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## Examination of gills from salmonids with bacterial gill disease using monoclonal antibody probes for *Flavobacterium branchiophilum* and *Cytophaga columnaris*

D. J. Speare, R. J. F. Markham, B. Despres, K. Whitman, N. MacNair

**Abstract.** Bacterial diseases of the gills of commercially reared salmonids in freshwater are common problems. They accounted for 18% of all diagnostic submissions to the Atlantic Veterinary College from commercial fish hatcheries. Definitive diagnosis is difficult because of the growth characteristics of the putative bacteria in culture. Research into the pathogenesis of these diseases has also been similarly limited. Monoclonal antibodies (MAbs) were developed to 2 globally significant gill bacterial pathogens, *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease, and *Cytophaga columnaris*, the causative agent of columnaris disease of salmonids. These MAbs were then used as the basis for an indirect fluorescent antibody test to assess archived cases of gill disease in our region. *Flavobacterium branchiophilum* was the dominant bacterium detected in the biofilm of diseased gills in our study region. Of the cases tentatively diagnosed based on histopathology as bacterial gill disease, 76.2% tested positively with the MAbs to *F. branchiophilum*. Also present within 18.7% of these cases were bacteria which reacted positively to the MAbs for *C. columnaris*. We conclude that the MAbs produced are valuable diagnostic and research probes for common bacterial diseases of the gills of salmon and trout in Atlantic Canada. This study also adds further proof that *F. branchiophilum* acting alone can be sufficient cause for bacterial gill disease.

Bacterial gill disease (BGD) and columnaris disease of salmonids are recognized globally as important contributors to death losses during the freshwater stages of salmonid production.<sup>3,4,18,19</sup> A preliminary review of diagnostic case submissions to the Fish Health Unit Diagnostic Services of the Atlantic Veterinary College (AVC) indicated to us that bacterial gill disease was the leading problem prompting diagnostic submissions from fish farmers or fish health professionals in Atlantic Canada.<sup>12</sup>

Recent work has pointed to *Flavobacterium branchiophilum* as the causative organism of BGD in several parts of the world.<sup>4,9,19</sup> This disease is characterized by explosive morbidity and mortality rates attributable to massive bacterial colonization of gill lamellar surfaces and progressive branchial pathology stemming from high rates of lamellar epithelial necrosis.<sup>14,16</sup> Columnaris disease of salmonids is recognized to be caused by *Cytophaga columnaris* (historically referred to as *Flexibacter columnaris*).<sup>1,13</sup> Classically, during outbreaks, its morbidity and mortality rates escalate more gradually than for BGD. Additionally, unlike the pattern of necrosis in BGD, fish with columnaris will have severe necrosis of all parts of the gill as the bacterium invades inwardly.<sup>14</sup>

For both diseases, there is low culture recovery rate of these bacteria, particular *F. branchiophilum*,<sup>6,9</sup> from infected gills. Therefore our major goal was to develop a monoclonal antibody (MAb)-based test for BGD and columnaris which could be used diagnostically thus eliminating the need for primary isolation. Use of polyclonal antibodies against *F. branchiophilum* has been reported by Japanese researchers to improve the sensitivity and specificity of detection methods for BGD in rainbow (*Oncorhynchus mykiss*) and yamame (*Oncorhynchus masou*) trout.<sup>6,7</sup>

The second goal of this research was to use the MAbs as a probe to evaluate the extensive bacteria-rich biofilm characteristic of the clinical stages of BGD.<sup>15</sup> In most diseases in which biofilms develop, heterogenous populations are more common than homogenous populations.<sup>2</sup> Within these consortia, various different bacterial species contribute to the biofilm's ecology, which in total, results in damage to underlying host tissue.<sup>2</sup> The historical difficulties in experimentally recreating BGD with bath exposure of fish to *F. branchiophilum*, although recently resolved<sup>5</sup>, underlines the possibility that the gill colonization and progression to disease itself may require contributions from more than 1 genus or species of bacterium.

The ecology of the extensive biofilm, which develops during BGD<sup>15</sup> has not been well defined. One of the first descriptions involved an experimental exposure of healthy fish to a *Flavobacteria* sp. isolate which led to gill colonization (but not disease) associated with a

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biofilm exclusively composed of filamentous organisms.<sup>19</sup> Studies of the biofilm of numerous natural infections of BGD revealed the absence of bacteria in the biofilm of healthy fish and exclusively filamentous bacteria in the biofilm of fish with BGD.<sup>15</sup> These observations, taken in context with the recent reports describing experimental induction of BGD with *F. branchiophilum* isolates, has led to the assumption that the bacterial component of the diseased biofilm is exclusively composed of *F. branchiophilum*. However, both *F. branchiophilum* and *C. columnaris* are filamentous and are indistinguishable on light microscopy. Also, most of the ultrastructural features noted for *C. columnaris*<sup>10</sup> are also present in *F. branchiophilum*.<sup>15</sup> One reported distinguishing feature, the presence of mesosomal vesicles in *C. columnaris*,<sup>11</sup> was not noted in bacteria within the BGD biofilm.<sup>15</sup> However, it is controversial whether mesosomes are inherent functional structures or simply a feature arising from membrane damage or fixation.<sup>8</sup> Accordingly, they are an inherently unreliable means of differentiation. We therefore decided to develop and use MAbs to the 2 filamentous bacteria which colonize gills during disease as a probe to investigate the biofilm ecology of the gills of fish from natural outbreaks of BGD in Atlantic Canada. Determining the biofilm composition can provide important direction for further research on the initiation, promotion, and sequelae of BGD infections.

### Materials and methods

**Monoclonal antibody production-bacterial strains.** An isolate of *F. branchiophilum* (ATCC<sup>a</sup> #35036) and an isolate of *C. columnaris* (ATCC<sup>a</sup> #43622) were used to immunize mice. The *F. branchiophilum* isolate was reconstituted in a 50-ml broth solution of modified Shieh medium. The suspension was placed on a shaker and incubated at 20 C for 5 days. An inoculum of the suspension was grown on Cytophaga agar to ensure purity. An aliquot of the suspension was inoculated into 200 ml of the Cytophaga broth and incubated for 4 days for use in this study. The isolate of *C. columnaris* was reconstituted in 50 ml of Cytophaga broth, placed on the shaker and incubated at 22 C. It was then handled similarly to the *F. branchiophilum* isolate.

**Immunization of mice.** Bacteria were killed by suspension in 0.4% formal saline, washed, and resuspended in saline, and 0.1 ml of an OD<sub>540</sub> suspension was injected intraperitoneally (IP) into Balb/c mice. This was repeated again at 3 wk, and 2-3 wk later blood was collected and tested for specific reactivity. If necessary an additional injection was given. Three days before fusion, a final IP injection was given.

**Fusion protocol.** Mice were anesthetized by administration of halothane, then were killed by cervical dislocation and exsanguinated by transcutaneous cardiac puncture. The spleen was aseptically removed from each mouse and fused with the myeloma partner Sp2/0 (ATCCB) following standard protocols utilizing RPM1 medium (20% heat-inactivated fetal bovine serum, 1% L-glutamine, 1% penicillin/strepto-

**Table 1.** Diagnostic case totals (=outbreaks) per year of gill disease based on diagnostic submissions from salmonid hatcheries in Atlantic Canada to the Fish Health Unit, AVC (1989-1991).\*

1989	1990	1991	Combined cases 1989-1991	Gill disease cases 1989-1991	BGD cases 1989-1991	columnaris cases 1989-1991
93	155	107	355	126†	59‡	3§

\* A case or outbreak is defined as a collection of fish sampled from a tank or raceway affected by disease.

† Proportion of cases involving gill disease (1989-1991) = 35.7%.

‡ Proportion of gill disease cases involving BGD (1989-1991) = 46.9%.

§ Proportion of gill cases involving columnaris disease (1989-1991) = 2.4%.

mycin, 1% sodium pyruvate, and 0.1% mercaptoethanol)<sup>b</sup> with HAT (10 mM hypoxanthine<sup>a</sup> 4 x 10<sup>-7</sup> M aminopterin<sup>a</sup>, and 1.6 x 10<sup>-5</sup> M thymidine<sup>a</sup>) medium supplement.

**Screening of hybridoma cultures.** Hybridomas that developed were screened by fluorescent antibody (FA) technique. Briefly, 10 µl of bacteria suspended in phosphate-buffered saline (PBS) were spotted onto 12-well multispot slides<sup>c</sup> and allowed to air dry. They were fixed in acetone-methanol (60:40) and hybridoma supernatant was added and incubated for 30 min in a humidified chamber at room temperature. The slides were washed in PBS. Then 10 µl of a solution containing antibodies to mouse IgM and IgG conjugated to fluorescein isothiocyanate<sup>d</sup> were added and the slides incubated for another 30 min. Slides were then washed by two 10-min immersions in PBS, mounted in FA mounting fluid,<sup>e</sup> and examined using fluorescence microscopy. On each slide, positive and negative mouse serum, PBS, and culture supernatant from the Sp2/0 cells were used as controls.

**Cloning procedure.** Selected hybridomas were subcloned once by use of a limiting-dilution method. Clones were screened as above, and strong positive clones, specific for each bacteria, were selected and isotyped using a mouse MAb typing kit.<sup>f</sup> Specific clones were grown in RPM1 medium<sup>b</sup> (with 10% fetal bovine serum), and supernatant fluid was retained for use in testing without any purification.

To determine the specificity of the reaction, each monoclonal supernatant (MAb) was tested against the homologous organisms as well as against isolates of the following fish pathogens and/or potential mucous-film inhabitants. These included isolates of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Vibrio alginoliticus*, *Vibrio ordalii*, *Yersinia ruckeri*, *Cytophaga psychrophila*, and *Cytophaga aquatilis* collected from Atlantic Canada.<sup>g</sup> MAbs to *F. branchiophilum* were also tested against the type culture of *C. columnaris* and vice versa.

**Caseselection and indirect immunofluorescence of paraffin-embedded sections.** To provide a general guideline as to the relative importance of gill disease, and specifically BGD and columnaris, in freshwater aquaculture in Atlantic Canada, the archives of all cases submitted from salmonid hatcheries to the AVC diagnostic unit from 1989-1991 were reviewed (Table 1). It is acknowledged that this type of sampling is

**Table 2.** Provincial distribution of selected cases of BGD and columnaris selected for IFAT analysis.\*

	BGD	columnaris
Nova Scotia	3	0
Newfoundland	5	2
New Brunswick	0	1
Prince Edward Island	34	3
Total	42	6

\* A case or outbreak is defined as a collection of fish sampled from a tank or raceway affected by disease.

not representative of the general population and is prone to Berkson's bias<sup>20</sup> and therefore is presented only as an empirical trend.

To assist with case selection for the immunofluorescent examination of the biofilm of gills with either BGD or columnaris disease, a computer search was conducted of the cases archived at AVC from Prince Edward Island (PEI), New Brunswick, Nova Scotia, and Newfoundland, Canada, from 1989-1992 (Table 2). Archived histopathology slides were re-examined. Appropriate specimens from cases of typical bacterial gill disease and branchial columnaris disease were selected based on published morphologic diagnostic criteria.<sup>14</sup> Specifically, those cases which had little or no autolytic change, typical morphologic criteria for the diseases in question, abundant bacteria, and no morphologic evidence of common secondary pathogens<sup>14</sup> were selected. Additionally, for inclusion, the presenting history had to indicate that the outbreak was acute and the fish had not yet been medicated.

The final selection involved 42 outbreak cases of BGD and 6 outbreak cases of columnaris disease (Table 2). Histology sections were recut from archived paraffin blocks and then used for the indirect immunofluorescent assay (IFAT) examination. Specifically, 5 µm paraffin-embedded sections were permanently fixed to glass microscope slides by heating overnight at 37 C. They were then stored at 4 C until required for use. Before staining, sections were dewaxed with xylene, then transferred to a series of decreasing alcohol baths, and finally transferred to distilled water until reaction with antibody. The primary and secondary antibodies were each sequentially incubated with the tissue on the slide for 30 min, and washes between incubations were for 10 min each. The primary antibody consisted of the hybridoma tissue culture supernatant. The second antibody was a rabbit anti-mouse IgG (H + L) conjugated with fluorescein isothiocyanate.<sup>d</sup> After rinsing and application of coverslips, sections were examined using epifluorescent microscopy.

## Results

*Significance of gill diseases due to bacterial pathogens in Atlantic Canada.* Gill diseases accounted for over one-third of all diagnostic submissions to the AVC from commercial salmonid hatcheries in Atlantic Canada (Table 1). Of these gill disease cases, almost one-

half were identified as BGD and a negligible proportion as columnaris disease (Table 1).

Based on the case selection criteria used in this study, the majority of cases examined involved BGD outbreaks from PEI, however lesser numbers of BGD cases were also examined from Nova Scotia and Newfoundland (Table 2).

*Monoclonal antibody for F. branchiophilum.* This monoclonal antibody reacted negatively to the panel of bacterial fish pathogens including the *C. columnaris* isolate.

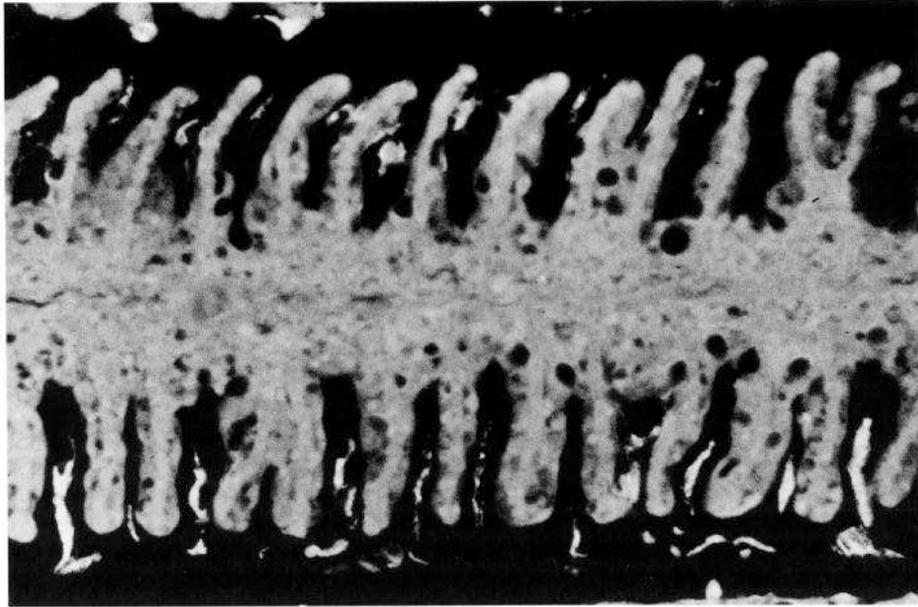
Overall, of the 42 cases deemed to have BGD, 32 of them (76.2%) tested positively with the monoclonal antibody. The MAb reacted both with individual filamentous bacteria and those which had developed into microcolonies (Figs. 1, 2). Microcolony formation is a characteristic of BGD. Positively fluorescing samples were present from each of the 3 provinces from which BGD cases were selected.

Fluorescence was limited to bacteria on the gill surface and in the mucous film, part of which was spatially dislodged from the gill surface (Figs. 1, 2). Chloride cells, particularly in the interlamellar regions of the gill had variable and sporadic degrees of autofluorescence (Fig. 3). There was no evidence of reactive bacteria within the gill structure and no evidence of reactive bacteria in other tissue sites of the fish, including the gastrointestinal tract.

*Monoclonal antibody against C. columnaris.* This MAb was reacted against the same panel of fish pathogens as described for the *Flavobacterium* MAb. There was no reaction to any of these. Additionally, it did not react against the *F. branchiophilum* isolate.

Of the 6 cases deemed to have columnaris disease, 4 of them (66.6%) tested positive with the MAb. The bacteria were strongly reactive and were noted in several locations. These included the gill surface, internal gill tissue associated with necrotic lesions, and on food particles and debris present between gill filaments. Reactive bacteria were also occasionally noted on food material within the gastrointestinal tract. Three of the four positive cases also showed the presence of lesser numbers of *F. branchiophilum*.

*Examination for mixed populations of bacteria in diseased gill biofilms.* Overall, of the cases presumptively diagnosed by histopathology as BGD there were no instances in which the samples reacted positively for *C. columnaris* but lacked *F. branchiophilum*. Of the 32 cases that showed *F. branchiophilum*, 6 of them (18.7%) also showed the presence of *C. columnaris* organisms. In general, they were located between filaments, rather than between lamellae, and were associated with cellular and other debris entrapped in gill mucus (Fig. 3). Three of the four positive cases of



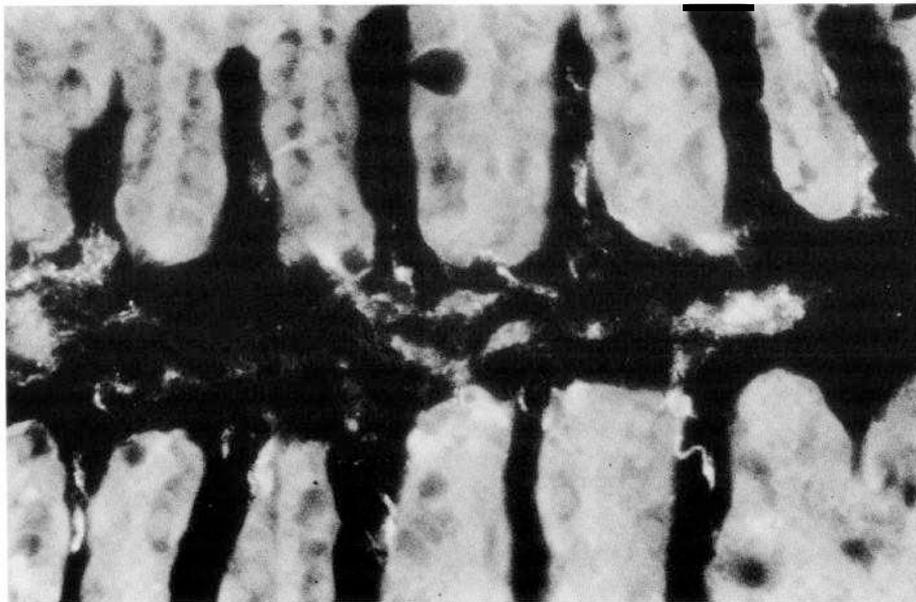
**Figure 1.** Gill filament from a rainbow trout with BGD. Indirect immunofluorescence technique demonstrating microcolonies of *Flavobacterium branchiophilum* between most of the gill lamellae. Epifluorescence x 175.

columnaris disease also showed the presence of small numbers of *F. branchiophilum*.

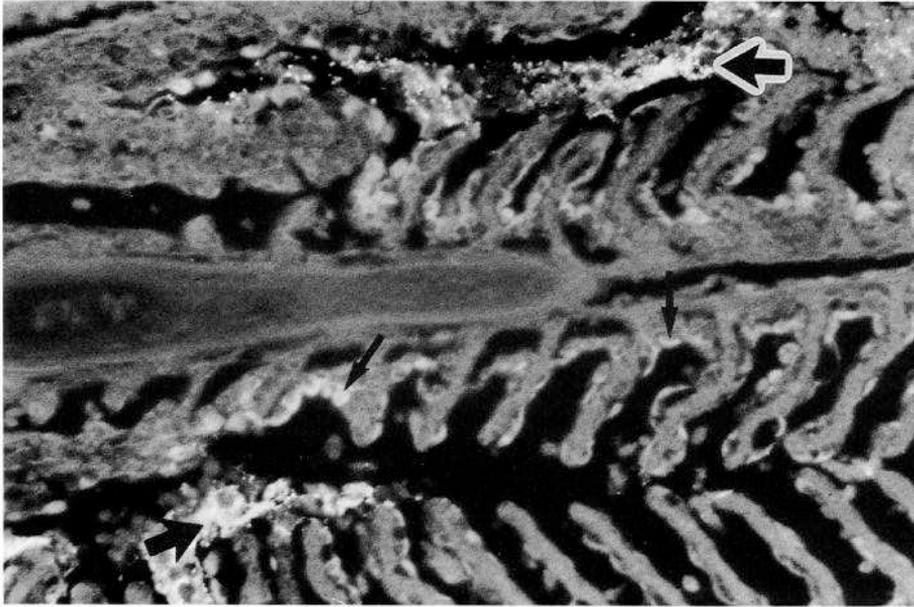
### Discussion

Based on the case survey results, gill diseases are a common reason fish farmers and attending health pro-

fessionals seek laboratory diagnostic assistance in Atlantic Canada. This is similar to the submission patterns in central Canada, and also for other regions where freshwater culture of salmonids is practised.<sup>14</sup> Infectious gill diseases characteristically present as outbreaks with rapid increases in proportional morbidity



**Figure 2.** Closer detail of gill lamellae from a rainbow trout with BGD. Indirect immunofluorescence technique demonstrating *Flavobacterium branchiophilum* occurring both individually and aggregated in close approximation to lamellar epithelial cells. Epifluorescence x 350.



**Figure 3.** Gill filaments from a rainbow trout with BGD. Indirect immunofluorescence technique demonstrating microcolonies of *Cytotophaga columnaris* (large arrows) associated with accumulated debris between filaments. The cytoplasm of some lamellar epithelial cells and intralamellar chloride cells displayed a degree of autofluorescence (small arrows). Epifluorescence x 125.

and mortality.<sup>14</sup> Accordingly, there is a need for accurate and rapid etiological diagnosis of these cases. Bacterial gill disease was far more common than columnaris disease in our study region. The relationship between columnaris disease and warmer water temperature is a likely factor reducing the role of this disease in our region.

The preponderance of cases from PEI in the profile of selected cases, reflects the role of extension services offered by the AVC for PEI fish farmers. This has facilitated more case submissions from PEI farmers. Also the quality of the submissions, specifically the fixation quality and completed extensive case history forms, made these cases more compliant with our selection criteria.

The reactivity of a large proportion of presumptive cases of BGD from Atlantic Canada with MABs created from the type culture of *F. branchiophilum* is further evidence of the global antigenic similarity of bacteria associated with BGD,<sup>6,9,19</sup> Whereas some antigens may be peculiar to regional isolates, the reactivity of our MAB suggests that we are detecting antigens common in several geographical locations. The lack of reactivity of some cases may be partially due to differences in tissue fixation (10% formalin, Bouin's, Davidson's fixatives) and processing (for example, time of storage in fixative before dehydration). These are inherent features of an archival collection of tissue within a diagnostic facility that receives both fresh and fixed tissues for diagnostics. Nevertheless, use of these MABs on archivally stored tissues has been shown in this

study to be relatively effective. No attempt was made in this study to reverse the masking effects of tissue fixatives on antigenic sites. However, such methods may make archival tissues even more suitable for retrospective epidemiological studies on this disease. MABs were produced against formalized bacteria and reacted well in paraffin sections. However, it is possible that additional processing could have destroyed antigens in some isolates. Interestingly, the MABs prepared against formalized bacteria also reacted against gill impression smears fixed only in acetone/alcohol. The sporadic autofluorescence of chloride cells has not been previously reported. This did not interfere with interpretation of slides since the pattern of fluorescence clearly depicted the cell outlines and was not part of the overlying acellular biofilm of interest in this study.

Use of these MABs has provided further details about the characteristics of the biofilm arising during BGD. In the majority of cases of BGD there was no evidence of *C. columnaris*. If we assume that there is no, as yet unidentified, gram-negative filamentous bacterium with morphologic similarities to *F. branchiophilum*, the following comments regarding the pathogenesis of BGD are appropriate. This study supports a previous tentative conclusion that a single bacterial species dominates the biofilm associated with BGD during its sequential development.<sup>16</sup> Furthermore it adds further support to the hypothesis that *F. branchiophilum*, acting alone, can be sufficient cause for BGD.<sup>5</sup>

The presence of *C. columnaris* within the biofilm of almost 20% of fish with BGD is, however, noteworthy.

Although we conclude that *C. columnaris* is not an inherent component of the BGD biofilm and therefore is not likely a requirement in the disease pathogenesis, its sporadic presence in the biofilm is further evidence of the reported ubiquitous and persistent nature of *C. columnaris* in freshwater.<sup>18</sup> Its role during BGD may be as a secondary invader, colonizing the abundant necrotic exfoliated lamellar epithelial cells which are trapped in gill mucus.<sup>16</sup> The BGD biofilm also includes particulate matter, such as small feed particles and other organic debris from the water column, which becomes trapped in the abundant mucus found on gills with BGD.<sup>15,16</sup> This may serve as an additional medium promoting the opportunistic growth of *C. columnaris* within the BGD biofilm, particularly since this bacterium has been shown to grow on particulate fish meals dispersed in water.<sup>17</sup> If these hypotheses are borne out with further research, it points to *C. columnaris* having a potential role as a secondary pathogen arising during BGD much like the various *Saprolegnia* spp. of fungus.<sup>14</sup> Accordingly, although *C. columnaris* may be frequently detected within the general biofilm in cases of BGD, its specific role in each case will have to be determined through other means such as histopathology, which can detect its characteristic pattern of necrosis subsequent to its direct attachment to and invasion of gill epithelium.<sup>14</sup> There may, for example, be a role for *C. columnaris* as a coinfecting agent during BGD. It could, for example, contribute to the pathogenesis and pathophysiology of some cases of BGD. Similarly of interest was the presence of *F. branchiophilum* within the biofilm of gills with columnaris disease. However we did not have sufficient numbers of samples to draw any conclusions other than noting that the biofilm was mixed.

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#### Sources and manufacturers

- a. ATCC, Rockville, MD.
- b. GIBCO BRL, Burlington, Ontario, Canada.
- c. John's Scientific, Dartmouth, Nova Scotia, Canada.
- d. Zymed Laboratories, San Francisco, CA.
- e. Difco Laboratories, Detroit, MI.
- f. ICN Immunobiologics, Costa Mesa, CA.
- g. Fish Health Unit and Diagnostic Services, Atlantic Veterinary College, Charlottetown, Prince Edward Island, Canada.

#### References

1. Bernardet, JF: 1989, *Flexibacter columnaris*: first description in France and comparison with bacterial strains from other origins. *Dis Aquat Org* 6:37-44.
2. Costerton JW, Cheng K-J, Geesey GG, et al.: 1987, Bacterial biofilms in nature and disease. *Ann Rev Microbiol* 41:435-464.
3. Daoust PY, Ferguson HW: 1983, Gill diseases of culture salmonids in Ontario. *Can J Comp Med* 47:358-362.
4. Farkas J: 1985, Filamentous *Flavobacterium* sp. isolated from fish with gill diseases in cold water. *Aquacult* 44:1-10.
5. Ferguson HW, Ostland VE, Byrne P, Lumsden JS: 1991, Experimental production of bacterial gill disease in trout by horizontal transmission and by bath challenge. *J Aquat Anim Hlth* 3:118-123.
6. Heo GJ, Kasai K, Wakabayashi H: 1990, Occurrence of *Flavobacterium branchiophila* associated with bacterial gill disease at a trout hatchery. *Fish Pathol* 25:99-105.
7. Huh GJ, Wakabayashi H: 1987, Detection of *Flavobacterium* sp., a pathogen of bacterial gill disease, using indirect fluorescent antibody technique. *Fish Pathol* 22:215-220.
8. Milton RJ, Salton RJ, Kim KS: 1991, Structure. *In: Medical microbiology*, ed. Brown S, 3rd ed., pp. 37-54. Churchill Livingstone, New York, NY.
9. Ostland VE, Lumsden JS, MacPhee DD, Ferguson HW: 1994, Characteristics of *Flavobacterium branchiophilum*, the cause of salmonid bacterial gill disease in Ontario. *J Aquat Anim Hlth* 6:13-26.
10. Pate JL, Ordal EJ: 1967, The fine structure of *Chondrococcus columnaris* 111. The surface layers. *J Cell Biol* 35:37-51.
11. Pate JL, Ordal EJ: 1967, The fine structure of *Chondrococcus columnaris* 1. Structure and formation of mesosomes. *J Cell Biol* 35:1-7.
12. Powell MD, Speare DJ, MacNair N: 1994, Effects of intermittent chloramine-T exposure on the growth, serum biochemistry and fin condition of juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum). *Can J Fish Aquat Sci* 51:1728-1736.
13. Reichenbach H: 1989, Genus 1. Cytophaga Winogradsky 1929, 577, <sup>A1</sup>emend. *In: Bergey's manual of systematic bacteriology*, ed. Staley JT, vol. 3, pp. 2015-2050. Williams and Wilkins, Baltimore, MD.
14. Speare DJ, Ferguson HW: 1989, Clinical and pathological features of common gill diseases of cultured salmonids in Ontario. *Can Vet J* 30:882-887.
15. Speare DJ, Ferguson HW, Beamish FWM, et al.: 1991, Pathology of bacterial gill disease: ultrastructure of branchial lesions. *J Fish Dis* 14:1-20.
16. Speare DJ, Ferguson HW, Beamish FWM, et al.: 1991, Pathology of bacterial gill disease: sequential development of lesions during natural outbreaks of disease. *J Fish Dis* 14:21-32.
17. Sugimoto N, Kashiwagi S, Matsuda T: 1981, Pathogenic relation between columnaris disease in cultured eel and the formula feeds. *Bull Jap Soc Sci Fisheries* 47:716-725.
18. Wakabayashi H: 1991, Effect of environmental condition on the infectivity of *Flexibacter columnaris* to fish. *J Fish Dis* 14: 279-290.
19. Wakabayashi H, Egusa S, Fryer JL: 1980, Characteristics of filamentous bacteria isolated from gill diseases of salmonids. *Can J Fish Aquat Sci* 37:1499-1504.
20. Walter SD: 1980, Berkson's bias and its control in epidemiological studies. *J Chron Dis* 33:721-725.