiple range test demonstrated a significant differ-



Figs. 5-6. Fig. 5. Gill filament from an infected fish with clusters of long, filamentous bacilli (b). Fig. 6. Section through gill area showing fungal mycelia (arrows).

Table 2. Comparison of the composition of lymphocytes, granulocytes and thrombocytes in blood smears of moribund (groups 1 and 2) and uninfected (groups 3 and 4) fish.

		Lymphocytes %			Granulocytes %			Thrombocytes %		
Group	*N*	Range	Mean[a]	SE	Range	Mean[a]	SE	Range	Mean[a]	SE
1	6	77-85	80.7A	1.4	8-15	11.8B	1.2	6-9	7.3A	0.5
2	7	72-82	77.3A	1.3	13-19	15.4A	0.9	4-10	7.3A	0.8
3	5	66-79	71.4B	2.2	13-22	18.4A	1.1	7-13	10.4B	1.4
4	5	68-75	71.0B	1.5	12-21	16.4A	1.2	11-15	12.6B	0.8
	F value		8.4			4.2			7.9	
	P value		0.009			0.0189			0.0013	

[a]Means within same designator (i.e., A or B) are not significantly different (P>0.05), Duncan's multiple range test.

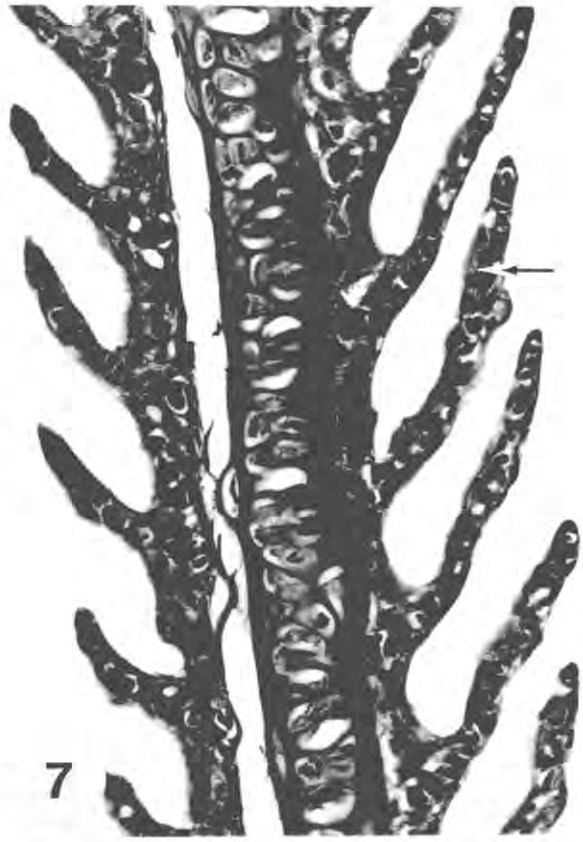


Fig. 7. Normal gill filament. Note the close adherence of epithelium (arrow) to the capillary bed.

ence in the means between the infected and control groups, but the differences in the means within the infected and control groups were not significant.

DISCUSSION

Previous experience with experimental transmission of *Flexibacter columnaris* has been highly variable, and often unsuccessful, because the virulence of the organism is rapidly attentuated in culture. In the present study, a frozen but viable sample, when thawed and subcultured, was still virulent. We think that the organism retained its virulence because the culture used for transmission was only 18-h old.

Transmission of columnaris requires a susceptible host and an exogenous (environmental) source of the bacterium; injured (scratched) controls did not develop columnaris infections. *F. columnaris* is not commonly found on the epidermis of fish because the mucous covering inhibits bacterial growth (Seiburth, 1975). Mechanical injury seems to be a predisposing factor in the presence of the virulent pathogen. A scratch or abrasion on the integument allows the pathogen to penetrate and infect the underlying tissue, as reported by Morrison et al. (1981) on Atlantic salmon *Salmo salar.*

Major pathological alterations were noted in sections of the skin of group 1 fish. Severe tissue damage indicates that mechanical injury can lead to focal areas of necrosis and bacteremia (Morrision et al. 1981; Ferguson 1981). Pacha and Ordal (1967), while studying the virulence of *F. columnaris* in sockeye salmon (*Oncorhynchus nerka*), suggested that a diffusible necrotising (proteolytic) substance was produced during growth of the bacteria on the skin leading to death of the fish. Morrison et al. (1981) recorded saddleback lesions when the organism was introduced by abrading the skin of Atlantic salmon, but we could not see such lesions in fingerlings walleye. The results of the present study demonstrate that skin abrasions from rough handling or from body contact with rough surfaes can be responsible for an outbreak of columnaris disease in walleye after harvest, handling, transportation, unloading and stocking in rearing tanks. Harvesting and transporting fish may cause surface damage in the form of abrasions or lost scales, and physiological trauma manifest in a decrease in the blood osmolality (loss of salt) (Lewis 1971).

Extensive gill damage was obvious in 3 fish (2 in group 1 and 1 in group 2). Gill tissue was disorganized; there was lamellar separation, areas of hemorrhage and infarcts. These findings are similar to those described for columnaris disease in sockeye salmon (Pacha and Ordal 1967).

Myxobacterium responsible for "cold water" disease of fish has a marked affinity for the renal glomeruli (Wood and Yasutake, 1956), but the kidney is uncommonly infected with *F. columnaris* (Morrison et al. 1981). In the present study, columnaris was isolated from the kidney, but only in 1 fish and histologically the kidneys of infected fish were similar to the controls.

Differential blood cell count showed significant increase in the relative abundance of lymphocytes, a common host response to infections (McCarthy et al., 1973; Alexander et al., 1980). The relative abundance of thrombocytes decreased. This may be a response to the abundance of lymphocytes or a rapid exhaustion of thrombocytes used to stem the hemorrhage caused by the lesions.

Antibiotic sensitivity tests showed that the *F. columnaris* isolate was resistant to triple sulpha (i.e., no measurable inhibition) and most sensitive to tetracycline. The antibiotic sensitivity test were similar to findings by Bootsma and Clerx (1976). The isolate studied by Fijan (1972) was resistant to ampicillin, and ampicillin also was the least effective antibiotic we tested. Fortunately, tetracycline, the most effective antibiotic tested, is an approved antibacterial for use in feed of food fish (Schnick 1988). Moore et al. (1990) found that vaccination, by immersion in a dilution of a bacterin, reduced the severity of but did not eliminate *F. columnaris* epizootics in channel catfish.

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