PATHOGENESIS AND TREATMENT OF *FLAVOBACTERIUM COLUMNARE*-INDUCED DERMATITIS IN KOI

by

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(Under the direction of KENNETH S. LATIMER)

ABSTRACT

Columnaris disease is a bacterial infection of fish caused by the Gram-negative bacillus *Flavobacterium columnare*. An experimental model of this disease was developed to reproduce *F. columnare*-induced dermatitis in koi with high (80% to 100%) mortality compared to uninfected controls (0% to 20% mortality). The protective mucus layer was wiped from the right side of the fish while leaving the left side intact. When fish were challenged with a virulent strain (strain 12/99) of *F. columnare*, cutaneous lesions consistently appeared on the right side of the body, but the left side of the body remained normal. This experimental model of columnaris disease differed from natural disease outbreaks in that cutaneous lesions were common while gill lesions were rare.

The antibacterial properties of the surface mucus layer in preventing *F. columnare* infection was demonstrated by incubating cultures of log-phase growth bacteria with isolated mucus from healthy koi. *F. columnare* viability decreased as quantitated by manual counting of bacterial colonies on agar plates and by differentiation of viable and dead bacteria using propidium iodide staining and fluorescence microscopy.

F. columnare in cytologic and histologic specimens appeared as long, thin, (5-12 μm x 0.5 μm) bacilli. Cytology was the best technique to tentatively diagnose

columnaris disease. *F. columnare* was more readily visualized by Giemsa, as opposed to hematoxylin and eosin, staining in histologic sections.

Both polymerase chain reaction (PCR) and DNA *in-situ* hybridization (ISH) diagnostic assays were developed to specifically detect *F. columnare* nucleic acid in fresh biological samples and in formalin fixed, paraffin embedded tissues. These techniques demonstrated that *F. columnare* infection in koi was restricted to the skin, fins, and, rarely, gills; systemic infection did not occur.

Hematologic and biochemical reference intervals were established to evaluate subsequent changes during experimental disease. Hematologic changes in *F. columnare* infected koi included a nonregenerative anemia and mild leukopenia with lymphopenia, neutrophilia, and monocytosis. Biochemical changes included significantly decreased sodium and chloride concentrations. Decreases in total serum protein and albumin concentrations were present but minimal.

A new proprietary solution (Tricide-Neo), was evaluated as a treatment for columnaris disease. Following experimental challenge with *F. columnare*, Tricide-Neo reduced mortality by 40% to 50% if treatment was performed before deep skin ulcers developed.

INDEX WORDS: *Cyprinus carpio*, carp, fish, koi, disease model, columnaris disease, *Flavobacterium columnare*, polymerase chain reaction, DNA *in-situ* hybridization, reference intervals, hematology, blood cell morphology, ultrastructure, cytochemistry, serum chemistry, serum electrophoresis, treatment

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DEDICATION

This dissertation is dedicated to my wife Tina and my family. Your constant love, support and encouragement led me through this walk of life.

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CHAPTER 1

INTRODUCTION

Within the past few decades, commercial production of koi (an ornamental strain of the common carp, *Cyprinus carpio*) has emerged as a major segment of the pet industry. In 1992, the value of international trade in the ornamental fish industry was estimated at 247 million dollars based on import statistics and 140 million dollars based on export statistics. Of this total, the United States was responsible for 26% of all imports and 11% of all exports. These figures reflect trade in both freshwater and marine fishes. Approximately 1 billion fish are exported annually, most of which are mass-cultured fish species such as guppies, tetras, angelfish, swordtails, platys, goldfish, and koi (Cheong, 1996). In 2001, the total value of ornamental fish farm sales in the United States was approximately 21 million dollars with total exports of 1.8 million dollars (Anonymous, 2003). Thus, outbreaks of classical or emerging diseases can have a severe economic impact on the ornamental fish industry. In 2003, the United States Department of Agriculture – Animal and Plant Health Inspection Service also recognized the importance of specific disease outbreaks in farmed carp and provided approximately 11.7 million dollars to implement a control and indemnity program for spring viremia of farm-raised carp (Anonymous, 2003). However, this program addresses only one of several important diseases of carp, including koi.

Generally, disease problems in fish are complex and result from the interaction of the disease agent, the fish, and the aquatic environment. Based upon etiology, fish

diseases can be broadly classified as either noninfectious or infectious. Noninfectious causes of disease may include environmental stressors such as water temperature changes, chemical pollutants, and poor management practices. Examples of poor management practices including overcrowding, malnourishment, improper diet, transportation effects, and lack of attention to water quality. Infectious causes of disease are diverse and include parasites, fungi, viruses, and bacteria. Bacteria that have been characterized as major piscine pathogens usually include *Flavobacterium* sp., *Edwardsiella* sp., and *Aeromonas* sp. Of these organisms, *Flavobacterium columnare*, the etiologic agent of columnaris disease, is one of the most common bacteria infecting carp, including koi.

Columnaris disease is one of the most common bacterial diseases of freshwater fishes. This disease was first reported by Davis in 1922. The name 'columnaris disease' was derived from the microscopic observation that the bacterium formed haystack-like columns in the wet mount preparations and stained cytologic smears from lesions of the skin and gills. The bacterium was designated '*Bacillus columnaris*' (Davis, 1922). This disease also is known by other common names including saddleback disease, cotton-wool disease, cotton mouth disease, and fin rot. Each of these common names reflects the gross lesions of affected fish including color changes over the dorsum, the presence of cottony white proliferations on the skin and fins, or loss of fin area (Davis, 1922; Pacha and Ordal, 1967; Griffin, 1987; Durborow *et al*, 1998). Since the original description of columnaris disease, this disease has been reported to infect most warm freshwater fish. In addition, columnaris disease has been reported to infect some cold water and marine fishes (Pacha and Ordal 1967; Morrison *et al*, 1981).

Columnaris disease is caused by a Gram-negative bacterium that was recently reclassified as *Flavobacterium columnare* (Bernardet *et al*, 1996). This bacterium is ubiquitous and frequently is associated with a mixed bacterial infection (Marks *et al*, 1980; Hawk and Thune, 1992). The clinical signs of disease are nonspecific and include lethargy, loss of appetite, and accelerated opercular movements. A tentative disease diagnosis can be made by microscopic examination of cytologic smears and imprints of skin or gill lesions. However, definitive disease diagnosis usually requires isolation and characterization of the bacterium. Isolation of *F. columnare* requires special culture medium with antibiotic additives that suppress the overgrowth of other common bacteria (Decostere, 1997). Griffin (1992) described a diagnostic procedure to identify *F. columnare* that used five biochemical and cultural characteristics that were unique to the bacterium. More specific and sensitive diagnostic techniques have been developed, but these techniques are primarily restricted to experimental use (Griffin, 1987; Bader and Shotts, 1998).

F. columnare is of very low pathogenicity and usually infects fish under stressful conditions. The pathogenesis of columnaris disease is not fully understood because it is difficult to infect healthy fish with the bacterium. Due to the lack of a reproducible model of disease, most of the previous research studies have been based on natural outbreaks of columnaris disease. Since natural infections frequently involve the gills, previous studies were focused on gill lesions where the bacterial capsule and adhesion proteins have been recognized as possible virulence factors (Decostere *et al*, 1998 and 1999). Extracellular proteases have been isolated from certain *F. columnare* strains and identified as virulence factors (Griffin, 1991; Bertolini *et al*, 1992; Newton *et al*, 1997).

In vivo studies have not been done to examine the progression of skin lesions. Although *F. columnare* has been cultured occasionally from the kidney of infected fish, systemic bacterial infection has not always been confirmed (Hawke and Thune, 1992)

Hematological and biochemical changes have not been studied in columnaris disease. Complete blood cell counts and serum biochemical profiles are important in the diagnosis of some piscine diseases; however, tremendous interspecies and intraspecies variations are apparent in these parameters due to various intrinsic and extrinsic factors such as age, gender, reproductive status, nutritional status, and water quality. In recent years, koi have become more popular as companion animals, promoting considerable progress in aquatic medicine and management practices. Despite these improvements in health care, lack of reliable reference intervals impedes interpretation of laboratory test results.

Previous studies of blood cell morphology (Imagawa, 1989), complete blood counts, and biochemical parameters (Groff and Zinkl, 1999; Rehulka, 1995) have been performed in common carp, catfish, and goldfish. Most published reports are primary or compiled experimental data where control groups were used for the comparison of laboratory test values. Reference intervals for various hematologic and biochemical parameters have not been established for koi. Accurate interpretation of hematologic and serum chemistry data may be helpful in the diagnosis, monitoring, and prognosis of disease. Identification of a disease process at an early stage of development will facilitate treatment in valuable ornamental fish.

Certain external chemical dips such as potassium permanganate and copper sulfate (Davis, 1922; Snieszko, 1981) have been used to treat the disease. Selected

antibiotics also have been used for this purpose (Nusbaum *et al*, 1981; Decostere, 2002). Chemical dips and antibiotic treatments have had limited success depending upon the severity and duration of columnaris disease. Both an efficacious treatment regimen and an effective vaccine are still needed to control and prevent columnaris disease. To date, there is no commercial vaccine to prevent this disease.

The present research studies were designed to attain the following objectives:

1). To gain clinical experience with natural outbreaks of columnaris disease in koi.

2). To establish hematologic reference intervals for koi, including the identification of leukocytes by cytochemistry and ultrastructural examination.

3). To establish biochemical reference intervals for koi.

4). To develop an experimental model of columnaris disease.

5). To develop polymerase chain reaction and DNA *in situ* hybridization techniques to specifically identify *Flavobacterium columnare* infection.

6). To investigate hematological and biochemical changes after infection with *F. columnare*.

7). To determine if columnaris disease is predominately a cutaneous or systemic bacterial infection in koi.

8). To evaluate the efficacy of a new treatment regimen using a combination of an antibiotic potentiator and an antibiotic.

References

Anonymous. Spring viremia of carp control program launched. *J Am Vet Med Assoc* 222:1332-1333, 2003.

Bader JA, Shotts EB. Identification of *Flavobacterium* and *Flexibacter* species by species-specific polymerase chain reaction primers to the 16S ribosomal RNA gene. *J Aquat Anim Health* 10:311-319, 1998.

Bernardet JF, Segers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P. Cutting a gordian knot: Amended classification and description of genus *Flavobacterium*, emended description of the family Flavobacteriaceae and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Internatl J Syst Bact* 46:128-148, 1996.

Bertolini JM, Rohovec JS. Electrophoretic detection of proteases from different *Flexibacter columnaris* strains and assessment of their variability. *Dis Aquat Org* 12:121-128, 1992.

Cheong L. Overview of the current international trade in ornamental fish, with special reference to Singapore. *Rev Sci Tech Off Int Epiz* 15:445-482, 1996.

Davis HS. A new bacterial disease of fresh water fishes. *Bulletin U. S. Bureau of the Fisheries* (Doc. 924), pp. 262-280, 1922.

Decostere A, Ducatelle R, Haesebrouck F. *Flavobacterium columnare* (*Flexibacter columnaris*) associated with severe gill necrosis in koi carp (*Cyprinus carpio* L). *Vet Rec* 150:694-695, 2002.

Decostere A, Haesebrouck F, Devriese LA. Characterization of four *Flavobacterium Columnare (Flexibacter columnaris)* strains isolated from tropical fish. *Vet Microbiol* 62:35-45, 1998.

Decostere A, Haesebrouck F, Devriese LA. Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare (Flexibacter columnaris)* from diseased fish. *J Clin Microbiol* 35:322-324, 1997.

Decostere A, Haesebrouck F, Driessche EV, Charlier G, Ducatelle R. Characterization of the adhesion of *Flavobacterium columnare (Flexibacter columnaris)* to gill tissue. *J Fish Dis* 22:465-474, 1999.

Durborow RM, Thune RL, Hawke JP, Camus AC. Columnaris disease: A bacterial infection caused by *Flavobacterium columnare*. *Southern Regional Aquaculture Center* publication # 479, 1998.

Griffin BR. A simple procedure for identification of *Cytophaga columnaris. J Aquat Anim Health* 4:63-66, 1992.

Griffin BR. Characteristics of a chondroitin AC lyase produced by *Cytophaga columnaris*. *Tran Am Fish Soc* 120:391-395, 1991.

Griffin BR. Columnaris disease: Recent advances in research. *Aquacult Mag* May/ June pp. 48-50, 1987.

Groff JM, Zinkl JG. Hematology and clinical chemistry of cyprinid fish. *Vet Clin North Am: Exotic Animal Pract* 2:741-776, 1999.

Hawke JP, Thune RL. Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel cat fish. *J Aquat Anim Health* 4:109-113, 1992.

Imagawa T, Hashimoto Y, Kitagawa H, Kon Y, Kudo N, Sugimura M. Morphology of blood cells in carp (*Cyprinus carpio* L.). *Jpn J Vet Sci* 51:1163-1172, 1989.

Marks JE, Lewis DH, Trevino GS. Mixed infection in the columnaris disease of fish. *J Am Vet Med Assoc* 177:811-814, 1980.

Morrison C, Cornick J, Shum G, Zwicker B. Microbiology and histopathology of 'saddle back disease' of underlying Atlantic Salmon, *Salmo salar L. J Fish Dis* 4:243-258, 1981.

Newton JC, Wood TM, Hartley MM. Isolation and partial characterization of extracellular proteases produced by isolates of *Flavobacterium columnare* derived from channel cat fish. *J Aquat Anim Health* 9:75-85, 1997.

Nusbaum KE, Shotts EB. Action of selected antibiotics on four common bacteria associated with diseases of fish. *J Fish Dis* 4:397-404, 1981.

Pacha RE and Ordal EJ. Histopathology of experimental columnaris disease in young Salmon. *J Comp Pathol* 77:419-423, 1967.

Rehulka J. Blood parameters in common carp with spontaneous spring viremia (SVC). *Aquacult International* 4:175-182, 1996.

Snieszko SF. Bacterial gill disease of fresh water fishes. *Fish Disease Leaflet 62*, U S Dept Interior, Washington DC, 1981.

CHAPTER 2

LITERATURE REVIEW

The etiological agent of columnaris disease is a long, thin, Gram-negative, gliding bacillus designated *Flavobacterium columnare*. This organism has been reclassified taxonomically and renamed several times since columnaris disease was first described. Despite recent advances in characterizing this bacterium, discrepancies regarding the nomenclature still exist. In this literature review, the organism will be addressed as *Flavobacterium columnare* for ease of discussion. The important aspects of columnaris disease that are related to this dissertation will be discussed below. In addition, an overview of columnaris disease in freshwater fishes is presented in detail in Chapter 1.

History of Columnaris Disease

In the summer of 1917 while studying protozoan parasites, Davis noticed a new bacterial disease in buffalofish (*Ictiobus bubalus* and *I. ciprinella*) fingerlings that were kept in aquaria (Davis 1922). This disease was easily identified and distinguished based on its presentation. First emergence of the disease was accompanied by one or more characteristic, dirty-white or yellowish areas on various parts of the body. These infected areas usually were conspicuous and increased rapidly in size, sometimes covering a large proportion of the body. Infected fish usually died within 24 to 72 hours after the first visible lesions appeared. Davis also observed that cutaneous lesions usually occurred at the base of caudal fin and spread to adjoining portions of the body. The following

summer, he observed an increased incidence of this disease in pond-reared fish. The disease had a presentation that was similar to that observed in the captive buffalofish fingerlings.

In subsequent years, more outbreaks of disease were observed in ponds and rivers involving many freshwater fishes including sunfish (*Lepomis incisor* and *L. humilis*), carp (*Cyprinus carpio*), black bass (*Micropterus salmoides* and *M. dolomieu*), crappies (*Pomoxis sparoides* and *P. annularis*), warmouth (*Chaenobryttus gulosus*), yellow perch (*Perca flavescens*), white bass (*Roccus chrysops*), brook trout (*Salvelinus fontinalis*), bluntnose minnow (*Pimephales notatus*), channel catfish (*Ictalurus punctatus*), and bullheads (*Ameeiurus nebulosus and A. melas*). Fish farmers frequently reported a disease with similar presentation in fishes from their ponds and sloughs near the Mississippi River. On microscopic examination of cytologic wet mounts from infected tissue, Davis observed long, thin, filamentous bacteria. He also reported that these bacteria were frequently arranged in haystack-like columns. Based upon the column-like arrangement of the bacteria, Davis proposed the name *Bacillus columnaris* for the organism. This new bacterial disease of freshwater fishes was later called columnaris disease (Davis 1922).

Initial observations indicated that the prevalence of columnaris disease increased when water temperature exceeded 80°F and fish were stressed by excessive handling. In contrast, columnaris disease spread more slowly and caused lower mortality when water temperatures were below 75°F. An increased incidence of infection with *F. columnare* (*Chondrococcus columnaris*) was reported in black bullheads (*Ictalurus melas*) from Clear Lake, Iowa during the spring when the lake was warming (Bowser,1973). This

outbreak of columnaris disease was associated with the production of white to yellow, cotton-like, proliferations over the fish's body. Prior to the initial description of columnaris disease, many cases of columnaris disease probably were misdiagnosed as a common fungal (*Saprolegnia* sp.) infection (Bowser,1973).

Columnaris disease was relatively uncommon in the United Kingdom; however, diseased fish were observed. To prevent the potential spread of columnaris disease, it was declared a reportable disease in Great Britain under the Diseases of Fish Act in 1937. In most instances, a subacute form of disease was observed in which the progression of lesions was slow and the duration of disease extended from 1 to 2 weeks. In 1976, columnaris disease was reported in rainbow trout (*Salmo gairdneri*) fingerlings in the United Kingdom (Ferguson, 1977). Currently, columnaris disease is distributed almost worldwide. It has been reported to infect most freshwater fishes and a few species of marine fish (Campbell and Buswell, 1982; Wakabayashi, 1991; Jeney and Jeney, 1995).

Since *F. columnaris* is generally of low pathogenicity, mixed bacterial infections frequently are observed (Marks et al,1980). Mixed bacterial and fungal infection of gills also was described in 1982 as bacterial gill disease (BGD) (Snieszko, 1981). In the earlier literature, BGD was described as a distinct entity wherein bacterial gill proliferation was observed as opposed to gill necrosis of fulminant columnaris disease (Snieszko, 1981). At that time the bacterium causing BGD was classified within the genus *Flavobacterium*. In contrast, the bacterium causing columnaris disease was classified in the genus *Cytophaga*. According to the most recent nomenclature, however, the bacterium responsible for columnaris disease has been reclassified as *Flavobacterium columnare*. Therefore, it is possible that BGD and columnaris disease represented

different clinical presentations of the same disease. In another report, a *Flavobacterium columnare*-like (*Flexibacter columnaris*-like), filamentous bacterium was isolated from Dover sole (*Mircostomas pacificus*) with black patch necrosis probably represents the same or similar bacterium infecting a species of marine fish. This bacterium was identical to *F. columnare* in morphology, biochemical characteristics, and antibiotic sensitivity pattern (Campbell and Buswell, 1982).

Identification and Classification of *Flavobacterium columnare*

Davis initially identified the etiological agent of columnaris disease in 1917 as a long, thin, bacillus that measured 0.5μm in width by 5 to 12 μm in length. However, he did not publish his findings on columnaris disease until 1922. He named this organism *Bacillus columnaris* based on the characteristic haystack-like arrangement of bacteria observed in cytological wet mounts (Davis, 1922). Although Davis was unable to isolate the bacterium in culture, he observed it consistently in stained cytologic smears of material from gill and cutaneous lesions. Thus, *F. columnare* subsequently was isolated by Ordal and Rucker in 1944 (twenty-seven years after its initial observation by Davis). Based on cellular morphology (long, thin, Gram-negative bacilli) and flexing movements on agar medium, this bacterium was classified under the order Myxobacterales and was renamed *Chondrococcus columnaris* (Ordal and Rucker 1944). At a later time, this organism was reclassified within the genus Cytophaga based on the production of microcysts and was renamed *Cytophaga columnaris*. In the 8th edition of Bergey's Manual of Determinative Bacteriology, this organism was placed in the order Cytophagales and renamed *Flexibacter columnaris* (Buchanan and Gibbon, 1974).

More recently, this bacterium was classified in the family Flavobacteriaceae and renamed *Flavobacterium columnare* based upon phylogenetic analysis of the 16S rRNA genes (Bernardet *et al*, 1996; Bader and Shotts, 1998; Jooste and Hugo, 1999; Boone and Castenholz, 2001). The polymerase chain reaction allowed almost complete amplification of the 16S rRNA gene. Sequence homology subsequently was studied by nucleic acid alignment. Certain other characteristics also were considered in this reclassification including the guanosine and cytosine content (G+C) of the bacterium's genome, DNA- ribosomal RNA (rRNA) hybridization, fatty acid profiles, and protein profiles (Bernardet *et al*, 1996).

F. columnare exhibits gliding movement in liquid medium and forms flat, rhizoid colonies on agar medium (Morrison *et al*, 1981; Bullock *et al*, 1986; Amin *et al*, 1988). In addition to cellular morphology, there are certain biochemical characteristics that allow definitive identification of *F. columnare*. These characteristics are its ability to grow in the presence of neomycin and polymyxin B; production of flat, yellow, rhizoid colonies on agar medium; production of a diffusible gelatin-degrading enzyme; production of chondroitin lyase; binding to aqueous congo red dye; production of flexirubin-type pigment and hydrogen sulfide; and reduction of nitrate (Griffin, 1992; Shamsudin and Plumb, 1996; Durborow *et al*, 1998).

F. columnare is a very slow growing bacterium and requires special media for its isolation. Cytophaga agar with neomycin (5µg/ml), Hsu-Shotts medium with neomycin (4µg/ml), or Shieh medium supplemented with tobramycin (1µg/ml) have been used for selective isolation of *F. columnare* (Pacha and Ordal, 1967; Bullock *et al*, 1986;

Decostere *et al*, 1997). In addition to antibiotics, these media also contain a low concentration of nutrients to prevent the overgrowth of other bacteria.

Ultrastructure of *Flavobacterium columnare*

Ultrastructural studies, revealed that *F. columnare* lacked flagella but had specialized structures called mesosomes that are formed by the invagination of the cytoplasmic membrane. These mesosomes are intracytoplasmic, tubular structures bound by unit membranes (Pate and Ordal, 1967a). Rhapidosomes also were identified and consisted of tubular structures with a central axial hole that ran throughout its length, widening and narrowing with regular periodicity. Rhapidosomes probably originated from mesosomal membranes (Pate *et al*, 1967). Peripheral fibrils were identified on the outer membrane of the bacterium and in combination with rhapidosomes are probably responsible for the gliding motion exhibited by this bacterium. A mucopolysaccharide substance was observed coating the surface of the bacterium and may be responsible for the adhesive properties of the bacterium (Pate and Ordal, 1967b).

Clinical Signs of Columnaris Disease

The clinical signs of columnaris disease are nonspecific and include lethargy, listlessness, swimming near the surface of the water, increased opercular movements, and inappetence. Columnaris disease can have different clinical presentations with various combinations of gill, skin and fin lesions. Disease with primary gill involvement is acute and significant skin lesions are not observed. Fish with acute columnaris disease may be observed lying on their sides and exhibit marked opercular movements. Death often

occurs within 48 hours of the appearance of clinical signs (Decostere *et al*, 2002a). In general, acute disease is observed in younger fish and characteristic skin discoloration and ulcers usually are not present. Death apparently occurs before external manifestations of the disease have time to develop (Pacha and Ordal, 1967; Decostere *et al*, 2002a).

The lesions on the external body surface and gills tend to vary with the species of fish. In scaleless fish, skin lesions begin as areas of discoloration. Hyperemia usually occurs just beneath the epidermis and is observed as a reddish zone around the area of discoloration (Davis, 1922; Jeney and Jeney, 1995). Discoloration typically begins at the base of the dorsal fin but also may be seen on the head and craniodorsal portion of the body (Bullock *et al*, 1986; Bullock and McCraren, 1989; Wakabayashi, 1991). Discoloration spreads from the base of the dorsal fin and extends laterally to form a pale white band around the body called a saddleback lesion (Davis, 1922; Griffin, 1987). The origin of skin lesions at the base of the dorsal fin probably represents injury to the skin and fins during netting and handling. Saddleback lesions are observed more frequently in certain species of fish such as catfish and salmonids (Pacha and Ordal, 1967; Morrison *et al*, 1981; Bullock *et al*, 1986; Griffin, 1987). A yellowish-white ulcer often develops in the center of the saddle as the lesion progresses (Griffin, 1987; Durborow, 1998; Decostere *et al*, 1999a). Skin lesions typically spread outwards. Extensive skin ulcers may develop involving one-half to two-third of the body. The bacteria that are attached to the surface penetrate the epidermis and corium, forming deep skin ulcers. Necrosis of the underlying muscle occurs frequently and may expose underlying bones (Bullock *et al*, 1986; Griffin, 1987; Kent *et al*, 1988; Decostere *et al*, 1998; Decostere, 2002b).

In scaled fish, gill and fin lesions usually are prominent but skin ulcers also can develop with or without gill and fin lesions. Progression of gill and fin lesions is similar to that observed in skin. Bacteria first attach to the surface of the gills and fins, appearing as cotton-like proliferations. This results in congestion of branchial blood vessels and proliferation of branchial epithelial cells that obliterate the spaces between secondary lamellae, impeding gaseous exchange. In advanced stages of the disease, extensive necrosis of gill epithelium and blood vessels occurs leaving visible skeletal structures (Davis, 1922; Bullock and McCraren, 1989; Wakabayashi, 1991; Decostere *et al*, 2002a). Gill necrosis is observed as yellowish-white spots that start on the distal tips of the gills and extend proximally. Necrosis may ultimately involve the entire gills. Necrosis of the fins begins at the peripheral margins and extends proximally toward the body. As the fin epithelium is destroyed, only the cartilaginous rays remain. Skin lesions initially are less prominent in scaled fishes, but become obvious as they advance from mild hyperemia to deep ulcers. The scales become loosened and slough as the skin disintegrates (Davis, 1922).

Channel catfish (*Ictalurus punctatus*) have a slightly different presentation of columnaris disease. In this species, a yellowish-brown, mucus-like growth is seen inside the mouth (Durborow *et al*,1998). In contrast, naturally infected koi usually develop hemorrhagic ulcers around the mouth. Also, ulcers in koi are generally restricted to skin; gill necrosis is observed infrequently. In crappie, columnaris disease usually is confined to fins and gills and extensive skin lesions are not observed (Davis, 1922).

Bacterial Virulence Factors and Pathogenesis of Columnaris Disease

High and low virulence strains of *F. columnare* have been isolated from several species of fish (Amin *et al*, 1988, Decostere *et al*, 1998; Decostere *et al*, 1999b; Decostere, 2002b). Occasional natural outbreaks of columnaris disease have occurred in the absence of any obvious stressors and it was assumed that highly virulent strains of the bacterium were involved (Decostere *et al*, 2002a). However, the severity of disease also may be affected by certain host factors such as age and species. In experimental infection, young carp were more susceptible to columnaris disease than adults which were generally resistant. Also, certain species of fish, such as buffalofish and crappie, were more susceptible to spontaneous columnaris infections. Other species of fish, including catfish and carp, were only moderately susceptible to this disease (Davis, 1922). The difference in disease resistance may be due to compactly arranged layers of scales or tougher skin in adult carp and other resistant fishes. Alternatively, it also is possible that strains of *F. columnare* infecting different species may vary in their virulence.

Because natural outbreaks of columnaris disease frequently were associated with gill necrosis, most experimental studies have focused on gill infection (Decostere *et al*, 1999c; Decostere *et al*, 1999d; Decostere, 2002b; Decostere *et al*, 2002a). The ability of *F. columnare* to adhere to gill tissue has been investigated as the initial step of disease development in most pathogenesis studies. In experimental infections, highly virulent strains of *F. columnare* attached to the gill tissue more closely than did less virulent strains of this bacterium (Decostere *et al*, 1998; Decostere *et al*, 1999b; Decostere *et al*, 1999c; Decostere, 2002b). A gill perfusion model has been developed to study the

adhesion of *F. columnare* to gill tissues and evaluate the effects of various treatments on bacterial adhesion (Decostere *et al*, 1998; Decostere *et al*, 1999c, Decostere, 2002b). Adhesion of the bacterium to gill tissues was studied after treatment with different carbohydrates (glucose, glucosamine, N-acetylglucosamine, galactose, galactosamine, N-acetylgalactosamine, sucrose, mannose, fucose, fructose), sodium metaperiodate, pronase, and trypsin. Significantly reduced bacterial adherence was observed following treatment of gill arch explants with glucose, galactose, N-acetylglucosamine, sucrose, and sodium metaperiodate. In contrast, bacterial adherence was unaffected by treatment with pronase and trypsin. Furthermore, bacterial adherence correlated well with the hemagglutination capacity of chicken and guinea pig red blood cells. Higher hemagglutination titers were observed with high virulence as compared to low virulence strains of *F. columnare*. Hemagglutination of highly virulent bacterial strains was enhanced in the presence of divalent cations and was inhibited following incubation of bacterial cells with sodium metaperiodate, glucose, and N-acetylglucosamine. Hemagglutination also was reduced by heat treatment of bacterial cells at 41°C for 10 minutes and was completely impaired by treatment at 60°C for 30 minutes (Decostere, 2002b). On transmission electron microscopy, a thick (120-130 nm), dense capsule was observed in highly virulent strains of *F. columnare*. Low virulence strains of this bacterium had a thinner (80-90 nm) and less dense capsule. Highly virulent strains of *F. columnare* lost their capsule after minor heat and sodium metaperiodate treatment. Loss of the capsule correlated well with a reduction in bacterial adherence to gill tissues. These results indicate that a lectin-like carbohydrate-binding substance incorporated into the capsule is responsible for the attachment of *F. columnare* to gill tissue (Decostere *et*

al, 1999c). Similar virulence factors also might be responsible for adherence of the bacterium to the skin and fins. Comparative experimental studies have not been performed to evaluate the adherence properties of less virulent strains of *F. columnare*.

The variable presentation of columnaris disease in some experimental studies could be due to decreased bacterial virulence related to isolation on artificial media. Media used for the isolation of *F. columnare* usually contain very low concentrations of nutrients to prevent overgrowth of other opportunistic bacteria. Sniezsko observed lower mortality in experimentally inoculated fish than in fish that developed spontaneous infections of *F. columnare* (Sniezsko, 1981). In an experiment with Atlantic salmon, a loss of virulence was observed when a highly virulent strain of *F. columnare* was isolated on artificial medium. Bacterial virulence also was reduced by inactivation of proteolytic enzymes that are synthesized by bacteria (Bullock *et al*, 1986). These findings indicate that the two different presentations of columnaris disease (gill-oriented versus skin-oriented disease) are most likely due to loss of virulence after isolation and propagation of *F. columnare* on artificial media. This may explain why skin infections are more common in experimental infections where bacterial isolates have reduced virulence. In contrast, naturally occurring infections of *F. columnare* are probably caused by more virulent bacteria resulting in gill lesions.

Although bacterial virulence factors and the pathogenesis of gill infection have been investigated, little is known about the development of skin lesions during *F. columnare* infection. Some extracellular proteases produced by *F. columnaris* have been isolated from cell-free culture medium. These proteases display lytic activity for chondroitin sulfates A and C as well as hyaluronic acid. These latter substances are

complex polysaccharides that are abundant in the connective tissue (Griffin, 1991; Bertolini and Rohovec, 1992; Newton *et al*, 1997). These extracellular proteases may degrade the skin and connective tissue, initiating the establishment of cutaneous infections by *F. columnare* (Griffin, 1991; Stringer *et al*, 2000). These proteases were constitutively produced by all 16 strains of *F. columnare* (previously *C. columnaris*), but by none of the non-columnaris, yellow pigmented bacteria of aquatic origin. There was no correlation between host origin, geographical distribution, and amount of enzyme produced by different bacterial isolates (Griffin, 1991). Similar proteases have been identified in cell-free media of *F. columnaris* as evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Bertolini and Rohovec, 1992; Newton *et al*, 1997). *F. columnaris*-derived proteases produced by bacteria from various species of fish of diverse geographical regions showed great uniformity, but differed from the protease profiles of other gliding bacteria. Experiments with protease inhibitors indicated that zinc metalloproteases constitute a major component of the extracellular proteases of *F. columnaris* (Bertolini and Rohovec, 1992). Newton and coworkers identified 58 kDa and 54 kDa proteases by SDS-PAGE that were expressed in media as early as 1 day after bacterial inoculation (Newton *et al*, 1997). More proteases were produced in media with low nutrient concentrations than in media with higher nutrient concentrations. Bader and coworkers also isolated this 58 kDa protease and observed that it was always associated with virulence and was immunogenic to channel catfish (Bader *et al*, 1999). The proteolytic activity of the 58 kDa protease was destroyed by heat treatment at 65°C for 15 minutes. Therefore, it was unsuitable for bacterin preparation; however, it does appear to be a good candidate for future subunit vaccine development (Bader *et al*, 1999). Since

both of these research studies involved strains of *F. columnare* isolated from channel catfish, there is the possibility that these proteases may be species-specific.

Highly virulent strains of *F. columnare* have been isolated from the kidney of some infected fish (Decostere *et al*, 1999a; Hawke and Thune, 1992); however, the incidence and importance of systemic bacterial infection has not been evaluated critically. Amin and coworkers reisolated *F. columnare* from cultured Nile tilapia (*Tilapia nilotica*) after intramuscular inoculation and demonstrated that systemic infection occurs after intramuscular inoculation with the bacterium (Amin *et* al, 1988). However, more rapid death occurred following bacterial inoculation by immersion. In this study, severe gill necrosis was observed but systemic infection was not found (Amin *et* al, 1988). These observations suggested that gill necrosis was the primary factor contributing to the death of infected fish (Amin *et al*, 1988).

Diagnosis of Columnaris Disease

A presumptive diagnosis of columnaris disease is based on the characteristic morphologic features of *F. columnare* in cytologic wet mounts or stained smears as well as in histologic preparations. Definitive disease diagnosis requires more extensive or sophisticated laboratory testing. The following discussion provides an overview of various laboratory techniques to diagnose columnaris disease

Cytology: Cytologic wet mount preparations are inexpensive, rapid, and simple to perform. This technique provides the easiest way to obtain a presumptive diagnosis of columnaris disease. *F. columnare* bacteria appear as long, thin bacilli, measuring 5 to 12 µm in length and 0.5µm in width, that frequently are arranged in hay-stack-like columns.

Occasionally, these bacteria may exhibit gliding movements (Davis, 1922; Ferguson, 1977; Durborow *et al,* 1998; Decostere *et al,* 1999). Cytologic smears made from skin, gill, or fin lesions may reveal an almost pure population of characteristic bacilli (Davis 1922; Morrison *et al,* 1981; Decostere *et al,* 2002a). However, small numbers of other bacteria frequently are mixed with the *F. columnare* (Marks *et al,* 1980). *F. columnare* stain very lightly with Romanowsky-type stains. They have a homogenous appearance but rarely may exhibit a slightly granular texture (Davis, 1922). Infrequently, microcysts may be present and appear as round spherical bodies (Farkas and Olah, 1986).

Histopathology: Histologic lesions vary according to the presentation of disease. In most cases of columnaris disease, characteristic, long, filamentous bacteria may be observed attached to the surface erosions and ulcerations of the skin, gills, or fin. In some instances, the gills may appear relatively normal. In other cases, the gill lesions vary from dilation of blood vessels to severe necrosis. Clubbing and fusion of secondary lamellae may be seen due to hyperplasia of branchial epithelial cells and goblet cells in early columnaris disease. This results in obliteration of the interlamellar spaces that are essential for gaseous exchange (Pacha and Ordal, 1967; Amin *et al,* 1988). In advanced columnaris disease, the gill epithelium may detach from blood vessels resulting in accumulation of edema fluid. Large, globose masses of necrotic epithelium, inflammatory cells, and bacterial colonies frequently are observed (Pacha and Ordal, 1967; Decostere, 1999a).

In early skin disease, scattered bacteria are observed in the epidermis and corium from foci of gross discoloration. Inflammatory cell infiltrates are minimal. In scaled fish, bacterial colonies may be observed in the scale pockets. In advanced skin lesions,

epithelium is lost and numerous bacteria are present in the dermis and extending into the underlying skeletal muscle. Necrotic muscle appears pale and muscle fibers are disrupted. Inflammatory infiltrates vary from minimal to severe and consist of neutrophils admixed with monocytes, basophils, and lymphocytes. Hemorrhage also may be apparent. *F. columnare* are better observed in the deeper lesions such as skin ulcers. In many cases, few bacteria may be present because of detachment and loss of organisms during routine tissue processing. The bacteria are difficult to visualize with routine hematoxylin and eosin (H&E) stain or Gram's stain. Organisms stain faintly pink with H&E and Gram's stain. Romanowsky stains, such as Giemsa stain, provide better visualization of these bacteria because they stain dark blue in tissue sections.

Neither lesions nor bacilli have been reported in histologic specimens of internal organs from fish with columnaris disease. Dilation of Bowman's capsule with accumulation of eosinophilic material has been reported previously, but the significance of this observation is unknown since similar glomerular lesions have been observed in fish without cutaneous lesions. In addition, Geimsa stained tissue sections have failed to reveal bacteria in internal organs (Pacha and Ordal, 1967; Morrison *et al*, 1981).

Hematology and clinical chemistry: Hematologic and biochemical parameters are frequently used to detect disease processes in domestic animals and human beings. Although these changes are often nonspecific, they often can detect disease-induced changes in major organ systems. Hematologic and clinical chemistry parameters are not evaluated routinely in aquatic medicine. When such laboratory testing is performed, interpretation of the data is difficult because reference intervals are unavailable for many species of fishes. Piscine hematology and serum chemistry also are complicated by

difficulties in blood collection and contamination of blood samples with tissue fluid during venipuncture. A few reports of hematologic and biochemical changes in carp with disease or toxicosis have been published (Liewes *et al*, 1982; Wlaswo and Dabrowska, 1990; Carbis *et al*, 1996; Rehulka, 1996; Karan *et al*, 1998; Schwaiger *et al*, 2000); however, these changes have not yet been evaluated for columnaris disease.

Detailed experimental studies involving *F. columnare* infection of the skin and gills may be helpful in understanding the pathogenesis of this disease. LaFrentz and coworkers studied some serum biochemical changes, serum and mucosal antibody responses, and percentage of survival in rainbow trout (*Oncorhynchus mykiss*) following immunization and challenge with *Flavobacterium psychrophilum* (LaFrentz *et* al, 2002). A similar approach can be used to evaluate the effectiveness of vaccines against *F. columnare*.

Bacterial culture: Bacterial culture is the initial step in identifying a bacterium. Definitive identification of a given bacterium usually relies on biochemical characterization. Culture and identification of *F. columnare* is more problematic because of other contaminating bacteria with subsequent overgrowth. This bacterium does not grow on blood agar, beef-broth agar, fish agar, or fish serum agar (Davis, 1922). Although *F. columnare* may be cultured between 20°C and 30°C, the optimal temperature for bacterial growth is 25°C. During culture, *F. columnare* exhibits a swarming phenomenon and forms small (1-3 μm diameter), round, flat colonies with rhizoid edges on agar plates (Morrison *et al*, 1981; Amin *et al*, 1988; Bullock *et al*, 1986). Characteristic yellow-pigmented colonies are observed within 48 to 72 hours when appropriate agar plates are incubated at 25°C. These colonies are adherent to the

agar plate; therefore, it is difficult to recover a single colony. Usually, the colony is scraped off the agar plate and used to inoculate additional agar plates or liquid media for bacterial culture. Bacterial culture in liquid medium requires constant stirring at 150 to 250 rpm. Yellow turbidity will be apparent in the culture medium within 24 to 48 hours when incubated at 25°C (Shamsudin and Plumb, 1996; Farkas, 1985; Decostere *et al*, 1998). Gliding movement can be observed in liquid media examined microscopically. Small filamentous aggregates appear in the culture and sediment at the bottom and sides of the culture flask. Bacilli start to disintegrate in 5- to 7-day-old cultures and produce small, spheroid bodies known as microcysts (Farkas, 1985; Farkas and Olah, 1986).

A few published reports mention the isolation of *F. columnare* from the kidneys of infected fish (Pacha and Ordal, 1967; Morrison *et al*, 1981; Hawke and Thune, 1992), but associated histopathologic lesions have not been mentioned. Although bacterial isolation ensures proper identification of a given bacterium, it is very difficult to avoid contaminating bacteria while collecting diagnostic specimens from the surface of the body fins and gills. Although previous researchers suspected systemic infection with *F. columnare* based upon microbiological culture (Pacha and Ordal, 1967; Morrison *et al*, 1981), the presence of opportunistic bacteria suggests that the material obtained from the kidneys and saddle area of the dorsum was contaminated with surface bacteria.

Molecular diagnostic tests: Recently, more sensitive and less time consuming diagnostic techniques have been developed to accurately diagnose *F. columnare* infections. A fluorescent antibody test has been developed to detect *F. columnare* (Griffin, 1987). A monoclonal antibody against *F. columnaris* (*Cytophaga columnaris*) has been developed, labeled, and used to detect this organism in frozen tissue sections (Speare *et al*, 1995).

Fluorescein-labeled antibody has been used to detect *F. columnare* in fresh salmonid gill tissue via an indirect fluorescent antibody method. In a retrospective study, nineteen percent of the tissues from salmonids that were tentatively diagnosed as bacterial gill disease caused by *Flavobacterium branchiophilum* were positive for *F. columnare* (*C. columnaris*) by fluorescent antibody testing (Speare *et al*, 1995).

The polymerase chain reaction (PCR) has been used for the detection of *F. columnare* nucleic acid in infected skin samples and in bacterial cultures. This technique used species-specific primers to amplify the 16S rRNA gene (Bader and Shotts, 1998; Tiirola *et al*, 2002; Bader *et al*, 2003). Combined PCR and restriction fragment length polymorphism (RFLP) allows a more accurate identification of *F. columnare*. Most *F. columnare*–like sequences were identified by direct molecular analysis, but most of the strains isolated on artificial media belonged to a phylogenetically heterogeneous group of Flavobacteria that clustered with *F. hibernum*. This difference in PCR and RFLP analyses was theorized to occur because *Flavobacterium* species replication may be retarded by saprophytic species of the same genus or by other antagonistic bacteria such as *Pseudomonas* sp. It has been reported that *F. columnare* may be differentiated from related bacteria (*F. psychrophilum*, *F. aquatile*, *F. branchiophilum*) and other fish pathogens like *Edwardsiella* sp., *Aeromonas* sp. and *Streptococcus iniae* by using species-specific 16S rRNA gene-based primers (Bader *et al*, 2003). Selected primer pairs can be used in the PCR technique to specifically identify various pathogens, including *F. columnare*. However, *F. columnare* is ubiquitous and may vary in its virulence or pathogenicity (Tiirola *et al*, 2002; Bader *et al*, 2003). Theoretically, a qualitative PCR diagnostic test is a very sensitive technique that may amplify a single DNA target

sequence up to1 million fold. Therefore, even a few *F. columnare* on the skin or gill will result in a positive PCR test result. In the case of columnaris disease, detection of a few *F. columnare* on surface tissues does not necessarily indicate disease. Numerous bacteria usually are present in natural or experimental columnaris disease.

DNA i*n-situ* hybridization (DNA-ISH) is another molecular test that has the potential to identify *F. columnare* specifically. This technique has the advantage of specifically identifying and localizing a bacterium within histologic lesions. A suitable DNA probe is identified that will detect a conserved target sequence of the genome of the desired bacterium. The tissue section is digested briefly to facilitate penetration of the probe. The probe is applied and allowed to hybridize to any target DNA sequences under carefully controlled conditions of temperature, organic solvent concentration, and ion concentration. The probe subsequently is localized and visualized using high affinity immunohistochemistry. Such assays often use an anti-digoxigenin antibody that is conjugated to alkaline phosphatase as the indicator system. The chromagen solution is a light yellow solution of nitroblue tetrazolium dye that is reduced to insoluble blue-black formazan pigment by the action of alkaline phosphatase.

Prevention and Treatment of Columnaris Disease

According to Davis, the saying ‘An ounce of prevention is worth a pound of cure’ is particularly applicable in this disease (Davis, 1922). Removal of stressors is a primary objective in successful prevention and treatment of columnaris disease. Some common stressors include overcrowding, excessive handling, high water temperature, high ammonia concentration, high organic waste concentration, and poor management

practices such as insufficient temperature and water equilibration when transferring fish from one location to another. Regulation of water temperature below 20°C and maintenance of optimal water quality may reduce the risk of columnaris disease or decrease its severity. Various chemicals, such as potassium permanganate, copper sulfate and Diquat cations, have been used to prevent and treat columnaris disease (Davis, 1922; Snieszko, 1981; Bullock *et al*, 1986; Bullock and McCraren, 1989). It is difficult to prevent exposure to the organism because of its ubiquitous nature. As another preventive measure, potassium permanganate or copper sulfate can be added to ponds at a final concentration of 0.5 ppm. These chemicals dissipate slowly, prolonging the preventative or therapeutic effect. However, these chemical concentrations may not be effective in treating the disease once bacterial infection has been established. The use of antibiotics as a preventive measure is impractical because of drug costs and labor expenses. Furthermore, long-term, indiscriminate use of antibiotics may result in development of antibiotic-resistant strains of bacteria.

Attempts at producing an effective vaccine to prevent columnaris disease have been largely unsuccessful in catfish. While evaluating the efficacy of a formalin-inactivated bacterin in combination with antibiotic administration, the immunized group had lower mortality and required fewer hours of antibiotic treatment. However, higher total mortality was recorded for the immunized group during three years of a five-year study period (Moore *et al*, 1990). Bader and coworkers compared whole-cell, pressure-killed, bacterial lysate and whole-cell, formalin-killed, bacterial lysate by SDS-PAGE and performed western blotting using sera from naturally infected and immunized channel catfish (Bader *et al*, 1997). Sera from immunized catfish reacted only to

pressure-killed lysate antigens. These observations suggest that formalin treatment either inactivates or modifies the 60 kDa protein antigen, rendering it unrecognizable to the antibodies in immunized channel catfish. Thus, formalin-killed bacterins are ineffective against *F. columnare* infection. At the present time, an effective experimental or commercial bacterin has not been developed to prevent columnaris disease.

Low concentrations of potassium permanganate (at a final concentration of 2 to 4 ppm) or copper sulfate (at a final concentration of 0.5 ppm) can be added to outdoor ponds for long-term treatment in instances of less severe columnaris disease. Potassium permanganate (20 ppm) or copper sulfate (30 ppm) also can be used as a dip to treat columnaris disease. Infected fish can be dipped in these solutions for up to 20 minutes without deleterious effects. Other reportedly effective treatments for columnaris disease include a 1 to 2 minute dip in potassium permanganate solution (1000 ppm) or a 1-hour treatment with Diquat cation (2 ppm of active ingredient) (Davis, 1922). Lysol and formalin are toxic to fish even at dilutions of 200 ppm. These compounds should be avoided (Davis, 1922).

Chemical dips largely have been replaced by sulfa drugs and antibiotics for the treatment of columnaris disease. However, antibiotics are more expensive and proper sensitivity testing should be done because *F. columnare* may be resistant to certain antibiotics. Oxytetracycline (Terramycin), when given orally at a dosage of 8g /100 kg body weight per day for up to 10 days in the feed, was found to be effective in treating both early and late outbreaks of columnaris disease (Bullock *et al*, 1986; Bullock and McCraren, 1989; Durborow *et al*, 1998; Srisopaporn *et al*, 2002). With this treatment regimen, there is a 21-day withdrawal period before fish can be sold as food for human

consumption. Oxytetracycline also may be used as an additive to water at a dosage of 2 gm oxytetracycline /100 L of water for 3 days (Decostere *et al*, 2002a). Significantly decreased mortality was recorded by Decostere and Haesebrouck when infected fish were treated with sodium benzyl penicillin at a dosage of 10,000 IU/L added to water for 24 hours (Decostere and Haesebrouck, 1999a). This treatment was repeated after 2 days. A two-step treatment for columnaris disease also can be done by adding either sulfamerazine or oxytetracycline in the feed at a dosage of 220 mg/kg/day for 10 days, followed by 50 to 75 mg/kg/day for 10 days (Wakabayashi, 1981). *In vitro* experiments with bacterial cultures of *F. columnare* indicated that the minimal inhibitory concentration (MIC) of oxytetracycline, erythromycin, and chloramphenicol was 5-6 μg/ml (Nusbaum and Shotts, 1981). Hawke and Thune reported MIC of oxytetracycline of < 1μg/ml for selected isolates of *F. columnare* (*Cytophaga columnaris*) (Hawke and Thune, 1992). Although *F. columnare* is sensitive to neomycin, a higher minimum inhibitory concentration of 10-20 μg/ml is necessary for successful treatment of *F. columnare* infection (Decostere *et al*, 1998).

References

Amin NE, Abdallah IS, Faisal M, Easa ME, Alaway T, Alyan SA. Columnaris infection among cultured Nile Tilapia *Oreochromis niloticus*. *Antonie van Leeuwenhoek* 54:509-520, 1988.

Anonymous. Nonradioactive *In Situ* Hybridization Application Manual, 2nd ed. Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany, 1996.

Bader JA, Klesius PH, Vinitnantharat S. Comparison of whole-cell antigens of pressure- and formalin-killed Flexibacter columnaris from channel catfish (*Ictalurus punctatus*). *Am J Vet Res* 58:985-988, 1997.

Bader JA, Shotts EB. Determination of phylogenetic relationships of *Flavobacterium psychrophilum* (*Flexibacter psychrophilus*), *Flavobacterium columnare* (*Flexibacter columnaris*), and *Flexibacter maritimus* by sequence analysis of 16S ribosomal RNA genes amplified by polymerase chain reaction. *J Aquat Anim Health* 10:320-327, 1998.

Bader JA, Vinitnantharat S, Klesius PH. An inducible protease in *Flavobacterium columnare*: a possible role in virulence? 24th Annual Eastern Fish Health Workshop, Atlantic Beach, NC. March 9-11,1999.

Bader JA, Shoemaker CA, Klesius PH. Rapid detection of columnaris disease in channel catfish (*Ictalurus punctatus*) with a new species-specific 16-S rRNA gene-based PCR primer for *Flavobacterium columnare*. *J Microbiol Methods* 52: 209-220, 2003.

Bernardet JF, Segers P, Vancanneyt M, Berthe F, Kersters K, Vandamme P. Cutting a gordian knot: emended classification and description of genus *Flavobacterium*, emended description of the family Flavobacteriaceae and proposal of *Flavobacterium hydatis* nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). *Interntl J Syst Bact* 46:128-148, 1996.

Bertolini JM, Rohovec JS. Electrophoretic detection of proteases from different *Flexibacter columnaris* strains and assessment of their variability. *Dis Aquat Org* 12: 121-128, 1992.

Boone DR, Castenholz RW, Garrity GM (eds): Bergey's Manual of Systematic Bacteriology: Volume 1: The *Archaea* and the Deeply Branching and Phototrophic *Bacteria*, 2nd ed. New York, Springer-Verlag, p.139-140, 2001.

Bowser PR. Seasonal prevalence of *Chondroccocus columnaris* infection in black bullheads from Clear lake, Iowa. *J Wildlife Dis* 9:115-119, 1973.

Buchanan RE, Gibbons NE (eds): Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins, Co., Baltimore, MD, p.1246, 1974.

Bullock GL, Hsu TC, Shotts EB. Columnaris disease of fishes. Fish Disease Leaflet # 72, *United States Dept of Interior*, Washington DC. 1986.

Bullock GL, McCraren JP. Columnaris disease of fishes. *Fish health bulletin no. 3*, United States Dept of Interior, Washington DC. 1989.

Campbell AC, Buswell JA. An investigation into the bacterial aetiology of 'black patch necrosis' in Dover sole, *Solea solea L. J Fish Dis* 5: 495-508, 1982.

Carbis CR, Mitchell GF, Anderson JW. The effects of microcystins on the serum biochemistry of carp, *Cyprinus carpio* L., when the toxins are administered by gavage, immersion and intraperitoneal routes. *J Fish Dis* 19:151-159, 1996.

Davis HS. A new bacterial disease of fresh water fishes. *Bulletin U. S. Bureau of the Fisheries* (Doc. 924): 262-280,1922.

Decostere A, Haesebrouck F, Devriese LA. Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare (Flexibacter columnaris)* from diseased fish. *J Clin Microbiol* 35(1): 322-324, 1997.

Decostere A, Haesebrouck F, Devriese LA. Characterization of four *Flavobacterium Columnare (Flexibacter columnaris)* strains isolated from tropical fish. *Vet Microbiol* 62: 35-45, 1998.

Decostere A, Haesebrouck F. Outbreak of columnaris disease in tropical aquarium fish. *Vet Rec* 144: 23-24, 1999a.

Decostere A, Haesebrouck F, Charlier G, Ducatelle R. The association of *Flavobacterium columnare* strains of high and low virulence with gill tissues of black mollies (*Poecilia sphenops*). *Vet Microbiol* 67: 287-298, 1999b.

Decostere A, Haesebrouck F, Driessche EV, Charlier G, Ducatelle R. Characterization of the adhesion of *Flavobacterium columnare (Flexibacter columnaris)* to gill tissue. *J Fish Dis* 22: 465-474, 1999c.

Decostere A, Haesebrouck F, Turnbull JF, and Charlier G. Influence of water quality on temperature and adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches. *J Fish Dis* 22: 1-11, 1999d.

Decostere A, Ducatelle R, Haesebrouck F. *Flavobacterium columnare* (*Flexibacter columnaris*) associated with severe gill necrosis in koi carp (*Cyprinus carpio* L). *Vet Rec* 150: 694-695, 2002a.

Decostere A. *Flavobacterium columnare* infections in fish. the agent and its adhesion to the gill tissue. *Verh K Acad Geneeskd Belg*. 64: 421- 430, 2002b.

Durborow RM, Thune RL, Hawke JP, Camus AC. Columnaris disease: a bacterial infection caused by *Flavobacterium columnare*. *Southern Regional Aquaculture Center* publication No. 479, 1998.

Farkas J. Filamentous *Flavobacterium* sp. isolated from fish with gill disease in cold water. *Aquacult* 44: 1-10, 1985.

Farkas J and Olah J. Gill necrosis- a complex disease of carp. *Aquaculture* 58: 17-26, 1986.

Ferguson HW. Columnaris disease in rainbow trout (*Salmo gairdneri*) in Northern Ireland. *Vet Rec* 101: 55-56, 1977.

Griffin BR. Columnaris disease: Recent advances in research. *Aquacult Mag* May/ June pp 48-50, 1987.

Griffin BR. Characteristics of a chondroitin AC lyase produced by *Cytophaga columnaris*. *Tran Am Fish Soc* 120: 391-395, 1991.

Griffin BR. A simple procedure for identification of *Cytophaga columnaris*. *J Aquat Anim Health* 4: 63-66, 1992.

Hawke JP, Thune RL: Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel cat fish. *J Aquat Anim Health* 4: 109-113, 1992.

Jeney Zs, Jeney G. Recent achievements in studies on diseases of common carp (*Cyprinus carpio L.*). *Aquacult* 129: 397-420, 1995.

Jooste PJ, Hugo CJ. The taxonomy, ecology and cultivation of bacterial genera belonging to the family *Flavobacteriaceae*. *International J Food Microbiol* 53: 81-94, 1999.

Karan V, Vitorovic S, Tatundzic V, Poleksic V. Functional enzymes activity and gill histology of carp after copper sulfate exposure and recovery. *Ecotox Environ Safety* 40: 49-55, 1998.

Kent ML, Dungan CF, Elston RA, Holt RA. *Cytophaga* sp. (Cytophagales) infection in seawater pen-reared Atlantic salmon *Salmo salar*. *Dis Aquat Org* 4: 173-179, 1998.

LaFrentz BR, LaPatra SE, Jones GR, Congleton JL, Sun B, Cain KD. Characterization of serum and mucosal antibody responses and relative percent survival in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following immunization and challenge with *Flavobacterium psychrophilum*. *J Fish Dis* 25: 703-713, 2002.

Liewes EW, VanDam RH, Vos-Maas MG, Bootsma R. Presence of antigen sensitized leukocytes in carp (*Cyprinus carpio* L) following bath immunization against *Flexibacter columnaris*. *Vet Immunol Immunopathol* 3:603-9, 1982.

Marks JE, Lewis DH, Trevino GS. Mixed infection in the columnaris disease of fish. *J Am Vet Med Assoc* 177: 811-814, 1980.

Moore AA, Eimers ME, Matteo AC. Attempts to control *Flexibacter columnaris* epizootics in pond-reared channel cat fish by vaccination. *J Aquat Anim Health* 2: 109-111, 1990.

Morrison C, Cornick J, Shum G, Zwicker B. Microbiology and histopathology of 'saddle back disease' of underlying Atlantic Salmon, *Salmo salar L. J Fish Dis* 4:243-258, 1981.

Newton JC, Wood TM, Hartley MM. Isolation and partial characterization of extracellular proteases produced by isolates of *Flavobacterium columnare* derived from channel cat fish. *J Aquat Anim Health* 9: 75-85, 1997.

Nusbaum KE, Shotts EB. Action of selected antibiotics on four common bacteria associated with diseases of fish. *J Fish Dis* 4: 397-404, 1981.

Ordal EJ, Rucker RR. Pathogenic myxobacteria. *Soc Exp Biol Med Proc* 56: 15-18, 1944.

Pacha RE and Ordal EJ. Histopathology of experimental columnaris disease in young Salmon. *J Comp Pathol* 77: 419-423, 1967.

Pate JL, Ordal EJ. The fine structures of *Chondrococcus columnaris* I. Structure and formation of mesosomes. *J Cell Biology* 35: 1-13, 1967a.

Pate JL, Johnson JL, Ordal EJ. The fine structures of *Chondrococcus columnaris* II. Structure and formation of rhapidosomes. *J Cell Biology* 35: 15-35, 1967.

Pate JL, Ordal EJ. The fine structures of *Chondrococcus columnaris* III. The surface layers of *Chondrococcus columnaris*. *J Cell Biology* 35: 37-50, 1967b.

Rehulka J. Blood parameters in common carp with spontaneous spring viremia (SVC). *Aquaculture International* 4:175-182,1996.

Schwaiger J, Spieser OH, Bauer C, Ferling H, Mallow U, Kalbfus W, Negele RD. Chronic toxicity of nonylphenol and ethinylestradiol: haematological and histopathological effects in juvenile common carp (*Cyprinus carpio*). *Aquat Toxicol* 51: 69-78, 2000.

Shamsudin MN, Plumb JA. Morphological, biochemical, and physiological characterization of *Flexibacter columnaris* isolates from four species of fish. *J Aquat Anim Health* 8: 335-339, 1996.

Snieszko SF. Bacterial gill disease of freshwater fishes. Fish Disease Leaflet # 62, *United States Dept of Interior*, Washington DC. 1981.

Speare DJ, Markham RJ, Despres B, Whitman K, MacNair N. Examination of gill from salmonids with bacterial gill disease using monoclonal antibody probes for

Flavobacterium branchiophilum and *Cytophaga columnaris*. *J Vet Diagn Invest* 7: 500-505, 1995.

Srisopaporn P, Schwedler TE, Rice CD, Dorociak IR. Treatment of *Flavobacterium columnare* infection in channel catfish (*Ictalurus punctatus*). 27th Annual Eastern Fish Health Workshop, Mount Pleasant, SC. March 18-22, 2002.

Stringer K, Caslake L, Yunghans W. Analysis of chondroitin AC lyase activity and virulence of *Flavobacterium columnare*. 25th Annual Eastern Fish Health Workshop, Kerneysville, WV. 10-13 March, 2000.

Tiirola M, Valtonen ET, Kinnunen PR, Kulomaa MS. Diagnosis of flavobacteriosis by direct amplification of rRNA genes. *Dis Aquat Org* 51: 93-100, 2002.

Wakabayashi H. Effect of environmental conditions on the infectivity of *Flexibacter columnaris* to fish. *J Fish Dis* 14: 279-290, 1991.

Wlasow T, Dabrowska H. Hematology of carp in acute intoxication with ammonia. *Pol Arch Hydrobiol* 3: 419-428, 1990.

CHAPTER 3

COLUMNARIS DISEASE IN FRESHWATER FISH[1]

[1]Tripathi, N.K., K.S. Latimer and P.M. Rakich. Accepted by *Compendium on Continuing Education for the Practicing Veterinarian.* Reprinted here with the permission of publisher, 6/26/2003

Abstract

Columnaris disease is a bacterial infection of fish caused by the gram-negative bacillus *Flavobacterium columnare. F. columnare* usually is of low pathogenicity and infects fish under stressful conditions. However, some strains of this bacterium are highly pathogenic and may cause disease in the absence of documented stress. The early clinical signs of columnaris disease are nonspecific and include lethargy, inappetence, and increased opercular movements. Cotton-like lesions of the skin, fins, and gills; saddleback lesions originating in the dorsal skin; and/or gill necrosis are typical findings in columnaris disease. The pathogenesis of this disease is unclear, but hypoxia caused by gill necrosis and biochemical disturbances secondary to skin ulceration are probably responsible for death. Definitive diagnosis of columnaris disease depends on microbiologic culture using a selective medium (i.e., *Cytophaga* or Shieh). Columnaris disease can be treated successfully, provided it is diagnosed early.

Introduction

Columnaris disease was first reported by Davis in 1922 and remains one of the most frequently encountered and devastating bacterial diseases of freshwater fish.[1] This disease is also known as *saddleback disease*, *cotton-wool disease*, *cotton-mouth disease*, and *fin rot*. These names reflect the gross lesions of affected fish, including color changes over the dorsum (Figure 1), cottony white proliferations on the skin and fins (Figure 2), or loss of fin area (Figure 3).

Davis initially noticed this disease in the summer of 1917 in buffalo fish (sucker; *Ictiobus bubalus* and *Ictiobus ciprinella*) that were transferred to aquaria.[1] The following

summer, he observed additional disease outbreaks in ponds and rivers involving many freshwater fish, including sunfish (*Lepomis incisor* and *Lepomis humilis*), carp (*Cyprinus carpio*), black bass (*Micropterus salmoides* and *Micropterus dolomieu*), black and white crappie (*Pomoxis sparoides* and *Pomoxis annularis*), warmouth (*Chaenobryttus gulosus*), yellow perch (*Perca flavescens*), white bass (*Roccus chrysops*), brook trout (*Salvelinus fontinalis*), bluntnose minnow (*Pimephales notatus*), channel catfish (*Ictalurus punctatus*), and brown and black bullhead (*Ameiurus nebulosus and Ameiurus melas*). In subsequent reports, columnaris disease has proven to be a devastating disease in most freshwater fish[1–9] and has also been reported in marine fish (e.g., Atlantic salmon).[2,5] The disease occurs more commonly in warm-water fish (20°C and higher) but has also been reported in cold-water fish, such as sheatfish (*Silurus glanis*), silver carp (*Hypophthalmichthys molitrix*), and rainbow trout (*Oncorhynchus mykiss*), at temperatures of 6°C to 12°C.[10] Thus columnaris disease may cause major economic losses in commercially farmed fish raised for pets and human consumption as well as in wild fish in their natural environment.

Cause and Taxonomy

The causative organism of columnaris disease is a long, thin, gram-negative, aerobic, microaerophilic, or anaerobic gliding rod that has recently been reclassified as *Flavobacterium columnare*.[11,12] A similar filamentous bacterium, probably *F. psychrophilum*, has been isolated from cold-water fish in Hungary.[10] *F. columnare* is ubiquitous (i.e., found in water and soil, on the skin of healthy fish) and distributed worldwide.[13–15]

Davis could not isolate the pathogen but observed it in the wet mounts of affected gills and fins, forming column-like masses, and named it *Bacillus columnaris*.[1] Ordal and Rucker isolated the bacterium for the first time in 1944. They classified it under the order Myxobacterales and named the bacterium *Chondrococcus columnaris*. Organisms of the order Myxobacterales are long, thin, gram-negative rods that are motile on agar by flexing motion and have vegetative cells, microcysts, and fruiting bodies in their life cycle. In 1945, Garnjobst subsequently demonstrated that the organism produced microcysts but not fruiting bodies. Thus the bacterium was reclassified as *Cytophaga columnaris.* In 1974, Buchanan and Gibbons reported that this bacterium produced neither microcysts nor fruiting bodies. Therefore, it was removed from the order Myxobacterales, reclassified in the order Cytophagales, and renamed *Flexibacter columnaris.* Recently, the bacterium was reclassified and renamed *F. columnare*, reflecting the production of yellow pigment in agarose culture.[11,16] Reclassification of the bacterium to the phylum *Bacteroides* was based on phylogenetic studies of 16S rRNA genes.[12]

Pathogenesis

The pathogenesis of columnaris disease is not clear, but it is known that the disease process involves bacterial invasion and external tissue damage.[17] *F. columnare* is generally of low pathogenicity; however, bacterial strains differ in their virulence. In most instances, bacterial infection occurs in fish that are exposed to stressful conditions, such as high water temperature (greater than 20°C), overcrowding, excessive handling, and poor water quality, especially high ammonia concentrations or increased organic

waste content.[13,14,17–20] Jeney and Jeney[15] demonstrated that adherence of *F. columnare* to gill tissues was enhanced by high nitrite concentration (5 mg/L), high organic content (2 g/L), high temperature (28°C), and high salt concentration (0.3% sodium chloride) in the water. Sudden changes in water temperature of 5°C or more pose significant stress, predisposing fish to infection by *F. columnare*. Spontaneous, natural infections with *F. columnare* have been reported in the absence of any obvious stressors. Spontaneous infections usually involve highly virulent bacterial strains and are associated with high mortality.[21]

Columnaris disease is usually transmitted by direct contact with infected fish or contaminated water. Immersion in *F. columnare*–contaminated water is more effective in producing experimental infection than either IM or intraperitoneal injection of this bacterium.[5] Because significant systemic infection cannot be documented, vertical transmission seems unlikely.

The morbidity, mortality, and course of disease depend mainly on the water temperature and virulence of the bacterial strain.[22] In an experimental infectivity study, mortality did not occur at 5°C or 10°C. In contrast, 25% mortality was observed at 15°C, with a mean death time of 7 days; whereas, 100% mortality was recorded at 20°C and higher, with a mean death time of 1 to 3 days.[14] This study demonstrates that increasing temperature is associated with increased mortality during bacterial infection. As early as 1922, Davis reported differences in the susceptibility of various fishes to *F. columnare* infection.[1] For example, black bullheads were not infected when kept with buffalo fish and bluegill (*Lepomis macrochirus*) that had columnaris disease. However, black bullheads were readily infected if exposed to the same bacterium that had been

propagated on other black bullheads. These observations demonstrate that *F. columnare* can adapt to new hosts and display increased virulence.

Thus far, most pathogenesis studies have focused on the development of external skin or gill lesions. Gill infection results in acute infection of variable severity. Adhesion factors of the bacterial capsule are mainly responsible for the attachment of the organism to gill tissue. Decostere et al[23] compared low- and high-virulence strains of *F. columnare* in their ability to adhere to gill tissue, demonstrating that bacterial strains with the highest virulence adhered more avidly to gill tissue. Bacterial adherence of *F. columnare* was reduced significantly following treatment with sodium metaperiodate (periodic acid, an oxidizing agent) or incubation with sugars (i.e., D-glucose, D-galactose, D-sucrose). Bacterial adherence was not inhibited after treatment with trypsin or pronase, indicating that the major component of the adherence receptor is a carbohydrate and not a protein. Transmission electron microscopy also demonstrated that the capsules of *F. columnare* with high virulence were thick (i.e., 120 to 130 nm) and dense, whereas capsules of *F. columnare* of low virulence were thinner (i.e., 80 to 90 nm) and less dense. Highly virulent strains of *F. columnare* lost their capsule after mild heating or treatment with sodium metaperiodate. Loss of the bacterial capsule resulted in a significant reduction in adherence to gill tissues. These results indicate that attachment of *F. columnare* to gill tissue is mediated by a lectin-like, carbohydrate-binding substance incorporated into the bacterial capsule (lectins are primarily plant-derived proteins that specifically bind monosaccharides or oligosaccharides in cell walls or membranes).[23]

Following attachment of *F. columnare* to the gill, initial microscopic lesions include branchial epithelial cell and goblet cell hyperplasia. These lesions rapidly progress to

severe neutrophilic inflammation and gill necrosis (Figure 4). In acute bacterial infection, hypoxia and death may result from extensive damage to the gills.[2]

F. columnare infection with primary skin involvement has a longer clinical course (i.e., 2 to 7 days). Studies of the pathogenesis of skin infection have been largely restricted to the identification of extracellular proteases that assist in establishing bacterial infection. Extracellular proteases degrade chondroitin sulfates A and C and hyaluronic acid, the complex polysaccharides found in connective tissue stroma.[24–26] Bertolini and Rohovec[25] isolated four extracellular proteases from *F. columnaris* that degraded gelatin, casein, hemoglobin, fibrinogen, and elastin. These proteases had different molecular weights of 47, 40, 34 and 32 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Newton et al[26] also identified proteases with molecular weights of 58 and 53.5 kDa from 27 strains of *F. columnare* (derived primarily from channel catfish) and reported that these two proteases were produced as early as 1 day after bacterial inoculation. They also demonstrated, using an endpoint colorimetric assay, that increased protease activity was present in culture medium with lower nutrient and salt concentrations than in media with high nutrient or salt concentrations. In later experiments with catfish, Bader et al[27] demonstrated proteolytic and immunogenic activities of a 58 kDa protease that was always associated with virulence. Because this protease was inactivated with formalin or heat treatment, it could not be used for preparing a bacterin using routine methods. Bacterial protease activity is important in the virulence of the organism and the production of cutaneous lesions. An effective vaccine against protease activity is theoretically possible but will require recombinant technology.

Osmotic disturbance secondary to a compromised epidermal barrier is probably responsible for death in fish with columnaris disease presenting with primary skin involvement. Morrison et al[5] found that *F. columnare* generally was confined to the dermis and underlying muscle in necrotic foci. *F. columnare* has been isolated from the kidneys of some catfish, carp, and salmonids with severe gill and muscle destruction.[5,28] Pacha and Ordal[2] isolated small numbers of bacteria from the kidneys of infected salmon, but significant lesions were not observed in internal organs except for enlargement of Bowman's capsule caused by deposition of an eosinophilic material around the glomerular tuft. However, similar glomerular changes have also been reported in fish that lack skin lesions. Thus the significance of septicemia or bacteremia as the cause of death is questionable.

Clinical signs and physical findings

The initial clinical signs of columnaris disease are nonspecific and include listlessness, lethargy, inappetence, swimming near the water surface, and accelerated opercular movement. The disease can have different clinical manifestations, with various combinations of gill, skin, or fin lesions. Columnaris disease associated with primary gill involvement is acute, and the course of disease ranges from 2 to 5 days.

Fish with peracute columnaris disease may be observed lying on their sides. They have marked opercular movement and often die within 48 hours of the appearance of clinical signs.[21] Characteristic skin discoloration and ulcers are not usually observed. Peracute columnaris disease is usually observed in younger fish that die within 1 to 4 days without visible lesions.

Fish with established columnaris disease usually have lesions on the external body surface and gills. The distribution of these lesions tends to vary with the species of the fish. In scaleless fish, the skin lesions begin as areas of discoloration, primarily at the base of the dorsal fin. However, lesions may also be seen on the head and craniodorsal part of the body.[14,28–30] As skin lesions spread from the base of the dorsal fin, a pale white band extends laterally and encircles the body to form a characteristic, pale white, "saddleback" lesion that has also been described in Atlantic salmon.[5] A yellowish-white ulcer often develops in the center of the "saddle" as the lesion progresses.[9,13,20] In advanced disease, extensive and deep skin ulcers may develop, exposing underlying muscle and bone (Figure 5).[28]

In scaled fish, prominent gill and fin necroses are usually observed; however, skin ulcers may also be present in the absence of gill and fin lesions. Necrosis of gills and fins begins at the outer margins and extends from the distal end toward the body. Gill necrosis is observed as yellowish-white spots on the tips of the primary lamellae of the gills. Initially, the skin lesions are less prominent in scaled fish but become obvious as the skin lesions advance from mild hyperemia to deep skin ulcers. The scales become loosened and slough off as the skin disintegrates.[1]

Grossly, bacterial mats can be seen attached to skin and/or fins and have a typical "cotton wool" appearance (Figures 2, 3, and 6). A yellowish-brown, mucoid-like growth is seen inside the mouth, mostly in catfishes.[20] In experimentally infected koi (*C. carpio*; an ornamental strain of common carp), hemorrhagic ulcers are seen more frequently around the mouth (Figure 7). Also in experimentally infected koi, lesions are restricted mostly to skin; gill necrosis is inconsistently seen in only a few cases.

Diagnosis

The diagnosis of columnaris disease, especially in pond outbreaks, is based on cytologic examination of smears from skin, gills, and fins; histopathologic examination of tissues obtained during necropsy; and microbiologic culture, which is the most reliable technique for the definitive diagnosis of *F. columnare* infection. Live fish with clinical signs of disease provide the best material for diagnostic testing. If diseased, live fish cannot be shipped to the diagnostic laboratory for testing, the fish can be euthanized with tricaine methanesulfonate (MS-222) and necropsy performed. Fresh tissue on ice and formalin fixed tissue can be submitted to the laboratory for analysis. Fresh tissue on ice is used for bacterial culture and formalin fixed tissue for histopathology.

The water should be tested for hardness and concentrations of chlorine nitrite and ammonia. Measuring water hardness is important because increased hardness can promote bacterial adhesion to gills.[19] Water quality can be evaluated in the field using commercially available test kits. If this is impractical, water samples can be submitted to the laboratory in clean containers for further analysis. A complete medical history, including any possible stressors to the fish, should be reported on the submission form.

Live diseased fish can be transported short distances in open containers with sufficient water to submerge them. Adding salt to the water (3 g/L) reduces stress by inhibiting the transport of nitrite across the gill epithelium. For overnight shipping, the fish should be packed in airtight plastic bags filled with oxygen and containing enough water to cover the fish. Ice packs can be used when necessary to maintain water temperature. Moribund fish should be euthanized with an overdose of tricaine methanesulfonate or other water-soluble anesthetic and shipped to the laboratory on ice to

delay decomposition. Fish that are found dead are usually decomposed and generally unsuitable for diagnostic purposes.

A tentative cytologic diagnosis of columnaris disease can be made by microscopic examination of wet mounts or Romanowsky-stained scrapings or imprints of affected skin and gill tissue. *F. columnare* is a filamentous bacterium (0.5 x 5 to 10 ìm) typically 10 to 20 times longer than its width (Figure 8). On wet mounts, *F. columnare* appear as long, thin bacilli arranged in haystacks. In Romanowsky stained preparations, the elongated bacilli are usually distributed singly, although organisms may be innumerable. Bacilli appear red or gram-negative following Gram's staining. Most of the pathogenic bacteria in fish are gram-negative bacilli; therefore, cytology alone cannot provide a definitive diagnosis.

F. columnare is fastidious and usually grows slowly. Culture and identification of this bacterium is frequently unsuccessful because of contamination and overgrowth by other bacteria. *F. columnare* does not grow on typical blood agar plates, and special media are required for bacterial isolation. Because this bacterium has the ability to grow on a medium that contains neomycin and polymyxin B, antibiotics are incorporated into selective media to isolate this organism. Thus to ensure that an appropriate culture medium is used to propagate the bacterium, the clinician must indicate to the bacteriology laboratory that *F. columnare* is the possible cause. Examples of appropriate media include Cytophaga agar with neomycin (5 ìg/ml agar) or Shieh medium supplemented with tobramycin (1 ìg/ml agar).[31] The plates are inoculated and incubated at 25°C. Visible bacterial growth is usually evident within 2 to 4 days. Because of swarming phenomenon on solid media, *F. columnare* forms small (i.e., 1- to 3-mm diameter),

yellow colonies that have rhizoid edges (i.e., a root-like appearance; Figure 9). It exhibits gliding movement in liquid media and produces a yellow turbidity containing small, filamentous aggregates.[32] Constant stirring at 150 to 250 rpm is required to promote bacterial growth in liquid media.[10,18] In 5- to 7-day-old cultures, sediment appears at the bottom of the culture flask and bacterial rods start to disintegrate, producing small spheroid bodies known as microcysts.[10,17] Certain biochemical characteristics aid in identifying *F. columnare*. Bacterial colonies produce gelatinase and chontroitin sulfatase. Furthermore, colonies of *F. columnare* bind Congo red dye.[33]

The primary histologic lesions associated with *F. columnare* infection are gill necrosis, skin ulcers and necrosis, and fin necrosis. In the initial skin lesions observed as areas of discoloration, the epidermal lining of the skin may be intact, but variable numbers of bacteria are present in the epidermis, dermis, and muscle layer, with minimal inflammation. In more advanced lesions, the epidermis may be hyperplastic to ulcerated, and scales, if present, may be lost. The hyperplastic epithelium is usually infiltrated with lymphocytes, plasma cells, and neutrophils. These inflammatory cells can infiltrate deep into the subjacent skeletal muscle, depending on the degree of damage. Cutaneous ulcers are usually covered by a thick mat of bacteria. The ulcer may extend into the underlying skeletal muscle. The epithelium covering the fins is lost, resulting in fin necrosis and leaving an exposed framework of cartilaginous rays. Gill lesions may include epithelial and goblet cell hyperplasia, neutrophilic inflammation of variable intensity, and an obliteration of interlamellar spaces (Figure 4). Gill necrosis is sometimes severe, and secondary gill lamellae are lost. Blood vessels supplying the gills are congested, and

accumulation of edema fluid sometimes results in separation of epithelium lining from the secondary lamellae.

F. columnare appear as long, thin bacilli in histologic sections, but organisms are superficial and may wash off of the gills, skin, and fins during routine tissue fixation and processing. In addition, necrotic and ulcerative lesions may also be colonized by other bacteria with similar morphology. Because most of the major bacterial pathogens of fish are gram-negative bacilli, routine histopathology of formalin fixed tissues can confirm the presence of a lesion compatible with columnaris disease, but it is not definitive for the diagnosis. The organism can be identified specifically in histologic sections using immunohistochemistry or DNA in situ hybridization, but these techniques are largely restricted to research laboratories.

Fluorescent antibody testing has been used to identify *F. columnare* in frozen sections of unfixed tissue.[13] Experimentally, polymerase chain reaction using species-specific primers has also been used experimentally to identify *F. columnare* grown on tryptone yeast plates.[16]

Treatment

Current methods of treatment include antibiotic administration and chemical dips or baths. Furthermore, the use of antibiotics may be governed by whether the fish are intended for human consumption or are being marketed for the tropical fish pet trade. Concurrent reduction of stressors to the fish should accompany any treatment regimen.

Sodium benzylpenicillin (10,000 IU/L of water),[9] oxytetracycline,[8,20] and chloramphenicol (to achieve a final concentration of 16 µg/ml of water)[34] have been

found to be effective in treating columnaris disease. In vitro experiments have demonstrated that a very low minimum inhibitory concentration of antibiotic is required to inhibit the growth of *F. columnare* on agar plates containing chloramphenicol, erythromycin, lincomycin, and oxytetracycline.[18,34] Neomycin was effective against the bacterium but required a higher minimum inhibitory concentration (10 to 20 µg/ml).[18] In contrast, special culture media for the isolation of *F. columnare* incorporate neomycin at a concentration of 5 µg/ml to select for the growth of this organism while suppressing the growth of other contaminating bacteria.

Sulfamerazine or oxytetracycline can be administered in the feed as a two-step treatment regimen (220 mg/kg/day for 10 days followed by 50 to 75 mg/kg/day for 10 days).[14] Oxytetracycline may also be used as an additive to water (2 g oxytetracycline/100 L of water for 3 days). Following treatment in this manner, infected fish had decreased mortality within 4 days followed by increased physical activity and appetite.[21]

For external infections with *F. columnare*, 2- to 8-hour baths may be used at a final concentration of 2 ppm of potassium permanganate ($KMnO_4$, based on the demand test) or 1 ppm of copper sulfate ($CuSO_4$). If the color of the bath water changes from wine red to yellowish brown in less than 4 hours, another treatment of $KMnO_4$ may be required. The color change indicates oxidation of the $KMnO_4$, the time for this alteration to occur depends on water quality. Davis[1] reported that fish without significant lesions can be treated with up to a 1:1000 concentration (1000 ppm) of $CuSO_4$ for 1 minute without causing injury to the fish. A complete water change should be done after bath treatment. Systemic infection, if present, can be treated with oral or parenteral antibiotic

administration (combined with therapeutic baths if external lesions also are visible). Dilute formalin dips are effective in treating surface parasites but are not recommended for treating columnaris disease.

Disease prevention and control

Columnaris disease is best prevented by good management practices that include minimal handling, attention to water quality and temperature, and prevention of overstocking. However, outbreaks of columnaris disease are difficult to avoid because of the ubiquitous presence of *F. columnare* in water sources. Because infection is less likely at lower temperatures, lowering water temperature (by adding cold water or ice) may help reduce the severity of disease. When fish are transferred from one aquatic environment to another, adequate time should be allowed for equilibration before release. The equilibration period ranges from 30 minutes to 1 hour, depending on the environmental temperature gradient and oxygen content of the water.

$CuSO_4$ or $KMnO_4$ has been advocated as a preventive treatment for fish or ponds. $CuSO_4$ has been used as a bath for fish (i.e., a 20-minute bath at a concentration of 37 ppm $CuSO_4$) or as a pond additive (to achieve a final concentration of 0.5 ppm $CuSO_4$). Alternatively, $KMnO_4$ has been added to ponds for the same purpose (to achieve a final concentration of 0.5 ppm $KMnO_4$). Other considerations in adding chemicals to ponds and dipping fish include increased cost and labor, added stress to the fish, and possible adverse environmental effects.

In an experimental study, Moore et al[35] evaluated efficacy *F. columnare* bacterin by immunizing catfish with and without antibiotic treatment. The vaccinated group had

decreased mortality and required fewer antibiotic treatment hours than the unvaccinated control group. However, the vaccinated group had higher total mortality than the control group during 3 of the 5 years of the study.[35] Experimental trials using vaccines for immersion and injection have also been evaluated,[36] but an effective commercial vaccine is not yet available to prevent columnaris disease.

Another preventive measure that has been suggested is competitive inhibition of *F. columnare* by a nonpathogenic bacterium, such as *Citrobacter freundii*.[14,37] The theory is that the nonpathogenic bacterium inhibits the natural attachment of the pathogenic bacterium, preventing tissue destruction and invasion. However, the practicality of this technique in large pond settings has not been evaluated.

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References

1. Davis HS: A new bacterial disease of fresh-water fishes. *Bulletin U. S. Bureau of Fisheries, Document 924*:261-280, 1921-1922.

2. Pacha RE and Ordal EJ: Histopathology of experimental columnaris disease in young Salmon. *J Comp Pathol* 77:419-423, 1967.

3. Bootsma R, Clerx JPM: Columnaris disease of cultured carp *Cyprinus carpio L*. Characterization of the causative agent. *Aquaculture* 7: 371-384, 1976.

4. Marks JE, Lewis DH, Trevino GS: Mixed infection in columnaris disease of fish. *JAVMA* 177:811-814, 1980.

5. Morrison C, Cornick J, Shum G, Zwicker B: Microbiology and histopathology of 'saddleback disease' of underyearling Atlantic salmon, *Salmo salar L*. *J Fish Dis* 4:243-258, 1981.

6. Campbell AC, Buswell JA: An investigation into the bacterial aetiology of 'black patch necrosis' in Dover sole, *Solea solea L*. *J Fish Dis* 5:495-508, 1982.

7. Foscarini R: Induction and development of bacterial gill disease in the eel (*Anguilla japonica*) experimentally infected with *Flexibacter columnaris*: Pathological changes in the gill vascular structure and in cardiac performance. *Aquaculture* 78:1-20, 1989.

8. Hawke JP, Thune RL: Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel catfish. *J Aquat Anim Health* 4:109-113, 1992.

9. Decostere A, Haesebrouck F: Outbreak of columnaris disease in tropical aquarium fish. *Vet Rec* 144:23-24, 1999.

10. Farkas J: Filamentous *Flavobacterium* sp. isolated from fish with gill diseases in cold water. *Aquaculture* 44:1-10, 1985.

11. Bernardet JF, Segers P, Vancanneyt M, et al: Cutting a gordian knot: Emended classification and description of genus *Flavobacterium*, emended description of the family Flavobacteriaceae and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Internatl J Syst Bact* 46:128-148, 1996.

12. Boone DR, Castenholz, Garrity GM (eds): Bergey's Manual of Systematic Bacteriology: Volume 1: The *Archaea* and the Deeply Branching and Phototrophic *Bacteria*, 2nd ed. New York, Springer-Verlag, 2001, pp. 139-140.

13. Griffin BR: Columnaris disease: Recent advances in research. *Aquacult Mag*, May/ June, pp. 48-50, 1987.

14. Wakabayashi H: Effect of environmental conditions on the infectivity of *Flexibacter columnaris* to fish. *J Fish Dis* 14:279-290, 1991.

15. Jeney Zs, Jeney G: Recent achievements in studies on diseases of common carp (*Cyprinus carpio L.*). *Aquacult* 129:397-420, 1995.

16. Bader JA, Shotts EB: Identification of *Flavobacterium* and *Flexibacter* species by species-specific polymerase chain reaction primers to the 16S ribosomal RNA gene. *J Aquat Anim Health* 10:311-319, 1998.

17. Farkas J and Oláh J: Gill necrosis - a complex disease of carp. *Aquaculture* 58:17-26, 1986.

18. Decostere A, Haesebrouck F, Devriese LA: Characterization of four *Flavobacterium columnare (Flexibacter columnaris)* strains isolated from tropical fish. *Vet Microbiol* 62: 35-45, 1998.

19. Decostere A, Haesebrouck F, Turnbull JF, Charlier G: Influence of water quality on adhesion of high and low virulence *Flavobacterium columnare* strains to isolated gill arches. *J Fish Dis* 22:1-11, 1999.

20. Durborow RM, Thune RL, Hawke JP, Camus AC: Columnaris disease: A bacterial infection caused by *Flavobacterium columnare*. *Southern Regional Aquaculture Center* Publication No. 479, September, 1998.

21. Decostere A, Ducatelle R, Haesebrouck F: *Flavobacterium columnare* (*Flexibacter columnaris*) associated with severe gill necrosis in koi carp (*Cyprinus carpio* L). *Vet Rec* 150:694-695, 2002.

22. Noga EJ: Fish Disease: Diagnosis and Treatment. St. Louis, Mosby-Year Book, Inc., 1996, pp. 123-126.

23. Decostere A, Haesebrouck F, Van Driessche E, et al: Characterization of the adhesion of *Flavobacterium columnare (Flexibacter columnaris)* to gill tissue. *J Fish Dis* 22:465-474, 1999.

24. Griffin BR: Characteristics of a chondroitin AC lyase produced by *Cytophaga columnaris*. *Tran Am Fish Soc* 120:391-395, 1991.

25. Bertolini JM, Rohovec JS: Electrophoretic detection of proteases from different *Flexibacter columnaris* strains and assessment of their variability. *Dis Aquat Org* 12: 121-128, 1992.

26. Newton JC, Wood TM, Hartley MM: Isolation and partial characterization of extracellular proteases produced by isolates of *Flavobacterium columnare* derived from channel catfish. *J Aquat Anim Health* 9:75-85, 1997.

27. Bader JA, Vinitnantharat S, Klesius PH: An inducible protease in *Flavobacterium columnare*: A possible role in virulence? 24th Annual Eastern Fish Health Workshop, Atlantic Beach, NC. March 9-11,1999.

28. Bullock GL, Hsu TC, Shotts EB: Columnaris disease of fishes. *Fish Disease Leaflet 72*, United States Department of the Interior, Washington, D.C., 1986, pp. 1-9.

29. Bullock GL, McCraren JP: Columnaris disease of fishes. *Fish Health Bulletin No. 3*,

United States Department of the Interior, U.S. Fish and Wildlife Service, Washington, D.C., 1989.

30. Decostere A, Haesebrouck F, Charlier G, Ducatelle R: The association of *Flavobacterium columnare* strains of high and low virulence with gill tissues of black mollies (*Poecilia sphenops*). *Vet Microbiol* 67:287-298, 1999.

31. Decostere A, Haesebrouck F, Devriese LA: Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. *Vet Microbiol* 35:322-324, 1997.

32. Shamsudin MN, Plumb JA: Morphological, biochemical, and physiological characterization of *Flexibacter columnaris* isolates from four species of fish. *J Aquat Anim Health* 8:335-339, 1996.

33. Griffin BR: A simple procedure for identification of *Cytophaga columnaris. J Aquat Anim Health* 4:63-66, 1992.

34. Nusbaum KE, Shotts EB: Action of selected antibiotics on four common bacteria associated with diseases of fish. *J Fish Dis* 4:397-404, 1981.

35. Moore AA, Eimers ME, Cardella MA: Attempts to control *Flexibacter columnaris* epizootics in pond-reared channel cat fish by vaccination. *J Aquat Anim Health* 2:109-111, 1990.

36. Newman SG: Bacterial vaccines for fish. *Ann Rev Fish Dis* 3:145-185, 1993.

37. Chowdhury MBR, Wakabayashi H: Effects of competitive bacteria on the survival and infectivity of *Flexibacter columnaris*. *Fish Pathol* 24:9-15, 1989.

Figure 3.1 Channel catfish (*Ictalurus punctatus*) fingerling with typical saddleback lesion of *Flavobacterium columnare* infection. Notice blanching of tissues around dorsal fin (arrows).

Figure 3.2 Koi (*Cyprinus carpio*) with columnaris disease. Cotton-like, white proliferations are present on the skin, tail, and fins.

Figure 3.3 Goldfish (*Carassius auratus auratus*) with partial loss of fins (fin rot) due to columnaris disease.

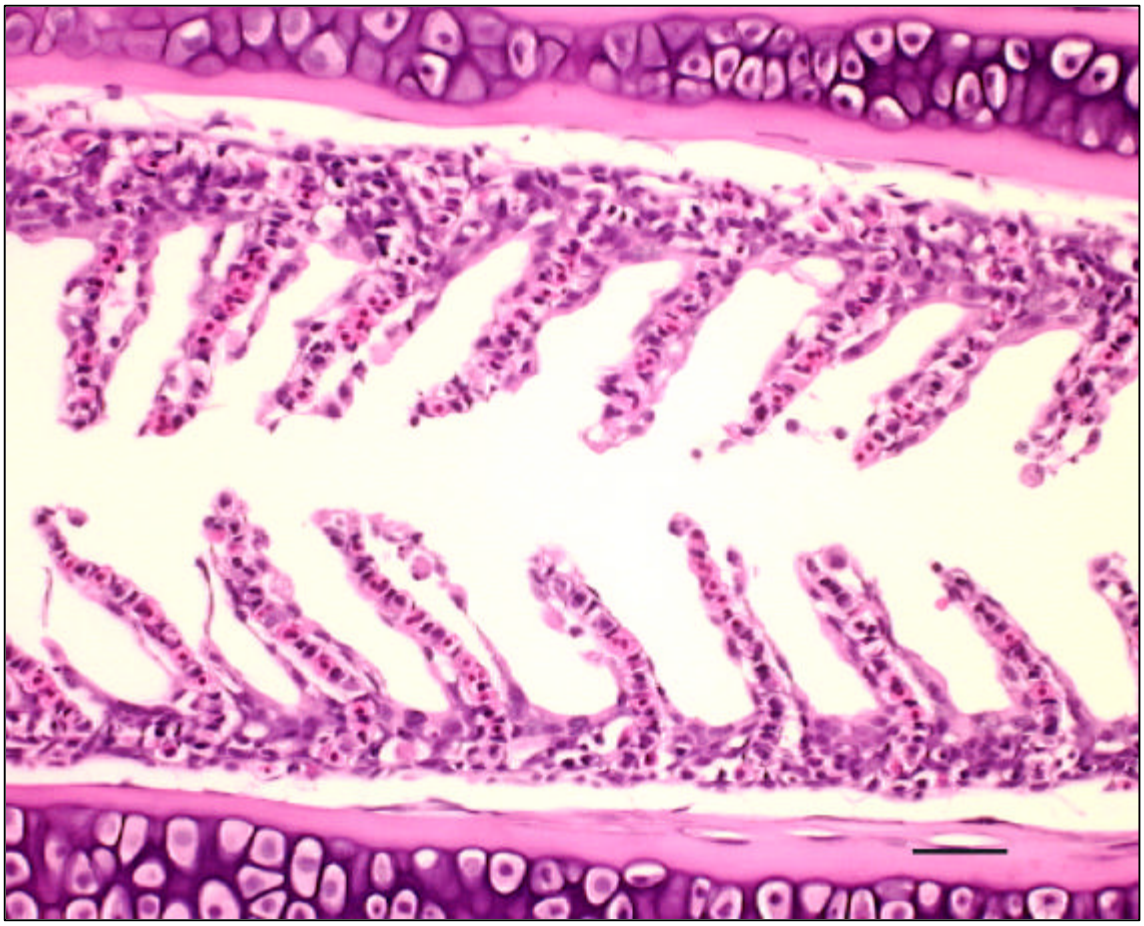

Figure 3.4 A. Histologic section of a healthy gill from a koi (*Cyprinus carpio*) showing secondary lamellae that are separated by interlamellar spaces that promote water flow and blood gas exchange (hematoxylin and eosin stain, bar = 20 µm).

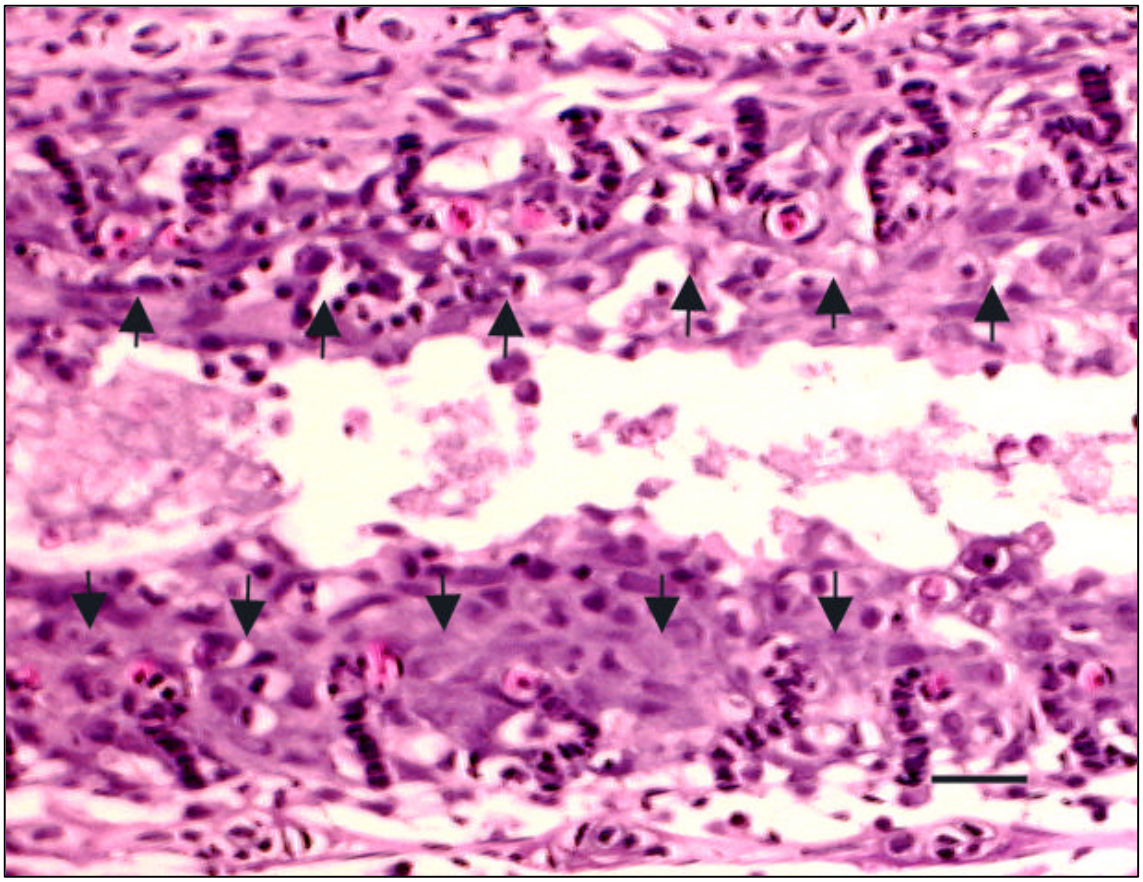

Figure 3.4 B. Histologic section of a diseased gill from a koi (*Cyprinus carpio*) with columnaris disease. The interlamellar spaces (arrows) are obliterated by epithelial cell and goblet cell hyperplasia as well as neutrophil infiltration. Bacteria have detached from the surface of the gill during fixation and tissue processing (hematoxylin and eosin stain, bar = 20 µm).

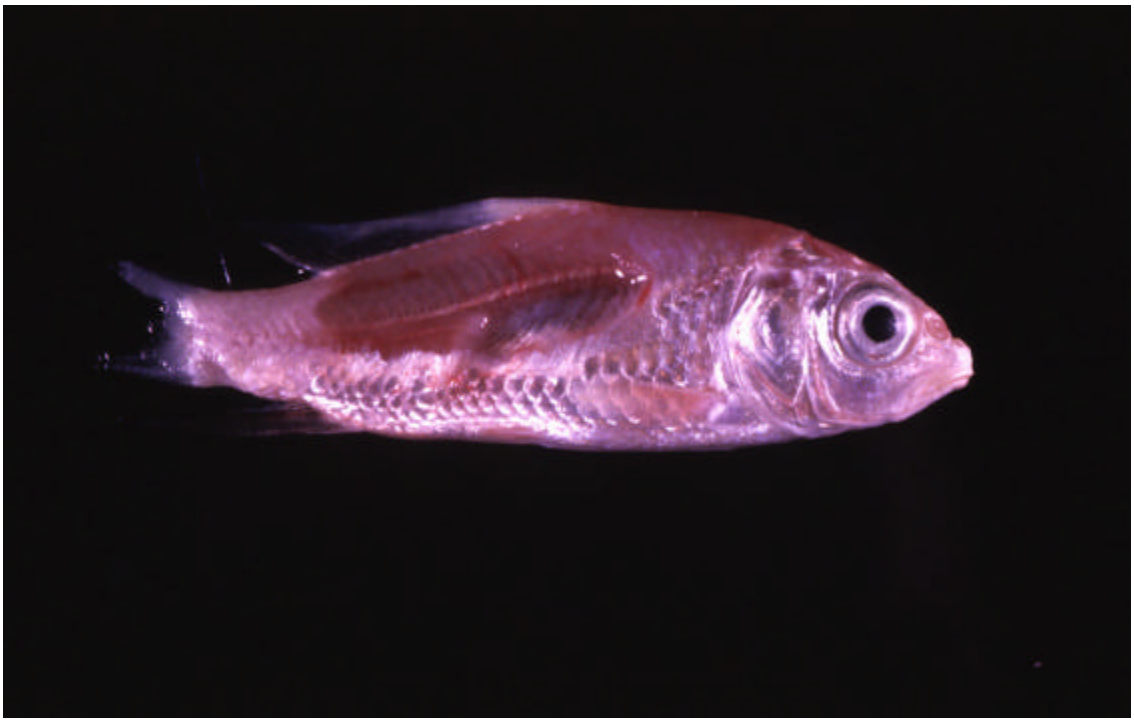

Figure 3.5 Young koi (*Cyprinus carpio*) with columnaris disease. A deep skin ulcer exposes underlying muscle and bone.

Figure 3.6 Koi (*Cyprinus carpio*) with cotton-like proliferations on the skin, tail, and fins that are suggestive of columnaris disease.

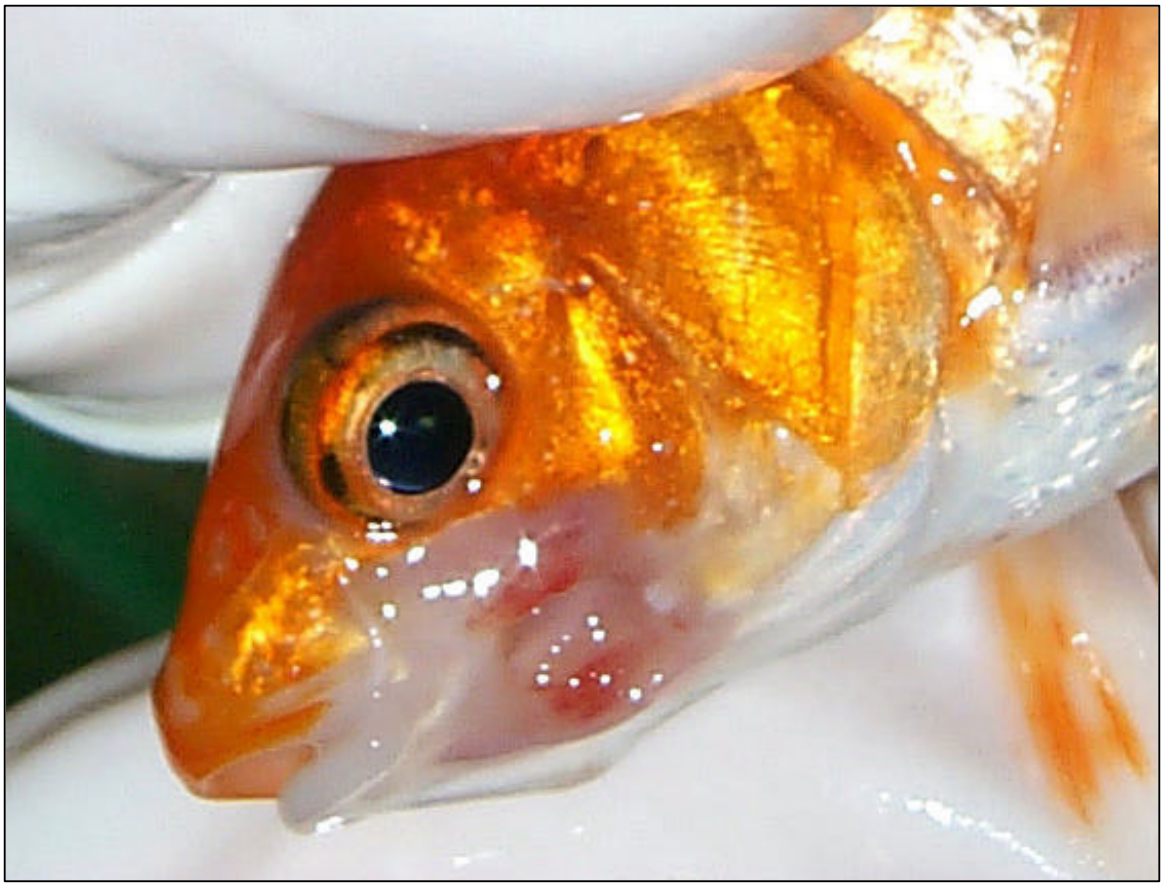

Figure 3.7 Koi (*Cyprinus carpio*) with columnaris disease. An ulcer is present on the head near the mouth.

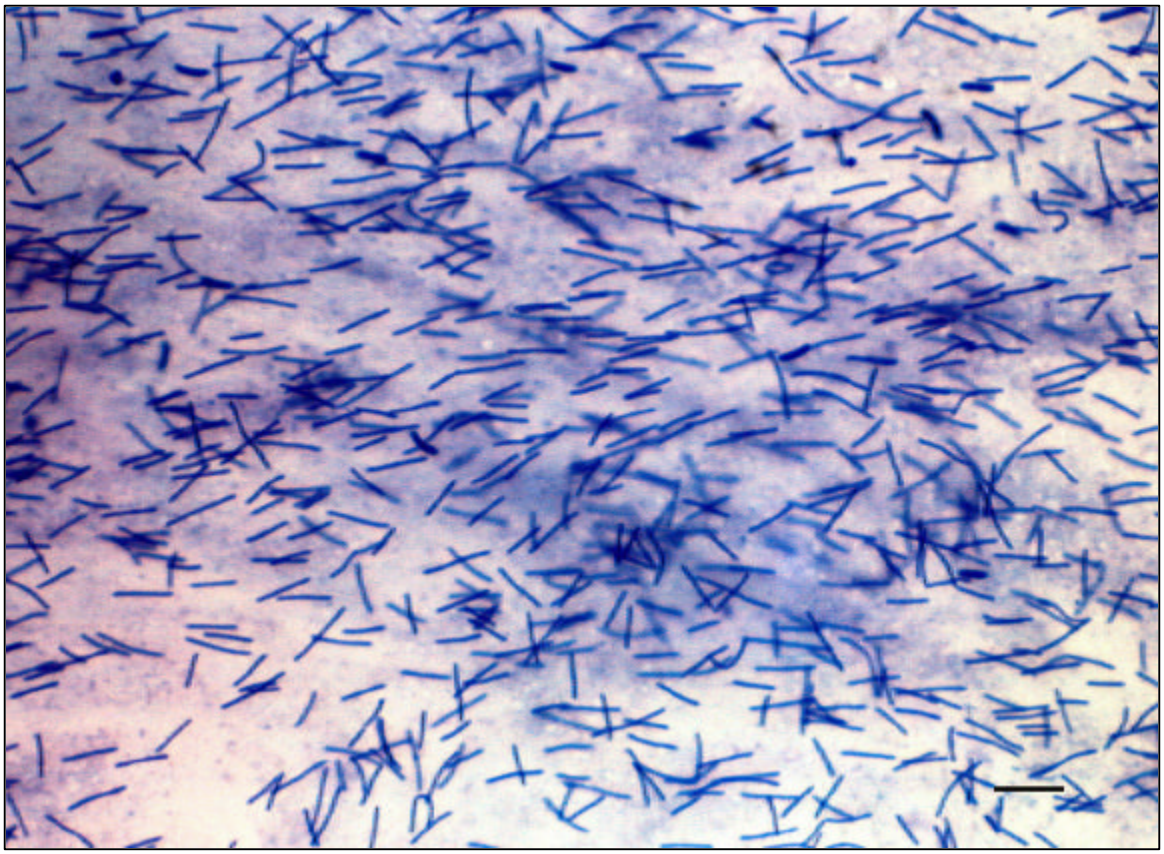

Figure 3.8 Cytology preparation of mucoid material from skin surface. *Flavobacterium columnare* appear as long, thin, blue bacilli with Romanowsky staining (bar = 10 µm).

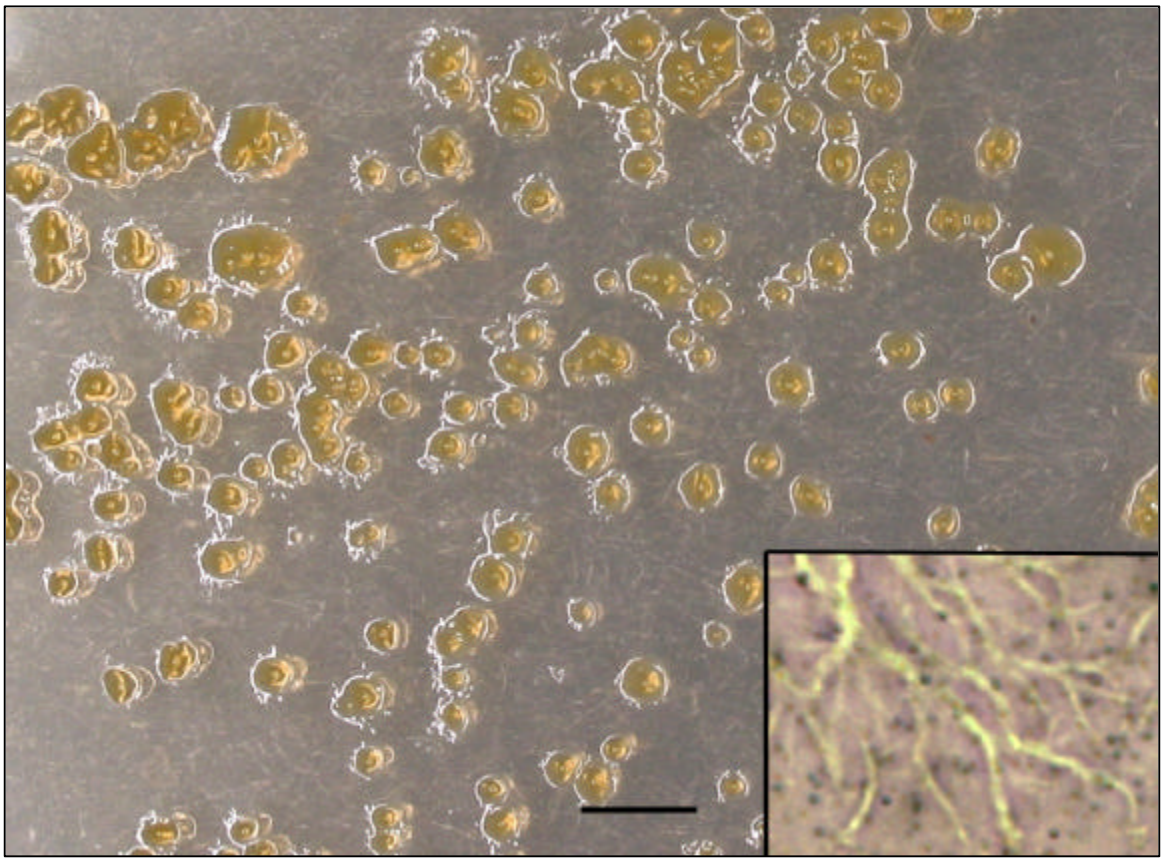

Figure 3.9 Culture of *Flavobacterium columnare* on Shieh agar. Colonies of *F. columnare* are yellow (bar = 1 mm). At higher magnification (inset, lower right), bacterial colonies have rhizoid or root-like edges.

CHAPTER 4

HEMATOLOGIC REFERENCE INTERVALS FOR KOI (*CYPRINUS CARPIO*), INCLUDING BLOOD CELL MORPHOLOGY, CYTOCHEMISTRY, AND ULTRASTRUCTURE[1]

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[1]Tripathi, N.K., K.S. Latimer and V.V. Burnley. Submitted to *Veterinary Clinical Pathology,* 6/09/2003

Background: Hematologic data are used routinely in the health care of humans and domestic mammals. Similar data for fish are largely fragmentary or have not been collected. **Objectives:** The primary purpose of this study was to derive hematologic reference intervals for koi, an ornamental strain of the common carp (*Cyprinus carpio*). Secondarily, the morphology, cytochemical reactions, and ultrastructure of koi blood cells were characterized. **Methods:** The complete blood count was performed manually on heparin-anticoagulated blood specimens using Natt and Herrick's diluent and a Neubauer-ruled hemacytometer. Leukocyte differential counts were done on Wright-Leishman-stained blood smears. Cytochemical reactions of koi leukocytes were determined using commercial kits. Transmission electron microscopy was performed to characterize the ultrastructural features of various blood cells of koi. **Results:** Hematologic reference intervals were established for koi. The parameters included packed cell volume, hemoglobin concentration, erythrocyte and leukocyte counts, erythrocyte indices, differential leukocyte counts, and absolute counts for various subtypes of leukocytes. In healthy koi, the PCV ranged from 30-34%, hemoglobin concentration ranged from 6.3-7.6 g/dl, erythrocyte counts ranged from 1.7-1.9 $x10^6/\mu l$, and leukocyte counts ranged from 30-35 $x10^3/\mu l$. Lymphocytes were the predominant leukocyte in healthy koi (accounting for up to 80% of all leukocytes), while eosinophils were rare. Cytochemical markers for koi neutrophils included acid phosphatase and peroxidase reactivity and well as positive staining for PAS reactivity and Sudan black B. Basophils were positive with PAS staining. Naphthol AS-D chloroacetate esterase activity was observed only in eosinophils. α-naphthyl butyrate esterase and β-glucuronidase activities were positive in monocytes. Some lymphocytes were reactive

for α-naphthyl butyrate esterase and acid phosphatase activity. Ultrastructurally, leukocytes, erythrocytes, and thrombocytes were identified based upon cytoplasmic organelles and granule appearance. **Conclusion:** Hematologic reference intervals and knowledge of the cytochemical reactions and ultrastructural characteristics of koi leukocytes will standardize hematologic studies in this species.

Keywords: *Cyprinus carpio*, carp, fish, koi, reference intervals, hematology, blood cell morphology, ultrastructure, cytochemistry

Hematology is a valuable routine diagnostic technique in the medical care of humans and domestic animals. Hematologic changes are often an early indicator of various pathologic changes and aid in disease diagnosis. Hematologic data often are not maximally utilized in fish medicine because of the lack of reference intervals and interpretive skills for these parameters in a wide variety of piscine species. Reference intervals are species-specific and, thus, cannot always be compared between species. Two reports have been published previously on hematologic reference ranges and morphology of blood cells of common carp.[1,2] However, these data were not from well-defined sample populations and cannot be used to construct meaningful reference intervals. The present study was performed to establish hematologic reference intervals for koi. In addition, koi blood cells also were characterized by their morphologic appearance in Wright-Leishman and Diff-Quik stained smears, cytochemical reactions, and ultrastructural features.

Materials and methods

Fish: Thirty clinically healthy koi (15-18 cm mean length and average wt 200g) were obtained from Blue Ridge Hatcheries (Kernersville, North Carolina, USA). These fish were randomly divided into three groups of 10 fish. Each group was housed in a 30-gallon glass aquarium with a flow-through water system. A carbon filtration system (US Filter Inc. Lowell, MA) was used to adsorb wastes and particulates from the water. Water temperature was regulated using a central water heater. The photoperiod averaged 10-12 hours of light per day. These fish were fed a commercial feed (Game Fish Chow, Melick Aquafeed Inc., Catawissa, PA) once daily. Chlorine and ammonia concentrations of

water were monitored daily. The koi were acclimatized for two weeks before sampling and were observed for development of clinical disease, gross lesions, or mortality each day. Visible lesions or gross abnormalities were not observed in any of the fish.

Blood collection procedure: The fish were anesthetized in aerated water containing tricaine methanesulfonate (Aquavet, Hayward, CA, USA) at a concentration of 100-125 mg/L. Blood was collected by venipuncture from ventral aspect of the caudal vein. Heparinized, sterile, disposable, 3 ml, plastic syringes and 22- to 23-gauge needles were used based on size of the fish. Heparinized 5 ml collection tubes (endotoxin-free sodium heparin, Sigma Chemical Co., St. Louis, MO, USA) were used for blood collection. Blood samples were immediately refrigerated until analyzed. When the blood was drawn, smears were prepared from Heparinized blood, air dried, and stained for the differential leukocyte count and morphologic examination. Total erythrocyte and leukocyte counts were performed within 2 hours of blood collection.

Blood cell counts: Natt and Herrick's[3] diluent was used for total erythrocyte and leukocyte counts. Total counts were done manually using an improved Neubauer-ruled hemacytometer (Bright-Line, American Opticals, Buffalo, NY) as described by Hrubec and Smith.[4] Briefly, the blood specimen was diluted 1:200 in Natt and Herrick's diluent. The hemacytometer was charged and allowed to stand for 4 minutes in a humid environment. Erythrocytes were counted in 5 secondary squares of the center primary square. The number of erythrocytes counted in the 5 secondary squares (raw count) was multiplied by 10,000 to calculate total erythrocyte count /μl. Total leukocyte counts were done at the same time by counting all leukocytes in the 4 corner primary squares. The total leukocyte count /μl was calculated by multiplying the number of leukocytes

observed in the 4 primary squares times 500 to calculate total leukocyte count /µl. To decrease the procedural variability, cells were counted in both chambers of the hemacytometer and the number was averaged to produce the raw WBC or RBC count.

Packed cell volume (PCV): The packed cell volume (%) was determined by the microcapillary centrifugation method. The microcapillary tubes were filled, plugged with clay, and centrifuged at 19,000 x g for 5 minutes. The height of the packed red cell column was expressed as a percentage of total blood column height.

Hemoglobin concentration (Hb): The hemoglobin concentration (g/dl) was done on a Baker 9000 hematology series cell counter (Baker Instrument Corporation, Allentown, PA) by the cyanmethemoglobin method. For each blood specimen, a prediluted sample was prepared by mixing 40 µl of blood with 40 µl of lysing solution. The specimen was centrifuged at 7000 rpm for 5 minutes to remove nuclear debris and cell membrane fragments. The supernatant subsequently was used for spectrophotometric determination of hemoglobin at a wavelength of 540 nm.

Erythrocyte indices: Erythrocyte indices were calculated from the total erythrocyte count, PCV, and hemoglobin concentration (Hb) using standard formulas. The calculations for mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were done as follows:

MCV (femtoliters) = PCV × 10 / RBC count (millions)

MCH (picograms) = Hb concentration × 10 / RBC counts (millions)

MCHC (g/dl) = Hb concentration × 100 / PCV

Differential leukocyte count and calculation of absolute cell counts: Fresh blood smears were prepared and stained with both Wright-Leishman and Diff-Quik stains for differential leukocyte counts and examination of blood cell morphology.
Diff-Quik and Wright-Leishman staining were performed simultaneously to discern alterations in tinctorial properties and possible stain-associated degranulation of basophils. Because of initial difficulties with preservation of basophil granules, additional blood smears also were treated with Basofix (Wescor, Logan, UT) in an attempt to stabilize the granule matrix. Smears of fresh blood without anticoagulant also were prepared. The various types of leukocytes were identified as lymphocytes, monocytes, neutrophils, basophils, and eosinophils. Absolute counts also were calculated for various leukocyte subtypes by multiplying the total leukocyte count times the percentage of a given leukocyte type.

Cytochemical staining

Cytochemical staining was performed using commercial kits (Sigma Diagnostic Inc, St Louis, MO, USA) designed for identification of human leukocytes. Similar kits previously have been used to identify leukocytes of domestic animals (dogs, cats, horse, cow, and chickens) and channel catfish.[5,6] The staining procedures performed included the following:

Peroxidase (PER) activity (Sigma procedure #390): Peroxidase activity was demonstrated following the manufacturer's recommendations. This technique is based on the interaction of phenylenediamine and catechol which form a brown-black insoluble product in presence of peroxidase. Peroxidase reactivity is observed in the granules as

diffuse black staining. Peroxidase activity usually is observed in myeloid cells including neutrophils and eosinophils.

Alkaline phosphatase (ALP) activity (Sigma procedure #86): Enzymatic activity was demonstrated using naphthol AS-BI phosphate and red violet LB salt with 2-amino-2-methyl-propendiol to produce a red, insoluble product at the site of alkaline phosphatase activity. A positive reaction is observed as red or blue (depending on the dye used) staining of cytoplasmic granules in leukocytes. Staining usually is observed in granulocytes and a subset of lymphocytes.

Sudan black B (SBB) (Sigma procedure #380): Staining to demonstrate neutrophil granules by Sudan Black B dye followed the manufacturer's directions. A positive staining reaction is visualized as black granules within the cytoplasm. Sudan black B staining is usually observed in neutrophils, eosinophils, azurophils, and occasional monocytes.

Acid phosphatase (ACP) activity (Sigma procedure #181-A): Demonstration of acid phosphatase activity is used primarily as a marker of lymphocytes. Enzymatic activity is demonstrated by a technique wherein naphthol AS-BI is released by enzymatic hydrolysis. This product couples with fast garnet GBC to form an insoluble, maroon dye at sites of ACP activity. Strong activity is observed in neutrophil granules as dark red staining. The ACP activity is tartrate sensitive in neutrophils. Tartrate resistant ACP activity has been reported in both eosinophils and basophils. Diffuse staining is seen in the cytoplasm of monocytes and focal or punctate staining is observed in the cytoplasm of lymphocytes.

Naphthol AS-D chloroacetate esterase (CAE) activity (Sigma procedure #91): CAE is referred to as specific esterase, because it is a reliable marker of human neutrophils. Naphthol AS-D chloroacetate, in the presence of diazonium salts, forms highly colored deposits at sites of enzymatic activity. This reactivity is observed in cytoplasmic granules. CAE activity is more specific for neutrophils than either PER reactivity or SBB staining.

α-Naphthyl acetate esterase (α-NAE) activity (Procedure #91): Enzymatic activity of α-NAE was demonstrated by a commercial kit. α-NAE, in the presence of diazonium salts, forms highly colored deposits at sites of cytoplasmic enzymatic activity. α-NAE also is referred to as nonspecific esterase because it will stain mammalian monocytes, T lymphocytes, and platelets. It is more sensitive but less specific than α-NBE in the identification of monocytes and T lymphocytes. Sodium fluoride inhibits α-NAE reactivity in monocytes and platelets, but does not affect staining within T lymphocytes.

α-naphthyl butyrate esterase (α-NBE) activity (Sigma procedure #181-B): α-NBE, in the presence of pararosaniline, forms a red-brown insoluble complex at sites of cytoplasmic enzymatic activity in mammals and chickens.[5] Diffuse brown-black, cytoplasmic staining also may be observed in monocytes. A subset of small T lymphocytes exhibits focal staining.

β-glucuronidase (β-G) activity (Sigma procedure #181-C): Naphthol AS-BI β-$_D$-glucuronide, in the presence of pararosaniline, forms a red, insoluble complex at cytoplasmic sites of β-G activity. Lymphocytes exhibit a focal reaction whereas monocytes have diffuse cytoplasmic staining.

Glycogen content: Glycogen content of blood cells was evaluated by the periodic acid-Schiff (PAS) reaction with and without diastase treatment. Focal to diffuse, magenta, cytoplasmic staining is observed if glycogen or glycoproteins are present. Diastase treatment degrades any glycogen and subsequent PAS staining is unreactive.

Ultrastructural studies

Blood for ultrastructural examination was collected in heparin, placed in Wintrobe tubes, and centrifuged for 10 minutes at 250 X g. After discarding the plasma, a layer of chilled (4ºC) gluteraldehyde-picric acid fixative was applied to the cells and the specimens were refrigerated overnight. Following fixation, the Wintrobe tubes were scored with a file, broken, and the fixed buffy coats were removed intact. The buffy coats were post-fixed in osmium tetraoxide, dehydrated in graded alcohols, and embedded in molten agar. Solidified agar specimens were infiltrated with plastic, thin sectioned, placed on copper grids, stained with uranyl acetate and Reynolds lead citrate, and viewed with a transmission electron microscope (JEM-1210, JOEL Ltd., Tokyo, Japan).

Statistical analyses

Results for various hematological parameters including WBC, absolute leukocyte counts, relative percentages of leukocyte types, RBC, Hb, PCV, and erythrocyte indices were analyzed by a statistical software program (JMP, Windows version 5.01, SAS Institute Inc., Cary, NC, USA). To establish the reference intervals, the data were fitted to a normal distribution. Outliers were identified using the statistical software program and

discarded. Normally distributed data were used to construct reference intervals with 95% confidence by using the 'goodness of fit' test (p = 0.05).

Results

Reference intervals: Reference intervals for the CBC are presented in Table 1. Reference intervals could not be established for eosinophil counts using a 95% confidence interval because these cells were rarely observed in Romanowsky-stained blood smears (0-2 eosinophils/smear). Because of variable aggregations of thrombocytes, neither counts nor estimates could be accurately determined.

Blood cell identification in Romanowsky-stained blood smears

Adequate cytologic details were observed by light microscopic examination of Wright-Leishman- and Diff-Quik-stained blood smears to readily identify erythrocytes, thrombocytes, lymphocytes and monocytes. Granulocytes were identified based on nuclear morphology and cytoplasmic characteristics.

Erythrocytes: On light microscopic examination, koi erythrocytes were round to oval and measured 10-12 μm in length by 3.0-4.0 μm in width. Erythrocytes had a central, round to oval nucleus and light, yellow-orange cytoplasm (Fig.1A). Erythrocyte precursors were slightly smaller in size and had round nucleus with a higher nuclear to cytoplasmic (N:C) ratio than mature erythrocytes (Fig.1B). The cytoplasm of erythroid precursors was blue and cytoplasmic basophilia decreased with maturity.

Thrombocytes: Thrombocytes were smaller than erythrocytes and measured 6-8 μm in length by 2.0-2.5 μm in width (Fig.1A). Thrombocytes typically were oval and had a

central, elongated nucleus and a rim of light gray cytoplasm. The N:C ratio was higher than that of erythrocytes.

Lymphocytes: Lymphocytes were categorized as small- (< 8μm), medium- (8-10μm) and large-sized (> 10μm) cells based on their diameter. Lymphocytes were round and had the highest N:C ratio of all leukocytes. The nuclear chromatin was condensed. Small lymphocytes had a very thin rim of basophilic cytoplasm around the nucleus (Fig. 1A). Large lymphocytes had more cytoplasmic volume than small lymphocytes. Occasionally, lymphocytes were distorted and appeared as oval to elongated cells.

Monocytes: Monocytes often were the largest leukocyte in the blood smear and ranged from 14-16 μm in diameter. Monocytes had a multilobed to indented nucleus, moderately condensed chromatin, and abundant basophilic cytoplasm (Fig.1C). A few clear, round vacuoles and fine azurophlic granules usually were present in the cytoplasm. More abundant cytoplasm, a lower N:C ratio, and cytoplasmic vacuoles helped to differentiate monocytes from large lymphocytes.

Neutrophils: Neutrophils were round and measured 10-12 μm in diameter. The nucleus was bi- to trilobed and contained moderately condensed chromatin. Neutrophils had abundant, lightly eosinophilic cytoplasm with numerous fine cytoplasmic granules (Fig.1D). The nuclear chromatin was more dispersed and fine compared to basophils.

Basophils: Basophils were less commonly observed than neutrophils. By light microscopy, these cells were round and had an average diameter of 8-10 μm. The nucleus often appeared eccentrically placed. The nuclear chromatin was more condensed than that of neutrophils. The cytoplasm was abundant, light blue-gray, and often appeared vacuolated (Fig.1E). A few purple granules were present in some basophils after

prolonged treatment in the Basofix solution and in direct smears of blood without anticoagulant.

Eosinophils: Eosinophils were observed rarely in stained blood smears. When present, eosinophils were round and had a diameter of 8-10 μm. The nucleus was irregular and contained fine, mildly condensed chromatin. The cytoplasm was moderately basophilic and contained a few large (0.5-1 μm long), rice-grain-shaped, red granules (Fig.1F).

Cytochemistry

The cytochemical staining patterns of the various leukocyte subtypes of koi are presented in Table 2. Leukocytes were uniformly unreactive with the ALP and α-NAE (nonspecific esterase) staining procedures. Small lymphocytes had focal cytoplasmic α-NBE activity (Fig.2A). Tartrate sensitive ACP activity was observed in lymphocytes. α-NBE (Fig.2B) and β-G reactivity in monocytes appeared as diffuse cytoplasmic staining. Rare PAS-positive granules were present in the cytoplasm of thrombocytes. Neutrophil granules stained positively with Sudan black B and exhibited reactivity for PER and ACP activities (Fig. 2C, D, E). Naphthol AS-D chloroacetate esterase (specific esterase), β-G and α-NBE reactivity was not observed in koi neutrophils. Basophils were unreactive in all cytochemical procedures except PAS staining. Strong CAE activity was observed in eosinophil granules (Fig. 2F), but PER activity was absent.

Electron microscopy

Erythrocytes, thrombocytes, lymphocytes, and monocytes had characteristic ultrastructural features consistent with other domestic species and were identified easily.

Granulocytes had more variable ultrastructural features and, therefore, were classified based on the characteristics of their granules.

Erythrocytes: Erythrocytes had a long, oval nucleus that was rich in heterochromatin. The cytoplasm was filled with electron dense hemoglobin (Fig.3A). A few mitochondria and profiles of endoplasmic reticulum were present in the cytoplasm of mature erythrocytes and erythroid precursors.

Thrombocytes: Thrombocytes had an elongated nucleus that was rich in heterochromatin and also contained a few strands of euchromatin (Fig.3A). The cytoplasm contained numerous microtubules and large canaliculi that appeared as clear vacuoles. Rare mitochondria, polyribosomes, and membrane-bound granules were observed in the cytoplasm. When cut in cross-section, thrombocytes had an eccentric, irregular nucleus and prominent canaliculi (Fig.3B).

Lymphocytes: The nucleus of lymphocytes had almost equal amounts of heterochromatin and euchromatin. The cytoplasm appeared dense due to presence of numerous free ribosomes. In addition, scattered mitochondria and rough endoplasmic reticulum were observed (Fig.3B). A few small (0.1-0.2 μm diameter) granules with fibrillar matrix were present in the cytoplasm. Microvilli were frequently observed on the surface of the cell membrane.

Monocytes: The nucleus was comprised of a peripheral rim and central aggregates of heterochromatin with strands of euchromatin. The cytoplasm was vacuolated and less dense than that of lymphocytes because fewer free ribosomes were present. Numerous mitochondria and profiles of rough endoplasmic reticulum were present in the cytoplasm (Fig.3C). Pseudopodia usually were present on the surface of the plasma membrane.

Neutrophils: Heterochromatin generally was arranged more peripherally in nucleus, while strands of euchromatin were more centrally oriented. The cytoplasm had numerous granules, mitochondria, and profiles of endoplasmic reticulum. Cytoplasmic granules were round to elongate and measured 0.1-0.2 μm in length (Fig.3D). These granules were composed of a homogenous matrix containing rare, thin, needle-shaped crystalloids. Basophils: The basophil nucleus had abundant heterochromatin with small strands of euchromatin(Fig.3E). Basophil granules were larger (0.2-0.4 μm diameter) than neutrophil granules and fewer in number. These granules were round to oval and had a pleomorphic core of dense crystalloid material. Scattered mitochondria, profiles of endoplasmic reticulum, numerous monoribosomes, and glycogen granules were distributed throughout the cytoplasm. Small folds were present in the cell membrane. Eosinophils: The nucleus contained primarily heterochromatin with small aggregates of euchromatin (Fig.3F). The cytoplasm was more dense than that of other granulocytes. A few villi were present on the cell membrane surface. The cytoplasmic granules were oval to elongate and measured 0.5-1.0 μm in diameter. These granules were composed of a fine granular matrix containing one or more dense, bar-shaped crystalloids. Mitochondria and rough endoplasmic reticulum were frequently observed in the cytoplasm.

Discussion

In the present study, hematologic reference intervals were established using a defined population of healthy koi (*Cyprinus carpio*). Previously published reference ranges for carp erythrocyte and leukocyte counts were based on a very small sample population and do not provide reliable reference intervals for research or clinical

purposes. Groff and Zinkl[1] reported more extensive hematologic values for common carp; however, most of their values were compiled or derived from different experiments. Thus, their data cannot provide meaningful reference intervals. Hematologic parameters frequently are affected by various intrinsic and extrinsic factors. Wlasow[7] reported significant changes in erythrocyte counts, PCV, and hemoglobin concentrations of common carp following exposure to high concentrations of ammonium chloride. Age,[8] breeding status, water temperature,[9] and oxygen content [10] frequently alter hematologic parameters. Therefore, hematologic values compiled from different experimental studies should not be combined to establish reference intervals unless the age, source, and health of the fish are identical and the husbandry practices and environment are similar.

We observed a wider reference interval for the total erythrocyte count and a narrower reference interval for the total leukocyte count compared to previously reported values for common carp.[11] Slight variation also was observed in the present reference intervals for hematocrit and erythrocyte count when compared to the data presented by Groff and Zinkl.[1] Mean corpuscular hemoglobin concentrations reported by Groff and Zinkl were markedly different than our data and possibly were miscalculated.[1] In previous publications, only the relative percentages of various leukocyte subtypes were presented; absolute values were not calculated.[1,11] In the koi of this report, higher absolute counts for various subtypes of leukocytes were present compared to a previous report for common carp.[2] The differences in values of the present study and those published previously could be due to physiological differences between koi and common carp as well as a variance in health status, husbandry practices, environmental factors, and laboratory techniques.

Human blood cells have been studied in most detail and provide a basis for the classification of similar cells in other species. Previous attempts have been made to identify and classify blood cells in many species of fish including the common carp (*Cyprinus carpio*).[11, 12] Erythrocytes, thrombocytes, lymphocytes, and monocytes usually are more readily identified by their characteristic appearance in Romanowsky-stained blood smears. Identification of nonhuman granulocytes may be more challenging. Human granulocytes are classified as neutrophils, eosinophils, and basophils based on the affinity of their granules for Romanowsky stains. In koi, granulocyte identification is based on both nuclear morphology and granule staining characteristics. All three granulocyte types have been reported previously in common carp.[12, 13] In koi, neutrophils were the most common granulocyte. These cells had a bi- to trilobed nucleus and abundant cytoplasm with fine granules that stained lightly eosinophilic with Romanowsky stains. The lightly eosinophilic tinctorial properties of the granules have sometimes led to the designation of these cells as heterophils. Heterophils are present in rabbits, rodents, birds, reptiles, amphibians, and some fishes and perform the functions of neutrophils.

Basophils were less common than neutrophils in stained blood smears. These granulocytes had a small, round to oval nucleus and a large amount of frothy cytoplasm. The vacuolated appearance of the basophil cytoplasm was interpreted to result from degranulation associated with exposure to anticoagulant and/or aqueous based stains. Granules and matrix were readily apparent in transmission electron micrographs of blood cells preserved in gluteraldehyde. Fine, purple-violet granules were observed in basophils by light microscopy when smears were made from freshly drawn blood without anticoagulant and prior fixation with Basofix. Basophils in blood smears treated with

Basofix also retained a few purple granules. This latter reagent is used primarily to improve the fixation of basophil granule matrix and prevent their dissolution during aqueous-based staining. Further studies should be performed to determine the best method to preserve the basophil granule matrix for successful Romanowsky staining.

Eosinophils were the third type of granulocyte, and were rarely observed in stained blood smears. Eosinophils had an oval to irregular nucleus with a moderate number of large, rice grain-shaped, red granules. The cytoplasm was abundant and light blue. Both Wright-Leishman and Diff-Quik staining were adequate for leukocyte identification; however, Wright-Leishman staining revealed neutrophil granules slightly better and resulted in less stain-induced degranulation of basophils.

Leukocytes also have been characterized by their cytochemical staining patterns. The commercial cytochemical staining kits used in this study were designed to identify human leukocytes, but have been used successfully to identify leukocytes in various domestic and exotic animals including catfish.[5] Because tremendous variability exists in the enzyme content of leukocytes among species of fish, cytochemical staining alone often cannot be used to identify piscine leukocytes. In the present study, some small lymphocytes of koi had focal or punctate cytoplasmic reactivity for α-NBE while monocytes had diffuse cytoplasmic staining. These observations are similar to α-NBE activity exhibited by T lymphocytes and monocytes of dogs, cats, horses, cattle, and chickens.[5] The α-NBE positive lymphocytes in koi may be similar to T lymphocytes of domestic animals that exhibit focal cytoplasmic reactivity. α-NBE reactivity has been reported previously in T lymphocytes and monocytes of channel catfish (*Ictalurus punctatus*).[5] Enzymatic activity in monocytes is inhibited by sodium fluoride treatment,

but that of T lymphocytes is unaffected. Sodium fluoride may inhibit α-NBE enzyme activity on the plasma membrane of monocytes but may not reach the intracellular organelles of lymphocytes. Blood lymphocytes of koi are unreactive for βG activity but diffuse β-G reactivity is observed in koi monocytes. β-G reactivity has only been reported in lymphocytes and monocytes of cats and neutrophils of channel catfish. [5]

Strong peroxidase (PER) activity was observed in koi neutrophil granules, as has been described previously for heterophils of common carp.[14] PER activity can be considered an identifying feature of neutrophils; neutrophils are PER positive but heterophils of chickens, yellow rat snakes, alligators, and green sea turtles are PER negative.[5] Sudan black B staining and acid phosphatase (ACP) activity were observed in koi neutrophils. The ACP reactivity was sensitive to tartrate inhibition. Sudan black B staining and ACP activity also have been reported in channel catfish neutrophils.[5] Sudan black B staining and ACP reactivity may be observed within neutrophils of dogs, cats, cattle, and horses.[5] Based on cytochemical reactivity, neutrophils of koi could be classified confidently by cytochemistry. In contrast to dogs, cats, cattle, and horses, neutrophils of koi were negative for CAE activity.[5]

Basophils of koi were negative for most of the cytochemical stains, except PAS staining. Peroxidase-negative basophils have been described in carp,[14] while PER- and alkaline phosphatase-negative basophils have been observed in the blood of goldfish (*Carassius auratus*).[15]

Eosinophil granules were strongly positive for CAE activity. CAE positive eosinophils also have been reported in the green sea turtle.[5] Generally, CAE activity is more specific but less sensitive for neutrophils of mammals when compared to PER and

SBB staining. However, these staining patterns are not consistent because channel catfish neutrophils also are negative for CAE reactivity.[5] Unlike human eosinophils, PER activity was not observed in koi eosinophils. Feline eosinophils also fail to stain with the PER and SBB techniques.[5] Less PER reactivity has been reported in goldfish eosinophils than in neutrophils.[15]

Ultrastructurally, koi erythrocytes had an oval nucleus with clumped chromatin, electron-dense hemoglobin, scattered mitochondria, and rough endoplasmic reticulum. The presence of organelles differed from mammalian erythrocytes. Immature erythrocytes of koi were slightly smaller than mature erythrocytes and contained more mitochondria and ribosomes. These cytoplasmic organelles were depleted with increasing cellular maturation. [10]

Imagawa and coworkers. studied the ultrastructural morphology of blood cells in common carp and subclassified the granulocytes into three classes designated as granulocyte types I, II, and III.[11] This subclassification was based on the ultrastructural characteristics of the cytoplasmic granules. Koi neutrophils in the present study had round to oval, membrane-bound, electron-dense granules similar to those described previously in the neutrophils of common carp.[12,13] Koi basophils had round granules with crystalloid cores. These cells were ultrastructurally similar to basophils of common carp that have electron–dense granules with a crystalloid core.[13] Eosinophil granules had bar-shaped crystalloids similar to those described in human eosinophils.[16] Similar granulocytes with rod-shaped granules were observed rarely in the pronephros of common carp and these cells were called "rodlet cells."[13]

Thrombocytes can be differentiated from small lymphocytes by the presence of characteristic vesicles (canaliculi), microtubules, lower nuclear to cytoplasmic ratio, and less dense cytoplasm. A surface connected canalicular system, similar to that of birds and amphibians, also has been described in carp thrombocytes.[17]

Compared to humans or other domestic animals, it is more challenging to study piscine hematology because of the presence of nucleated erythrocytes and thrombocytes. In fish, blood is collected most commonly from the caudal vein. Venipuncture is performed through the ventral midline musculature of the caudal peduncle. During venipuncture, tissue factor contaminates the blood specimen and may stimulate thrombocyte activation or clotting. Therefore, an increased concentration of anticoagulant generally is required to prevent blood clotting in fish blood as compared to blood specimens from other domestic animals. Heparin appears to be the anticoagulant of choice for koi because some blood samples turn brown and viscous when exposed to EDTA as is the case with blood from some avian species such as ratites, jays, and thrushes.[18] However, heparin is known to interfere with Romanowsky staining and, when used in large quantity, may also leave a pink film on blood smears following Romanowsky or Diff-Quik staining.

Erythrocyte and leukocyte counts can be performed manually using a hemacytometer but this procedure is labor intensive and time consuming. The manual counting procedure has 10% or more inherent error. Also, differentiation of thrombocytes from small lymphocytes is very difficult on the hemacytometer. An attempt was made to differentiate these two cell types by performing the counts at higher magnification (40X objective) compared to the usual practice of enumerating cells under low magnification

(10X). Small lymphocytes are distinguished from thrombocytes more reliably on stained blood smears. The cytoplasm of lymphocytes appears light to deep blue while the cytoplasm of thrombocytes is light gray. Automated cell counts are more accurate in mammals, but impedance counters cannot provide clinically useful cell counts in fish, except perhaps for erythrocytes. Newer generation automated counters use photometry to identify erythrocytes, thrombocytes, and various leukocyte subtypes based on size, density, and internal complexity of the cell, including granularity of the cytoplasm and lobulation of the nucleus.

In summary, reference intervals were established for koi that can be used to interpret hematologic data in this species when they are kept under similar conditions. Koi leukocytes also were characterized based upon their morphologic appearance in Wright-Leishman and Diff-Quik stained blood smears, cytochemical reactions, and ultrastructural features.

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References

1. Groff JM, Zinkl JG. Hematology and clinical chemistry of cyprinid fish. *Vet Clin North Am: Exotic Animal Pract* 1999; 2:741-776.

2. Rehulka J. Blood parameters in common carp with spontaneous spring viremia (SVC). *Aquacult International* 1996;4:175-182.

3. Natt MP, Herrick CA. A new blood diluent for counting the erythrocytes and leukocytes of the chicken. Poult Sci 1952;31:735-738.

4. Hrubec TC, Smith AS. Hematology of fish In: *Schalm's Veterinary Hematology*. 5th ed. Baltimore, MD: Lippincott Williams & Wilkins; 2000: 1120-1125.

5. Raskin RE, Valenciano A: Cytochemistry of normal leukocytes. In: *Schalm's Veterinary Hematology*. 5th ed. Baltimore, MD: Lippincott Williams & Wilkins; 2000: 337-346.

6. Hanson LP, Ainsworth AJ. Differential cytochemical staining characteristics of channel catfish leukocytes identify cell populations in lymphoid organs. *Vet Immunol Immunopathol* 2000;73:129-144.

7. Wlasow T, Dabrowska H. Hematology of carp in acute intoxication with ammonia. *Pol Arch Hydrobiol* 1990; 37:419-428.

8. Speckner W, Schindler JF, Albers C. Age-dependent changes in volume and haemoglobin content of erythrocytes in the carp (*Cyprinus carpio* L.). *J Exp Biol* 1989;141:133-149.

9. Hrubec TC, Robertson JL, Smith SA. Effect of temperature on hematologic and serum biochemical profiles of hybrid striped bass (*Morone chrysops* × *Morone* saxatilis). Am J Vet Res 1997;58:126-130.

10. Rothamann C, Levinshal T, Timan B, Avtalion RR, Malik Z. Spectral imaging of red blood cells in experimental anemia of *Cyprinus carpio*. *Comp Biochem and Physiol* A 2000; 125: 75-83.

11. Imagawa T, Hashimoto Y, Kitagawa H, Kon Y, Kudo N, Sugimura M. Morphology of blood cells in carp (*Cyprinus carpio* L.). *Jpn J Vet Sci* 1989; 51:1163-1172.

12. Ainsworth AJ. Fish granulocytes: Morphology, distribution and function. *Annual Rev of Fish Diseases* 1992;123-148.

13. Temmink JMH, Bayne CJ. Ultrastructural characterization of leukocytes in the pronephros of carp (*Cyprinus carpio* L.). *Dev Comp Immunol* 1987;11:125-137.

14. Bielek E. Developmental stages and localization of peroxidase activity in leukocytes of three teleost species (*Cyprinus carpio* L.; *Tinca tinca* L.; *Salmo gairdneri* Richardson). Cell Tissue Res 1981;220:163-180.

15. Garavini C, Martelli P. Alkaline phosphatase and peroxidase in goldfish (*Carassius auratus*) leukocytes. Bas Appl Histochem 1981;25:133-139.

16. Steffens WL. Ultrastructural Features of Leukocytes In: *Schalm's Veterinary Hematology*. 5th ed. Baltimore, MD: Lippincott Williams & Wilkins; 2000: 326-336.

17. Daimon T, Mizuhira V, Takahashi I, Uchida K. The surface connected canalicular system of carp (C*yprinus carpio*) thrombocytes: Its fine structure and three-dimensional architecture. *Cell Tissue Res* 1979;203:355-365.

18. Latimer KS, Bienzle D: Determination and interpretation of the avian leukogram. In: *Schalm's Veterinary Hematology*. 5th ed. Baltimore, MD: Lippincott Williams & Wilkins; 2000: 417-432.

Figure 4.1 Morphology of koi blood cells under light microscope. A. Erythrocyte is in the center and is a large elliptical cell with an elongated nucleus; thrombocyte is smaller than erythrocyte (on the right) with an elongated nucleus and light gray cytoplasm; small lymphocyte (on the left) with round nucleus and deep blue cytoplasm, B. Erythrocyte precursor (on the left) with round nucleus, less clumped chromatin and blue cytoplasm as compared to mature erythrocyte on the right. C. A monocyte with large lobulated nucleus and deep blue cytoplasm. D. Neutrophil with bilobed nucleus and abundant light pink cytoplasm(Wright's). E. Basophil with small eccentric nucleus and abundant frothy cytoplasm F. Eosinophil with large indented nucleus with moderately dispersed chromatin and light blue cytoplasm containing large, pink, rice-shaped granules (Wright's). (Diff-Quik stain unless mentioned) X1000.

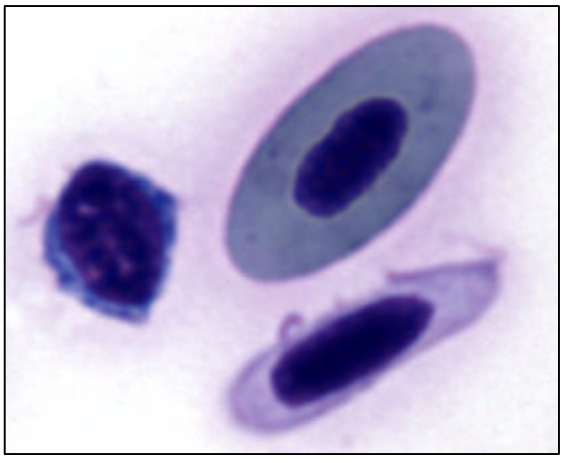
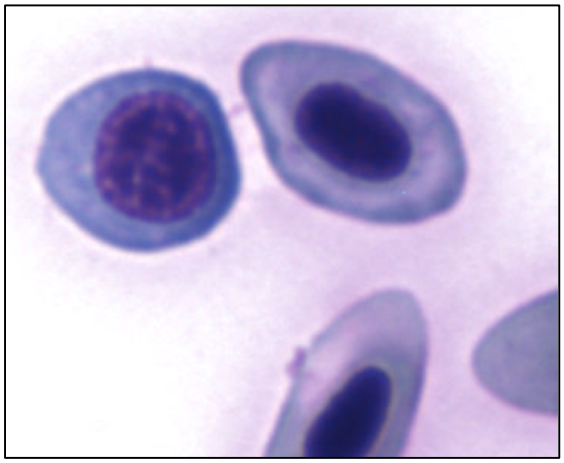
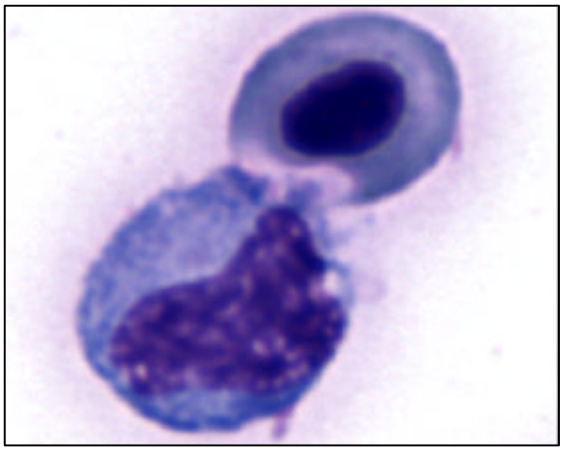
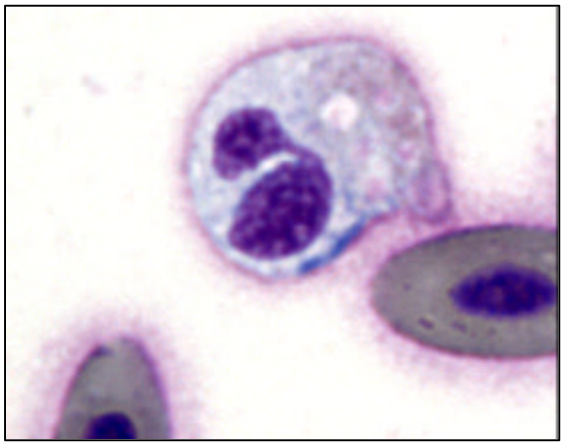
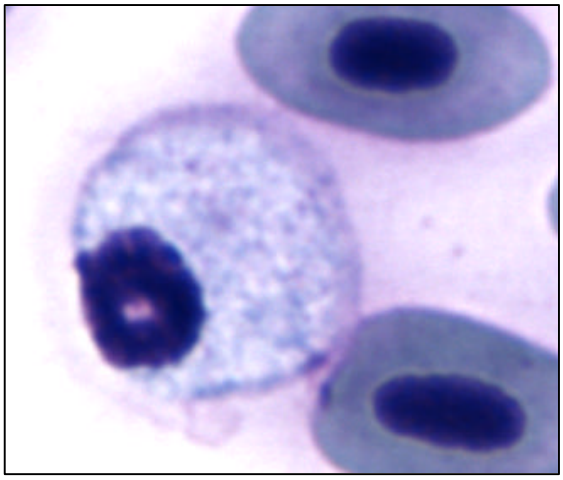
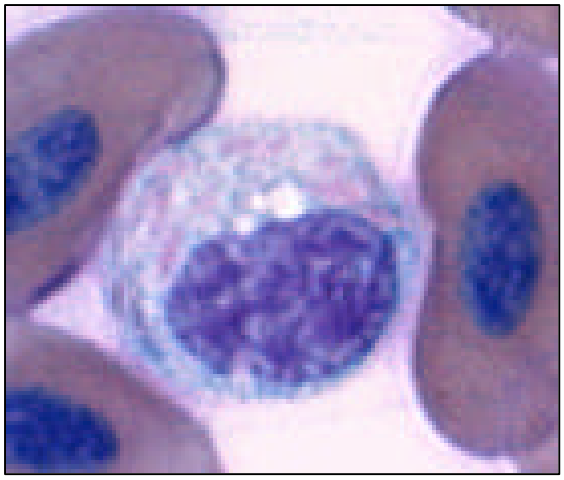
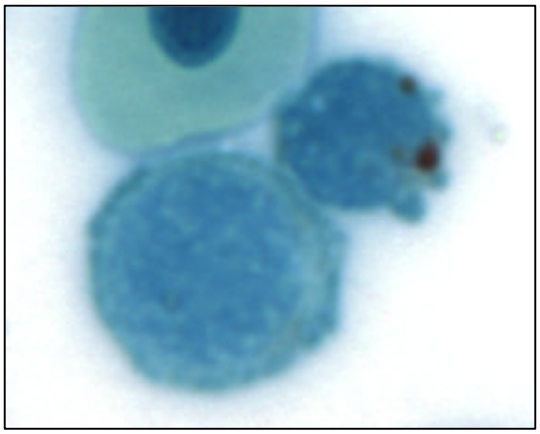
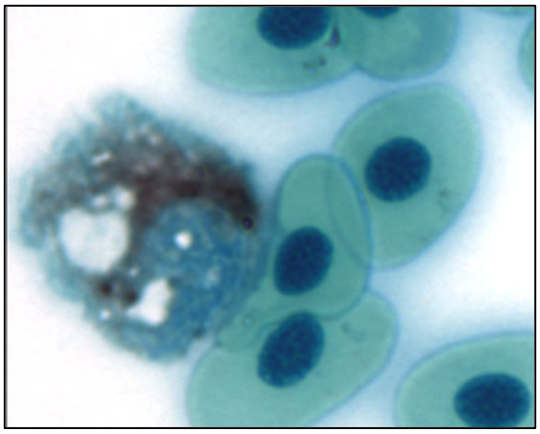
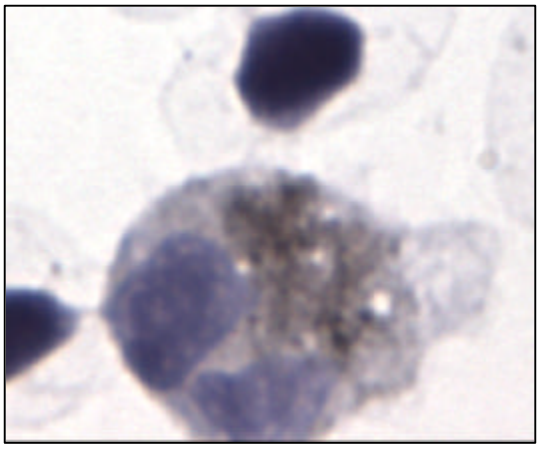
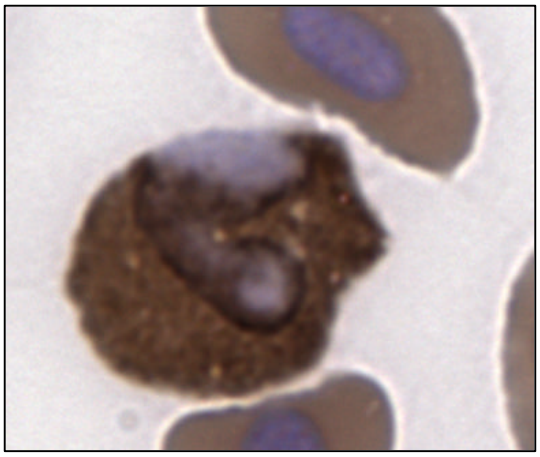







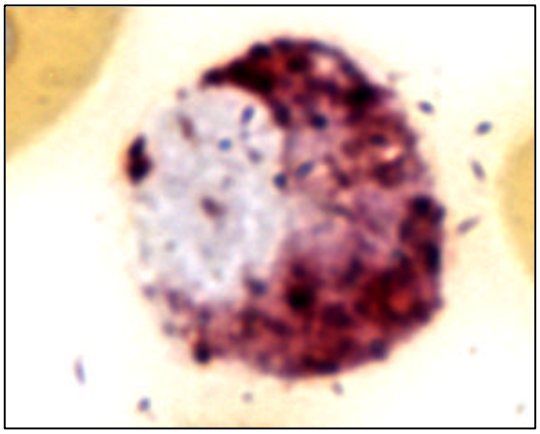
Figure 4.2 Cytochemical staining patterns of koi leukocytes. A. Focal staining in a small lymphocyte due to localized α-naphthyl butyrate esterase activity, B. Diffuse staining in cytoplasm of a monocyte showing α-naphthyl butyrate esterase activity, C. Sudan black B staining of granules in neutrophil cytoplasm, D. Peroxidase reactivity in neutrophil granules, E. Acid phosphatase activity in neutrophil granules, F. Naphthol AS D-chloroacetate esterase activity in granules of an eosinophil. X1000.











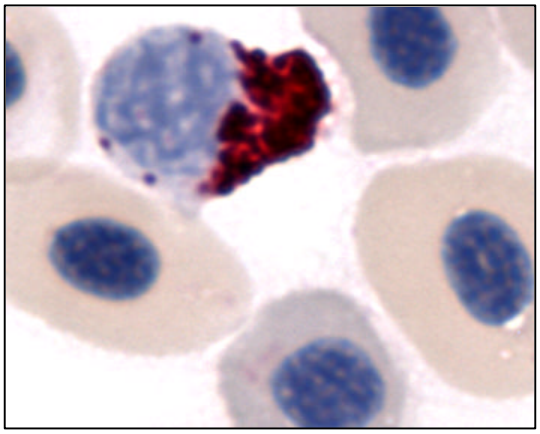


Figure 4.3 Ultrastructure of koi blood cells. A. An erythrocyte with dense cytoplasm and a thrombocyte with an elongated nucleus and cytoplasmic microtubules (magnification 10K, bar 1 ì m. B. Canaliculi are visualized as clear, membrane bound vacuoles in a cross section of a thrombocytes (left), a small lymphocyte (right) with a thin rim of cytoplasm containing a few mitochondria (magnification 10K, bar 1 ì m) C. A monocyte with large amounts of vacuolated cytoplasm containing scattered mitochondria and endoplasmic reticulum (magnification 10K, bar 1 ì m). D. Neutrophil with lobulated nucleus and numerous, small cytoplasmic granules (magnification 10K, bar 1 ì m). E. Basophil with numerous monoribosomes and fewer cytoplasmic granules with crystalloid cores (magnification 12K, bar 500 nm). F. An eosinophil with dense cytoplasm, scattered mitochondria, and large rice-grain shaped granules that have characteristic needle-shaped crystalloid (magnification 15K, bar 400 nm).

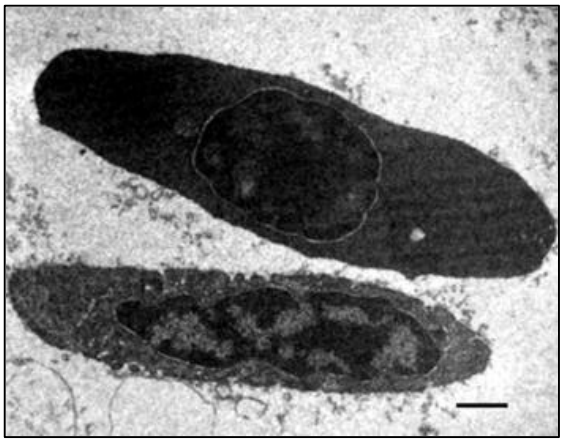
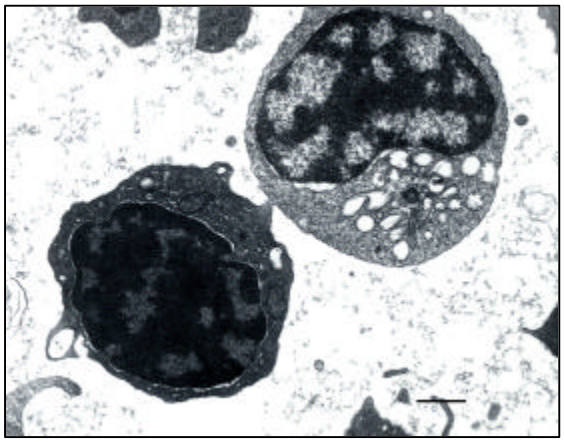
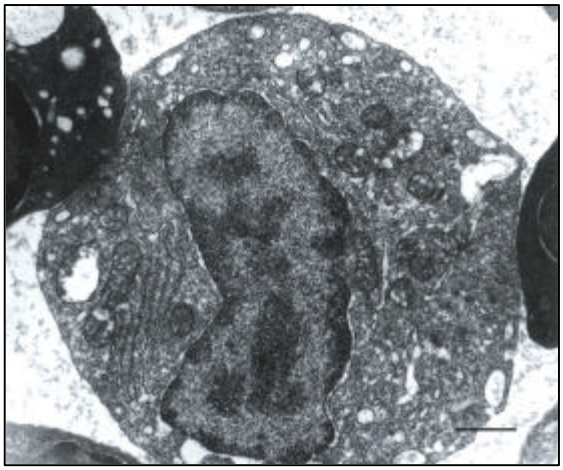
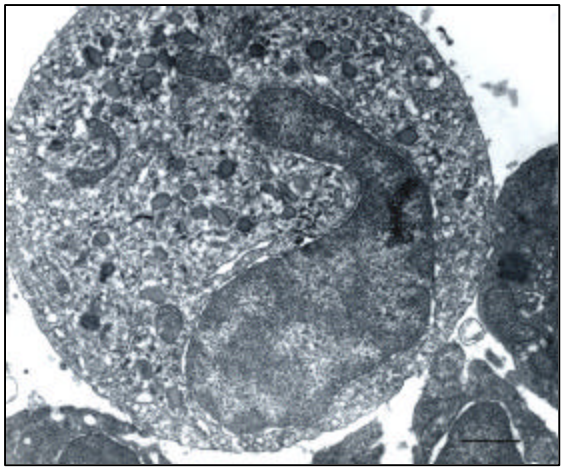
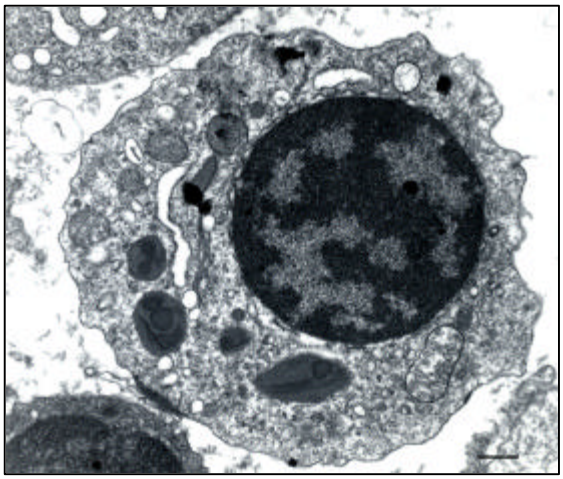
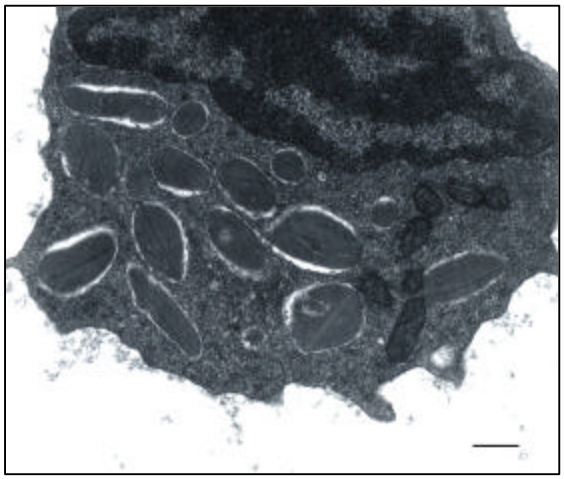







Table 4.1 Hematologic reference intervals for koi (*Cyprinus carpio*)

Parameter	N=	Mean	Std Dev	CI 95%	Prob<W
PCV %	30	31.8	5.5	29.73-33.86	0.12
Hb (g/dl)	30	6.94	1.6	6.32-7.55	0.62
RBC ($x10^6$/ul)	30	1.81	0.2	1.69-1.91	0.21
MCV (fl)	30	178.2	31.7	166.3-190	0.8
MCH (pg)	28	40.2	6.5	37.7-42.7	0.11
MCHC (g/dl)	30	21.6	3.3	20.4-22.9	0.05
WBC ($x10^3$/ul)	29	24	5.6	19.8-28.1	0.14
Lymphocyte %	30	79.1	12.4	74.5-83.7	0.1
Lymphocyte ($x10^3$/ul)	30	17.6	5.4	14.7-23.5	0.07
Monocyte %	22	2.9	1.3	2.3-3.4	0.05
Monocyte ($x10^3$/ul)	28	0.66	0.3	0.46-0.96	0.12
Neutrophil %	29	10.9	7.7	7.96-13.89	0.05
Neutrophil ($x10^3$/ul)	30	2.44	1.4	1.57-3.9	0.07
Basophil %	25	4.6	2.6	3.5-5.6	0.006
Basophil ($x10^3$/ul)	28	1.05	0.7	0.69-1.57	0.06

Table 4.2 Cytochemical staining reactions of koi leukocytes

	Neutrophil	Basophil	Eosinophil	Lymphocyte	Monocyte
Acid phosphatase	+	-	-	+	+
Alkaline phosphatase*	-	-	-	-	-
α-naphthyl acetate esterase*	-	-	-	-	-
α-naphthyl butyrate esterase	-	-	-	+/-	+
β-glucuronidase	-	-	-	-	+
Naphthol AS D-chloroacetate esterase	-	-	+	-	-
Periodic acid-Schiff stain	+	+	-	+	+
Peroxidase	+	-	-	-	-
Sudan black B	+	-	-	-	-

* Alkaline phosphatase and α-naphthyl acetate esterase stains were unreactive with all koi leukocytes tested.

CHAPTER 5

BIOCHEMICAL REFERENCE INTERVALS FOR KOI (*CYPRINUS CARPIO*) [1]

[1]Tripathi, N.K., K.S. Latimer, T.T. Lewis and V.V. Burnley. Submitted to *Comparative Clinical Pathology*, 1/31/2003

Abstract

Various biochemical parameters of koi were measured to construct biochemical reference intervals. Very low serum concentrations of creatinine, total bilirubin, uric acid and γ-glutamyl transferase activity were observed in koi. Increased activities of creatine kinase, lactate dehydrogenase, and aspartate aminotransferase were observed due to venipuncture through the skeletal muscle of the caudal peduncle; however, these changes were not statistically significant. Alanine aminotransferase and sorbitol dehydrogenase activities were affected the least and these enzymes may be considered relatively liver specific in koi. Cellulose acetate serum protein electrophoresis also was performed to confirm the presence of a serum albumin-like protein in koi. However, the resultant protein fractions were highly variable and the albumin protein-like fraction did not correlate well with that determined by the BCG dye binding assay.

Keywords: *Cyprinus carpio*, carp, fish, koi, reference intervals, serum chemistry, serum electrophoresis.

Introduction

Within the past few decades, commercial production of koi (an ornamental strain of the common carp, *Cyprinus carpio*) has emerged as a major segment of the pet industry with prices of individual koi ranging from a few dollars to $250,000.00. In 1992, the value of international trade in the ornamental fish industry was estimated at 247 million dollars based on import statistics and 140 million dollars based on export statistics. The ornamental fish trade in the United States was responsible for 26% of all imports and 11% of all exports in freshwater and marine fishes. Approximately 1 billion fish are exported annually, most of which are mass-cultured fish species such as guppies, tetras, angelfish, swordtails, platys, goldfish, and koi (Cheong, 1996). In 2001, the total value of ornamental fish farm sales in the United States was approximately 21 million dollars with total exports of 1.8 million dollars (Anonymous, 2003). Despite the individual value of koi, little research has been done to establish biochemical reference intervals for this species to improve routine healthcare.

Biochemical analysis is an essential component of disease diagnosis in human and veterinary medicine. Many pathologic changes are reflected in serum or plasma well before development of clinical disease. Thus, the biochemical profile may be a useful ancillary diagnostic technique. Unfortunately, serum biochemical testing is not used commonly as a diagnostic tool in piscine medicine because of expense and a lack of suitable reference intervals for interpretation of clinical laboratory data. Because of the value of many koi and concerns about their health as companion animals, a greater awareness of medical diagnosis and treatment exists. The present study was conducted to establish selected biochemical reference intervals for koi.

Materials and methods

Fish: Twenty clinically healthy adult koi were obtained from Blue Ridge Hatcheries in Kernersville, North Carolina. These fish measured 15-18 cm in length and had an average body weight of 200 gm. These fish were randomly assigned to one of four groups, each of which was housed in a 30-gallon glass aquarium with a temperature controlled flow-through water system. Because pH and hardness of the water was of consistent quality, only ammonia and chloride concentrations were monitored daily. The photoperiod ranged from 10-12 hours of light per day. The fish were fed a commercial, pelleted diet once a day (Game Fish Chow, Melick Aquafeed Inc., Catawissa, PA). The fish were acclimated for two weeks prior to the initiation of the study. During the acclimatization and study periods, the fish were observed daily for any clinical signs of disease including inappetence, lethargy, increased opercular movements, or visible lesions of the skin, tail, and fins. Signs of disease were not apparent during either period of observation.

Blood Collection Procedure: To facilitate venipuncture and blood collection, the fish were netted quickly and transferred to a 10-gallon aquarium containing aerated water with an aqueous anesthetic agent (MS-222, tricaine methanesulfonate, Aquavet, Hayward, CA). Once the fish were immobilized, they were removed from the aquarium and placed on a level surface. Venipuncture was performed rapidly using 20- or 22-gauge needles attached to 3 ml plastic disposable syringes. The blood specimen was collected from the caudal vein using a ventral approach through the midline of the caudal peduncle. After removing the needle, blood was transferred from the syringe into a sterile glass tube and allowed to clot for 15 minutes at room temperature. Subsequently, the clots were rimmed with an applicator stick and the specimen was centrifuged at 2,000

rpm for 10 minutes. The serum was collected and stored at 4°C until analyzed. Laboratory analysis was performed within 1-2 hours of collection. Following venipuncture, the fish were placed in a recovery aquarium with aerated water until completely recovered from the effects of anesthesia. The fish were then returned to their original aquarium.

To determine the effect of venipuncture through the musculature of the caudal peduncle on selected serum chemistry parameters, blood specimens were collected from five additional fish after intentional passage of the needle through the musculature. After venipuncture was performed, 1 ml of blood was drawn initially (muscle contamination specimen) and a fresh syringe was then placed on the needle. An additional 1 ml blood sample (control specimen) was obtained. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), sorbitol dehydrogenase (SDH), and creatine kinase (CK) were compared in the two sets of sera.

Serum Chemistry Analyses: Serum specimens were analyzed by automated methods and standardized protocols (Hitachi 912 Analyzer and Reference Manual, Boehringer Mannheim, Indianapolis, IN, USA). The serum parameters that were evaluated included the concentrations of total serum protein, albumin, glucose, creatinine, serum urea nitrogen (SUN), uric acid, total bilirubin, cholesterol, bile acids, sodium, potassium, chloride, calcium, magnesium, phosphorus, and total CO_2 . The activities of selected enzymes also were evaluated including ALT, AST, LDH, SDH, alkaline phosphatase (ALP), γ-glutamyl transferase (γ-GT), and CK.

Total serum protein was determined by the biuret reaction. Albumin-like protein was quantitated by bromcresol green (BCG) dye binding. Globulin concentration and

albumin to globulin (A:G) ratios ratio were calculated mathematically. To further evaluate albumin and globulin concentrations, cellulose acetate electrophoresis was performed. Glucose concentration was determined by the glucose hexokinase method, using a coupled reaction at 37°C with an endpoint reading at a wavelength of 340 nm. Serum urea nitrogen (SUN) concentration was determined by the method of Talke and Schubert using a coupled urease / glutamate dehydrogenase kinetic assay system with measurement in the ultraviolet wavelength range. Creatinine concentration was determined by Jaffe's method, a colorimetric technique resulting in the development of a yellow-orange colored complex in the presence of picric acid. Uric acid was determined using the endpoint colorimetric method of Town *et al* wherein uric acid is oxidized by uricase to form allantoin and hydrogen peroxide. An endpoint, colorimetric, modified diazo method was used for determination of total bilirubin. Cholesterol was determined by Liebermann's method, an endpoint coupled reaction in which cholesterol esters are hydrolyzed into free cholesterol and fatty acids by a microbial cholesterol esterase. Concentrations of bile acids were determined by an enzymatic reaction in which the bile acids were oxidized to 3-oxo bile acids with the subsequent generation of nicotinamide adenine dinucleotide (NAD) and reduction of nitroblue tetrazolium salt to formazan that was measured at a wavelength of 530nm. Concentrations of sodium, potassium, and chloride were determined by liquid/liquid interface ion-selective electrodes. Direct calcium concentration was determined by modified method of Sarkar and Chauhan. Magnesium concentration was determined by an endpoint assay based on the reaction of magnesium with xylidyl blue in an alkaline medium. Calcium masking in the reaction was accomplished using EGTA (ethylene glycol bis (2-aminoethyl ether)-N,N,N',N'-

tetraacetic acid), a calcium chelator. Inorganic phosphorus was determined by an endpoint method with sample blanking using ammonium molybdate reagent. Total CO_2 was measured by a coupled reaction in which bicarbonate reacts with phosphoenolpyruvate to produce oxaloacetate that acts as a hydrogen ion acceptor with the subsequent reduction of NADH to NAD^+.

All serum enzymatic activities were determined at 37°C. ALT, AST, LDH, and SDH activities were measured as indicators of hepatocellular damage and enzyme release. ALT activity was measured by a modification of the kinetic method of Wroblewski and LaDue using a coupled reaction with the ultimate generation of pyruvate and consumption of NADH. AST activity was determined by modifications of the method of Karmen, *et al*. This kinetic procedure uses a coupled reaction that generates oxaloacetate with the consumption of NADH. LDH was determined by a modification of the procedure of Gay, *et al* using Tris buffer (tris-hydroxymethyl-aminomethane buffer). This kinetic reaction involves the conversion of L-lactate to pyruvate with the formation of NADH. SDH activity was determined as a kinetic reaction wherein the generation of NAD was measured at an ultraviolet wavelength. Both ALP and γ-GT activities were determined as examples of "induction" enzymes. ALP activity was determined by the kinetic method of Hausamen, *et al* using p-nitrophenyl phosphate hydrolysis with the liberation of p-nitrophenol. γ-GT activity was determined in a kinetic procedure by the method of Szasz wherein the rate of release of 5-amino-2-nitrobenzoate is proportional to enzymatic activity. CK activity was assayed by Oliver's kinetic method using creatine phosphate and ADP as substrates with the subsequent generation of NADPH.

Serum Protein Electrophoresis: Electrophoresis separates serum proteins into different bands based on charged distribution. Cellulose acetate plates (Titan III, Helena Laboratories, Beaumont, TX, USA) were individually identified and soaked for 15 minutes in tris-barbital-sodium barbital buffer (pH 8.6 to 9.0). The plates were blotted and 3 µl serum samples were applied to the cellulose acetate plate using a sample applicator. The cellulose acetate plates were electrophoresed for 15 minutes at 180 volts. Subsequently, the plates were placed in Ponceau S stain for 5 minutes and treated with clearing solution. After drying for 5 minutes at 50-60°C, the stained plates were scanned in a densitometer (Cliniscan 2, Helena Laboratories, Beaumont, Texas,USA) using a 525 nm filter to obtain the relative concentrations (%) of the albumin and various globulin peaks. Specific "albumin" and globulin concentrations were determined by multiplying the relative percentage of the "albumin" or globulin peak by the total protein concentration as determined by the biuret reaction. In this study, the most anodal peak was assumed to be albumin-like protein.

Statistical analyses

Results for routine biochemical testing were analyzed using JMP statistical software program (Windows version 4.0, SAS Institute Inc., Cary, NC, USA). To establish the reference intervals, the data were fitted to a normal distribution. Any outliers were identified and discarded using the statistical software program. Reference intervals were constructed for different parameters with 95% confidence by using a 'goodness of fit' test applied to the normally distributed data.

To determine the effect of muscle enzyme contamination during venipuncture and blood collection, the two sets of data were analyzed by one-way analysis of variance (ANOVA) and comparisons between group means were made by t-test at a significance level of $p = 0.05$.

One-way ANOVA also was used for analysis of the two sets of albumin values derived by BCG dye binding and by cellulose acetate electrophoresis. These two sets of data subsequently were compared by t-test at the significance level of $p = 0.05$.

Results

Reference intervals for various serum chemical parameters are summarized in Table 1. Creatinine, total bilirubin, uric acid concentrations and γ-GT activity have very low values in healthy koi compared to those of mammals. The uric acid concentrations were often unmeasurable and the data appeared skewed.

Changes in different enzymatic activities caused by muscle penetration during venipuncture are presented in Table 2. Inspection of individual test values indicates that the most prominent changes were observed in creatine kinase activity. Moderate increases in LDH and AST activities also were observed in two of five samples. However, these changes in enzyme activity were not statistically significant when the group means were compared by t-tests at the $p = 0.05$ level.

Cellulose acetate electrophoresis was performed to confirm the presence of serum albumin-like protein and to compare “albumin” concentrations determined by electrophoresis with those obtained by BCG dye binding. The protein peaks obtained via electrophoresis were highly variable (Fig. 1). The most anodal peak was assumed to

represent an albumin-like protein. Good correlation was not seen between the "albumin" values determined by serum protein electrophoresis compared to those obtained by BCG dye binding.

Discussion

Previous studies have been performed to evaluate the effects of toxins (Carbis *et al*, 1996; Kakuta *et al*, 1994), herbicides (Neskovic *et al*, 1996; Poleksic and Karan, 1999), temperature, high ammonia concentrations (Hrubec *et al*, 1997a and 1997b), and infectious agents (Rehulka, 1996; Steinhagen et al, 1997) on various biochemical parameters of fish, especially common carp. In most of the studies, however, test values were evaluated by comparing those of the principal group were compared to those of a control group. Attempts to construct clinical reference intervals were not done. In a recent review article (Groff and Zinkl, 1999), reference ranges were published for various hematological and serum chemistry parameters for common carp but information is not available specifically for koi. Also, the reported values were compiled from different studies. Since many interspecies and intraspecies factors such as age, nutritional status, and environmental conditions can affect these parameters (Hrubec *et al*, 1996; Hrubec *et al*, 2000), the reliability of these values as reference ranges is questionable. To avoid the variables mentioned above, we sampled koi from a defined reference population that was maintained and handled under identical environmental conditions. In addition, laboratory procedures to analyze the serum specimens were performed by standardized techniques and protocols. In the present study, the reference population, husbandry, and analytical methods have been described in detail. Although these reference intervals may not be

uniformly applicable to koi worldwide, they may provide general guidelines for the interpretation of laboratory data from this species of fish.

Skin and gills are primary osmotic barriers in the fish body that help maintain water homeostasis. The gills also are responsible for major excretion of nitrogenous wastes and some degree of ion regulation. The kidneys only play a minor role in excretion of nitrogenous wastes, but are primarily a hematopoietic organ (especially the anterior or head kidney). Most of the biochemical parameters that could be affected by cutaneous and gill damage, such as concentrations of serum proteins, electrolytes, and nitrogenous wastes, were included in the study.

Low concentrations of creatinine and uric acid can be attributed to the unique excretory system of fish, in which most of the nitrogenous wastes are excreted via gills. Only a small fraction is excreted by the piscine kidney. Therefore, renal disease in fish probably would not be associated with life-threatening azotemia. The effect of gill disease on impairment of nitrogenous waste excretion has not been elucidated, but severe gill disease probably results in life threatening hypoxia before nitrogenous wastes can accumulate to toxic levels in the blood. Reference intervals could not be determined for uric acid concentration because the data were skewed. In such cases, uric acid concentration must be determined in 100 to 120 serum specimens to provide adequate sampling for nonparametric statistical analysis. Furthermore, the values for uric acid were so low that the test results may not be valid.

Hepatic and muscle enzyme activities also were determined to provide reference intervals in healthy koi and to determine the effect of venipuncture through muscle and fascial planes. CK appears to be the most specific indicator of muscle damage as is the

case in domestic animals. In contrast to mammals, ALT activity appears to be more liver-specific and its activity does not increase with muscle damage. SDH activity also appears to be a potential indicator of hepatocellular integrity in koi as is the case in domestic animals. Activity of LDH and AST appears to increase with muscle damage in koi. In mammals, increased LDH and AST activity may be observed with hepatic and muscle injury as well as with hemolysis. Because hemolysis was not observed in the serum specimens from koi, the increased activity of these enzymes is probably due to venipuncture through skeletal muscle (Kramer and Hoffmann, 1997). In contrast, ALT and SDH activities were less affected by venipuncture through skeletal muscle and, thus, may be more liver-specific enzymes in koi.

Negligible concentrations of total bilirubin in fish serum have been attributed to a lower activity of biliverdin reductase as compared to mammals (Fang and Bada, 1982). ã-GT activity also was found to be very low. Low concentrations of creatinine, total bilirubin, and uric acid, as well as low ã-GT activity, appear to exist in healthy koi, but changes in these parameters may prove to be significant in disease.

The presence of a human serum albumin equivalent protein in carp serum has been a topic of controversy for many years. In the present study, BCG dye binding and cellulose acetate electrophoresis were performed in an attempt to identify and quantitate carp serum albumin-like protein. In mammals, albumin is the most negatively charged serum protein and appears as a prominent peak at the anodal side (farthest from the site of application) of the serum electrophoretogram (Kaneko, 1997; Nagano *et al*, 1975). In the present study, the protein peaks observed in electrophoretograms were inconsistent. Therefore, correlation could not be made between the serum electrophoresis and BCG

dye binding in their abilities to detect an albumin-like protein in koi. In a previous study of piscine coccidiosis, carp serum was reported to separate into three protein fractions that were observed as distinct bands on cellulose acetate electrophoresis (Steinhagen *et al*, 1997). Another report demonstrated the separation of carp plasma proteins into five fractions, each of which exhibited the ability to bind with bromcresol green (BCG) and hydroxyazobenzene carboxylic acid (HABA) dyes (Yanagisawa *et al*, 1977). A more recent report, did not find a serum protein analogous to human serum albumin in carp serum; however, some high-density lipoproteins were present in high concentrations (De Smet *et al*, 1998). It was hypothesized that these lipoproteins were responsible for transport of free fatty acids in carp serum (De Smet *et al*, 1998). In summary, further studies are needed to confirm or refute the presence of an albumin-like protein in the serum of koi.

In comparison to total protein data previously reported from common carp, narrower intervals of total protein concentration were found in present study using the biuret reaction. This colorimetric reaction is based on the binding of a copper-containing reagent to peptide bonds (Kaneko, 1977). The test values obtained by this technique are relatively reliable, but may be influenced by the precision of the spectrophotometer and substances that interfere with light transmission within the specimen. Although the "A:G" ratios were slightly lower in the present study, this calculated value probably has considerable error because albumin-like protein could not be identified or quantitated accurately.

In the present study of koi, a minimal increase was observed in glucose, sodium, and calcium concentrations, whereas, chloride, potassium, phosphorus, and magnesium

concentrations were decreased when compared to a previous study in common carp (Groff and Zinkl, 1999). These earlier studies in common carp are of limited use for diagnostic purposes in koi because the test values were obtained from an undefined reference population and differences and similarities in laboratory values between common carp and koi were not investigated. In one of the experiments, crude methods of capture and restraint (stunning) were used (Rehulka 1996) that might impose significant stress on the fish and alter some of the biochemical parameters such as glucose, electrolyte, and total protein concentrations. In the present study, the koi were anesthetized to reduce the stress and trauma that can be caused by physical restraint of conscious animals. In fish, glucose concentration will increase in stress as is the case in mammals. Electrolyte exchange in fish is more complex than in mammals. Besides the usual considerations of acid-base regulation, electrolyte concentrations in fish are influenced by the mineral content of the water, adequate gill function, and alterations in water intake during stress (Groff and Zinkl, 1999).

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References

Anonymous. Spring viremia of carp control program launched. *J Am Vet Med Assoc* 222:1332-1333, 2003.

Carbis CR, Mitchell GF, Anderson JW et al. (1996) The effects of microcystins on the serum biochemistry of carp, *Cyprinus carpio* L., when the toxins are administered by gavage, immersion and intraperitoneal routes. J Fish Dis 19:151-159

Cheong L. (1996) Overview of the current international trade in ornamental fish, with special reference to Singapore. *Rev Sci Tech Off Int Epiz* 15:445-482

Fang L-S, Bada JL (1982) Biliverdin reductase activity in marine fishes. Marine Biology Letters 3:121-130

Groff JM, Zinkl JG (1999) Hematology and clinical chemistry of cyprinid fish. Vet Clin North Am: Exotic Animal Pract 2:741-776

Hrubec TC, Cardinale JL, Smith SA (2000) Hematology and plasma chemistry reference intervals for cultured tilapia (*Oreochromis* Hybrid). Vet Clin Pathol 29:7-12

Hrubec TC, Robertson JL, Smith SA (1997a) Effect of temperature on hematologic and serum biochemical profiles of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). Am J Vet Res 58:126-130

Hrubec TC, Robertson JL, Smith SA (1997b) Effects of ammonia and nitrate concentration on hematologic and serum biochemical profiles of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). Am J Vet Res 58:131-135

Hrubec TC, Smith SA, Robertson JL et al. (1996) Blood biochemical reference intervals for sunshine bass (*Morone chrysops* × *Morone saxatilis*) in three culture systems. Am J Vet Res 57:624-627

Kakuta I, Ishii K, Murachi S (1994) Effects of diluted sewage on biochemical parameters of carp, *Cyprinus carpio*. Comp Biochem Physiol 107C:289-294

Kaneko JJ (1997) Serum proteins and the dysproteinemias. Clinical enzymology. In: Kaneko JJ, Harvey JW, Bruss ML (eds): Clinical Biochemistry of Domestic Animals, 5th ed. Academic Press, San Diego, pp. 117-138

Kramer JW, Hoffmann WE (1997) Clinical enzymology. In: Kaneko JJ, Harvey JW, Bruss ML (eds): Clinical Biochemistry of Domestic Animals, 5th ed. Academic Press, San Diego, pp. 303-325.

Nagano H, Hosaka K, Shukuya R (1975) Comparative biochemistry of serum albumin. A serum albumin-like protein from carp, *Cyprinus carpio.* Comp Biochem Physiol 50B:573-578

Neskovic NK, Poleksic V, Elezovic I et al. (1996) Biochemical and histopathological effects of glyphosate on carp, *Cyprinus carpio* L. Bull Environ Contam Toxicol 56: 295-302

Poleksic V, Karan V (1999) Effects of trifluralin on carp: Biochemical and histological evaluation. Ecotoxicol Environ Safety 43:213-221

Rehulka J (1996) Blood parameters in common carp with spontaneous spring viremia (SVC). Aquaculture International 4:175-182

Smet HD, Blust R, Moens L (1998) Absence of albumin in the plasma of common carp *Cyprinus carpio*: binding of fatty acids to high density lipoprotein. *Fish* Physiology and Biochemistry19:71-81

Steinhagen D, Oesterreich B, Korting W (1997) Carp coccidiosis: Clinical and hematological observations of carp infected with *Goussia carpelli.* Dis Aquat Org 30:137-143

Yanagisawa T, Hashimoto K, Matsuura F (1977) Occurrence of multiple albumins in carp blood plasma. Bull Jap Soc Sci Fish 43:1219-1228

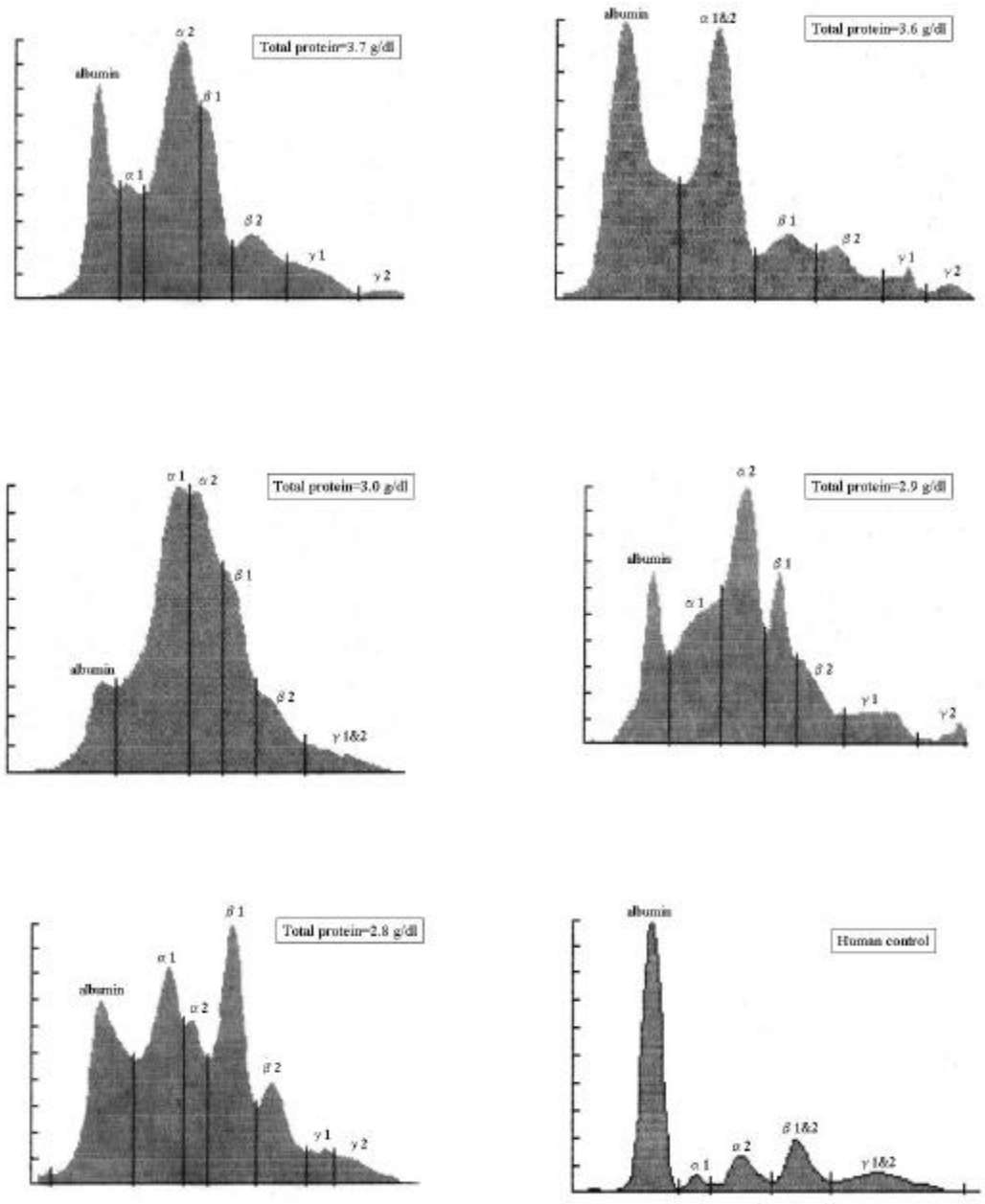

Figure 5.1 Serum electrophoretograms from five healthy koi demonstrate highly variable patterns of protein fractionation compared to human control.

Table 5.1 Serum chemistry reference intervals of koi (*Cyprinus carpio*)

Parameter	N =	Mean	Std Dev	CI 95%	Prob<W
A:G ratio	20	0.8	0.1	0.8-0.9	0.03
Alanine aminotransferase (U/L)	19	31	8	28-35	0.24
Albumin (g/dl)	19	1.4	0.1	1.3-1.4	0.043
Alkaline phosphatase (U/L)	19	11	4	9-13	0.0408
Aspartate aminotransferase (U/L)	19	85	28	71-98	0.22
Bile acids (µmol/L)	20	9	4	8-11	0.14
Bilirubin, total (mg/dl)	20	0.07	0.05	0.04-.10	0.0001
Calcium, total (mg/dl)	18	10.2	0.7	9.9-10.6	0.01
Chloride (mmol/L)	19	113	2	111-114	0.16
Cholesterol (mg/dl)	20	164	24	153-175	0.57
Creatine kinase (U/L)	17	5,855	2,992	4,317-7,392	0.01
Creatinine (mg/dl)	20	0.2	0.1	0.1-0.2	0.0001
Gamma glutamyltransferase (U/L)	18	0.22	0.42	0.01-.43	0.0001
Globulin (g/dl)	20	1.6	0.2	1.5-1.7	0.33
Glucose (mg/dl)	19	48	10	43-53	0.18
Lactic dehydrogenase (U/L)	17	207	136	138-277	0.21
Magnesium (mg/dl)	17	2.4	0.2	2.3-2.5	0.3
Phosphorus (mg/dl)	20	4.9	1.4	4.3-5.5	0.05
Potassium (mmol/L)	18	2.4	0.9	2.0-2.9	0.2

Sodium (mmol/L)	19	140	4	38-142	0.51
Sorbitol dehydrogenase (U/L)	20	2.7	1.7	1.9-3.5	0.53
Total CO_2 (mmol/L)	20	7	1	6-8	0.35
Total protein (g/dl)	20	3.0	0.3	2.8-3.1	0.2
Urea nitrogen (mg/dl)	19	2.4	0.5	2.2-2.7	0.363
Uric acid (mg/dl)	20	0.015	0.036	0.032-(–0.02)	0.0001

Table 5.2 Influence of venipuncture through ventral caudal peduncle on selected serum enzymatic activities of five koi (*Cyprinus carpio*). Syringes were replaced between first (before) and second (after) samples.

Alanine aminotransferase (U/L)		Aspartate aminotransferase (U/L)		Creatine kinase (U/L)		Lactic dehydrogenase (U/L)		Sorbitol dehydrogenase (U/L)	
Before	After	Before	After	Before	After	Before	After	Before	After
13	13	112	111	24465	25053	222	197	7.4	7
11	9	77	47	11336	3574	440	63	6	4.5
17	18	40	39	2911	2781	68	58	4.5	3.8
16	16	60	51	5964	3717	206	55	4.7	2.7
34	34	81	87	4044	5302	178	245	8.1	8

CHAPTER 6

DEVELOPMENT AND EVALUATION OF AN EXPERIMENTAL MODEL OF CUTANEOUS COLUMNARIS DISEASE IN KOI (*CYPRINUS CARPIO*) [1]

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[1]Tripathi, N.K., K.S. Latimer, C.R. Gregory, B.W. Ritchie, R.E. Wooley, and R.L. Walker. To be submitted to the *Journal of Veterinary Diagnostic Investigation*.

Abstract

Columnaris disease is a bacterial infection of fish caused by the Gram-negative bacillus *Flavobacterium columnare.* A reproducible experimental model was developed to study the cutaneous disease associated with *F. columnare* infection in koi (*Cyprinus carpio*). This model subsequently was used to investigate the pathogenesis and treatment of *F. columnare*-induced cutaneous lesions. Presumptive diagnosis of columnaris disease could be made by cytological and histopathological examination. Specific detection of *F. columnare* was done using the polymerase chain reaction (PCR) and DNA *in-situ* hybridization (ISH). PCR allowed the detection of *F. columnare* in fresh biological material and in formalin fixed, paraffin embedded tissues. The DNA ISH technique allowed the identification and localization of *F. columnare* in formalin fixed, paraffin embedded tissues. Using these molecular techniques, *F. columnare* was readily detected in skin specimens from infected fish; however, the bacterium was infrequently detected in specimens of liver, kidney and spleen. These observations suggest that columnaris disease generally presents as a cutaneous disease that is unassociated with systemic infection in koi. Hematologic studies indicated that most koi with *F. columnare* infection developed microcytic, normochromic, nonregenerative anemia and leukopenia characterized by lymphopenia, mild neutrophilia, and monocytosis. Biochemical changes included a significant increase in glucose concentration and a significant decrease in sodium and chloride concentrations. Total serum protein, albumin, calcium and magnesium concentrations were decreased but remained within the reference interval. A commercially available antibiotic potentiator (Tricide-Neo) was used to treat experimentally-induced cutaneous disease. Following bacterial infection, a 40% to 50%

reduction in mortality was observed in the treatment groups compared to infected but untreated control groups. In experimentally produced columnaris disease of koi, lesions were usually restricted to skin and fins; gill necrosis was not a consistent finding. In the few koi that developed gill infections, treatment was only effective if started in the early stages of *F. columnare* infection before severe gill necrosis occurred.

Keywords: *Cyprinus carpio*, carp, fish, koi, disease model, columnaris disease, *Flavobacterium columnare*, polymerase chain reaction, DNA *in-situ* hybridization, hematology, biochemistry, treatment

Columnaris disease was first reported by Davis in 1922, and it remains one of the most frequently encountered and devastating bacterial diseases of freshwater fishes.[7,12] This disease also is known by other colloquial names including saddleback disease, cottonwool disease, cotton mouth disease, and fin rot. Columnaris disease has been reported worldwide in most species of freshwater fishes with rare infections reported in marine fishes.[17,19,21,23] The etiologic agent of columnaris disease is a long, thin, Gram-negative, gliding rod that recently has been reclassified and renamed as *Flavobacterium columnare*.[4] This bacterium usually is of low pathogenicity and generally produces disease in fish that are stressed. Natural infections of *F. columnare* in fish occur most frequently at water temperatures > 20°C. Such disease outbreaks are associated with high mortality that may reach 100%. Columnaris disease also has been reported in coldwater fish at normal environmental temperatures ranging from 6°C to 12 °C.

The pathogenesis of columnaris disease is not well understood. Because of difficulty in reproducing the disease experimentally, previous studies usually relied upon natural infections. In addition, most of these pathogenesis studies focused on gill lesions; skin infections were not examined in much detail.[11] The present study was conducted to develop a reproducible model for the production of experimental columnaris disease in koi, to develop molecular techniques for specific detection of *F. columnare*, to characterize the development of cutaneous lesions, to evaluate hematologic and biochemical changes during disease, and to evaluate a proprietary treatment for columnaris disease.

Materials and methods

Fish: Two hundred clinically healthy koi, with a mean length of 12 to 18 cm and an average weight of 150gm, were obtained from Blue Ridge Hatcheries, Kernersville, NC. The fish were maintained in 10,000 gallon stocking tanks with a flow-through water system. They were randomly subdivided into smaller groups of 10 to 20 fish as needed. Each subgroup of 10 to 20 fish was housed in a 30-gallon glass aquarium with a flow-through water system equipped with a carbon filtration system.[a] The water temperature was regulated using a central water heater. Chlorine and ammonia concentrations of the water were monitored daily using commercial test kits.[b,c] A photoperiod of 12 hours of light was provided and the fish were fed a commercial feed[d] once daily. The fish were acclimatized for 2 weeks before initiation of any experiments. In addition, the koi were observed twice daily for clinical signs of disease or mortality during the study period. Neither clinical signs of disease nor mortality were observed prior to the initiation of these experiments.

Bacterial propagation: Three strains of *F. columnare*, designated 12/99, 7, and AL-94, were obtained from the Fisheries and Aquaculture Research Center, Pine Bluff, AR. Based upon preliminary infectivity studies in koi, strain 12/99 was the most virulent organism and was used for subsequent infectivity experiments. The stock *F. columnare* culture was plated on Shieh agar containing 1µg/ml tobramycin and incubated at 25°C. Bacterial colonies were visible after 24 to 48 hours of incubation. A single colony was sampled to inoculate 5 ml of Shieh broth with tobramycin. The inoculated broth was placed in a shaker incubator at 25°C and 200 rpm for 24 hours. This initial broth culture was used to inoculate an additional 500 ml of broth as described above. The final

inoculum for infectivity studies was produced by centrifuging the bacterial culture at 2000 rpm for 10 minutes, decanting the supernatant, resuspending the bacterial cell pellet, and diluting the bacteria to a final concentration of 10^6 to10^7 cfu /ml with 0.3% saline. All experiments used a 24-hour, log-phase growth bacterial culture. For long term storage of *F. columnare*, the bacterial cell pellet was reconstituted with fresh medium and the resulting bacterial culture was mixed with equal amounts of 10% sterilized glycerol and frozen at –80°C.

Experimental model of disease: Twenty clinically healthy koi, ranging from 8 to12 cm length and weighing approximately 100 gm each, were chosen at random and assigned to infected or control groups. Each group was housed in a 30-gallon aquarium and was acclimatized for 1 week before starting the experiments. The fish were anesthetized using tricaine methanesulfonate[e] at a concentration of 100 to 125 mg/L in aerated water and quickly laid on a flat surface. The right side of the fish was wiped gently with a Kimwipe[f] containing a 10-fold dilution of detergent.[g] The fish in the infected group were immersed for 1 hour in a bacterial culture of *F. columnare* that was diluted to 10^6to10^7 cfu/ml of inoculum with 0.3% saline. The control fish were immersed in aerated 0.3% saline for 1 hour. After exposure to the inoculum or placebo, the two groups were transferred to separate 30-gallon aquaria. The experiment was repeated three times on different groups of fish to determine the reproducibility of the model.

Evaluation of antibacterial properties of fish mucus: Mucus was gently scraped off the sides of healthy koi using a glass slide. The mucus was pooled in a small glass beaker, diluted 20-fold with deionized water, and filter sterilized through a 0.22 μm vacuum filter unit.[h] This filter-sterilized mucus was collected in sterile 1.5 ml Eppendorf tubes and

was concentrated using a vacuum concentrator.[i] The concentrated mucus was stored at 4°C until further use. The antibacterial properties of mucus subsequently were evaluated by plate counts of viable bacterial colonies and by a stain-based viability assay.

Viable plate count method - Cultures of *F. columnare* strain 12/99 were prepared by inoculating fresh growth medium and incubating at 25°C for 24 hours. Ten-fold serial dilutions of log phase growth bacterial cultures were made with sterile phosphate buffered saline. Twenty µl of concentrated mucus was added to 200 µl of bacterial culture. Three 20 µl drops of this mixture were plated on Shieh agar and incubated for 24 to 48 hours at 25°C. Bacterial colonies were counted on the agar plates after 24 to 48 hours of incubation.

Bacterial viability assay - Bacterial cultures were prepared as described above. Twenty µl of concentrated mucus was mixed with 200 µl of bacterial culture and incubated overnight at 25°C. The live/dead bacterial cell count was performed using a commercial bacterial viability kit[j] that contained two components. Component A was a 1.67 mM propidium iodide solution in dimethylsulfoxide and component B was a 18.3 mM propidium iodide solution in dimethylsulfoxide. After 12 hours incubation of a mixture of *F. columnare* culture and mucus, 1 µl each of components A and B of was added to 100 µl of the bacterial-mucus culture. The mixture was allowed to stand in the dark for 10 minutes at room temperature and then observed under fluorescent microscope using a triple filter.[k] With this dye, live bacteria stained red and dead bacteria stained green when observed by fluorescence microscopy. The live:dead bacterial ratios were determined from digital images.[l]

Cytology: Tissue imprint preparations were made from skin and gill lesions. These specimens were air-dried and stained with Wright-Leishman stain. Smears were examined for presence of characteristic long, thin bacilli suggestive of *F. columnare*.

Necropsy and histopathology: Moribund fish were euthanized and necropsied. Specimens of skin (including both wiped and unwiped skin), skeletal muscle, fins, gill, liver, anterior kidney, spleen, heart, brain, and eyes were obtained for histologic examination. These tissues were preserved in 10% neutral-buffered formalin solution, trimmed, and processed routinely. Paraffin-embedded tissues were sectioned at 3 µm, stained with hematoxylin and eosin, and examined microscopically for presence of bacteria. Giemsa- stained sections also were examined for better visualization of any bacteria.

Polymerase chain reaction (PCR): Initially, full length *F. columnare* 16 S rDNA was amplified using degenerate primers and *F. columnare* DNA as the template. The nucleic acid sequence of the forward primer was AGAGTTTGATCATGGCTCAG, while the sequence of the reverse primer was ACGGCTACCTTGTTACGACTT. Following sequencing of the initial PCR product, a specific primer set was designed to detect *F. columnare*. The sequence of the specific forward primer was CGATGGGTAGGGGTCCTGAG and the sequence of the reverse specific primer was GCTGCTGGCACGGAGTTAGC. The predicted amplicon was a 250 bp product that was amplified from a conserved region of the *F. columnare* genome. This PCR diagnostic assay was used to detect *F. columnare* in fresh, frozen, and paraffin embedded specimens of infected skin, kidney, spleen, and liver. DNA was extracted from these clinical specimens using a commercially available kit.[m] These DNA extracts were used

to detect *F. columnare*. A positive test result was indicated by the presence of a specific 250 bp replicon. Previous sequencing and BLAST searches indicated that the replicon was 100% homologous to the expected *F. columnare* genomic sequence.

DNA *in-situ* hybridization (ISH): Using *F. columnare*-specific primers, digoxigenin labeled 250 bp product was produced by PCR using digoxigenin labeled dNTPs. This labeled product (250 bp) was used as a probe for DNA-ISH using a standardized laboratory protocol. Briefly, the paraffin embedded tissues were deparaffinized in 3 washes in limonene for 5 minutes each, followed by 2 washes in serial dilutions of ethanol (100%, 95%, and 70%) for 2 minutes each. Pepsin digestion (50mg/ 20ml of automation buffer) was done to improve probe penetration of the tissue sections. DNA hybridization was performed after formamide treatment by incubating the tissue sections at 37°C for 1 hour in the probe solution. High stringency washes were done after hybridization to remove nonspecifically bound probes. Foci of DNA hybridization were identified by affinity cytochemistry using an antidigoxigenin antibody conjugated to alkaline phosphatase. The chromagen solution was nitroblue tetrazolium dye solution. A positive reaction was recognized as deep blue deposits of formazan pigment at the sites of DNA hybridization. Positive control specimens included tissues from fish with documented *F. columnare* infection. Tissues from fish that were inoculated intramuscularly with *Aeromonas* sp., *Pseudomonas* sp., and *E. coli* were used as negative control specimens. Hybridization solution with a nonspecific nucleotide was included as an additional negative control specimen to exclude nonspecific binding of the antidigoxigenin antibody.

Hematologic and biochemical changes during columnaris disease: Twenty five healthy koi were randomly divided into an infected group (n=10), an infected and treated group (n=10), and an uninfected control group (n=5). Each group of fish was housed in a 30-gallon aquarium and acclimatized for 1 week before the start of these experiments. The fish were then anesthetized and blood specimens were collected from the caudal vein for baseline hematology and biochemical determinations. For hematolgic parameters, the blood was placed in lithium heparin anticoagulant.[n] Smears of heparinized blood were prepared for leukocyte differential counts and the remaining blood specimens were refrigerated immediately. Complete blood counts were performed within 1 to 2 hours of blood collection. The determinations included packed cell volume, total and differential leukocyte counts with the calculation of absolute leukocyte values, erythrocyte count with calculation of erythrocyte indices, and morphologic examination of leukocytes, erythrocytes, and thrombocytes. For biochemical parameters, blood was collected without anticoagulant, placed in sterile glass tubes, and allowed to clot for 15 minutes at room temperature. The serum was separated by centrifugation at 1000 rpm for 10 minutes. After recovery from anesthesia, the fish were divided randomly into infected (n=20) and control groups (n=5). The fish in the infected and control groups were manipulated as described previously for the "experimental model of disease." Following bacterial inoculation or immersion in the placebo, both groups were transferred to their respective aquaria. All fish were observed for cutaneous lesions or other signs of disease twice daily. When individuals in the infected group developed skin lesions, they were anesthetized and additional blood samples were collected for determination of hematologic and biochemical values. Similar blood specimens were obtained from the

control group after 7 to 10 days. During the course of the experiment, any mortality was recorded and dead fish were necropsied as described above.

Treatment protocol: Koi were randomly divided in three groups. These groups were designated as infected and untreated (positive control), infected and treated (principals), and uninfected (negative control). Both infected groups were exposed to an inoculum of *F. columnare* as described in the experimental model of disease above. The uninfected fish were immersed in a placebo solution. Twenty four hours post infection, the treatment group was immersed in a solution of Tricide-Neo (containing 8mM USP disodium ethylenediaminetetraacetate dihydrate, 20 mM USP 2-amino-2-hydroxymethyl-1,3-propanediol, and 0.5 mg/ml of neomycin) for 3 to 5 minutes on alternate days. The infected untreated (positive control) and uninfected (negative control) groups were not treated in any way. All fish were monitored visually twice daily for the appearance of cutaneous lesions. Mortality was recorded for each group.

Results

The experimental model of columnaris disease was reproducible and produced cutaneous lesions repeatedly in *F. columnare*-infected koi. In three separate trials, mortality rate ranged from 80% to 100% in the infected group as compared to 0% to 20 % in the uninfected control groups (Table 1). Deep skin ulcers were observed in the koi from the infected group (Fig.1). These fish also had clinical signs of disease including lethargy, depression, and anorexia. Initially, cotton-wool like bacterial growth was observed on the skin from right side of the fish where the mucus had been removed with

detergent, but the contralateral side of the fish remained normal (Fig. 2A and 2B). Neither clinical signs nor skin lesions were observed in the uninfected control groups.

The natural antibacterial properties of cutaneous mucus were demonstrated by two techniques Using the viable plate count method, the average number of *F. columnare* colonies per agar plate was 7 in the culture that was incubated with mucus compared to 12 for *F. columnare* cultures without exposure to mucus. The stain-based bacterial viability assay also verified the antibacterial properties of cutaneous mucus. On florescent microscopy, the number of dead bacteria was higher in the *F. columnare* culture incubated with mucus compared to that of bacterial cultures without exposure to mucus (Fig. 3A and 3B).

Wright-Leishman-stained cytologic imprints of cutaneous and gill lesions contained a homogeneous population of slender, elongate bacilli (Fig. 4). Almost a pure population of *F. columnare* was recovered from most of the skin and fin lesions and some necrotic gills following bacterial culture.

Histopathologic study indicated that experimental *F. columnare* infection in koi was primarily associated with skin and fin ulcers, gill necrosis was rarely observed. Large numbers of long thin bacilli, characteristic of *F. columnare*, were observed in the skin ulcers and attached to the exposed layers of the skin and dermis. Occasionally, the cutaneous ulcers extended to the deep dermis and underlying skeletal muscle. Necrosis of skin and muscle was accompanied by infiltrates of neutrophils (Fig. 5). Bacteria usually were not observed associated with fin lesions and were probably lost during tissue processing. Neither bacilli nor microscopic lesions were observed in internal organs including liver, spleen, and anterior kidney.

The polymerase chain reaction amplified a 250 bp product in PCR gels (Fig. 6). Sequencing and BLAST search of the amplicon revealed 100% homology with *F. columnare.* Using the PCR assay, *F. columnare* nucleic acid was detected repeatedly in the skin of infected fish. However, most DNA extracts of the liver, kidney, and spleen specimens were negative for *F. columnare*. The specific 250 bp replicon was observed only in a few kidney and liver specimens.

DNA *in-situ* hybridization also demonstrated the presence of *F. columnare* in most of the cutaneous lesions. DNA hybridization was readily discerned because of blue-black formazan pigment deposition against a green counterstained background (Fig. 7). Reactivity was not observed in tissue sections of internal organs or sections of uninfected skin.

Values of hematologic parameters before and after infection with *F. columnare* are presented in Table 2. Significant decreases were noticed in the packed cell volume (PCV), hemoglobin concentration (Hb), red blood cell (RBC) counts, mean corpuscular volume (MCV), and absolute lymphocyte counts. White blood cell (WBC) counts were decreased slightly but remained within reference intervals. Mild neutrophilia and monocytosis were observed.

Pre- and post-infection values of biochemical parameters are presented in Table 3. Marked decrease was observed in sodium and chloride concentrations. Only a minor decline was observed in calcium and magnesium concentrations. The increase in anion gap was minimal. Mild decreases were observed in total serum protein and albumin concentrations with no change in the A:G ratios. A significant increase in glucose concentration was observed following infection with *F. columnare*. Alkaline phosphatase

(ALP), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK) activities were significantly increased. In contrast, changes observed in alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) activities were insignificant. Significant changes were not observed in the uninfected control group (Tables 4 and 5). Post-infection blood specimens could not be collected from 4 infected fish and were not included in the study.

Decreased mortality was observed following treatment of *F. columnare*-infected koi with Tricide-Neo. Total mortality ranged from 30% to 60% in treated group as compared to 80% to 100% mortality in the infected but untreated group (positive control group). The uninfected control group had very low mortality, ranging from 0% to 20% (Table 6).

Discussion

F. columnare-induced bacterial dermatitis in koi was studied using an experimental model of disease. This model was based on the assumption that the surface mucus layer is part of the innate host resistance of fish to disease and its removal would promote the establishment of bacterial infection. A previous study demonstrated that *Flexibacter columnaris* infection was not transmitted in healthy Atlantic salmon with intact skin but infection did occur following a breach in the skin surface.[19] That supposition provided the basis for the development of the current experimental model of columnaris disease where the mucus layer was wiped off the right side of the fish while the left side of the fish was left intact. In experimental *F. columnare* infection, cotton wool type bacterial colonies were attached to skin on the right side of infected fish where

the mucus layer had been removed but these lesions were absent on the contralateral side. These initial lesions subsequently developed into extensive skin ulcers on right side of the fish.

In the present experiments, visible lesions were restricted primarily to the skin and fins; gill involvement was rare. The duration of *F. columnare* infection was approximately 5 to 7 days. In early investigations of columnaris disease, the condition was less severe and had lower mortality when transmitted experimentally.[7] In most of natural outbreaks of columnaris disease, gill necrosis is the major lesion and death may occur before cutaneous lesions are evident.[7-9]

In the present experimental model of disease, cutaneous lesions were prevalent but gill lesions were rare. This observation may be explained because bacterial infection was established only on the skin where the protective mucus layer is compromised and not on the gills that were undisturbed. A previous study of experimental columnaris disease in salmonids also demonstrated primarily skin disease with inconsistent gill necrosis.[19]

In fish, the cutaneous mucus layer has a protective effect that resists the establishment of bacterial infection. The antibacterial properties of surface mucus in koi were demonstrated in 2 bacterial viability assays. The viability of *F. columnare* decreased following incubation with cutaneous mucus from healthy koi as determined by manual counts of bacterial colonies on agar plates and by propidium iodide staining. The antibacterial properties of mucus have been demonstrated previously in carp and other fishes. Two hydrophobic proteins (27 and 31 kDa) have been isolated from the mucus of carp. Both proteins had pore-forming activity that correlated well with strong

antibacterial action against several Gram-negative and Gram-positive bacteria.[13] Ultrastructurally, a damaged mucus coat also has been observed in association with skin ulcers in trout with spontaneous *F. columnare* infection.[26]

Most previous studies of the pathogenesis of columnaris disease have focused on gill infection, while the development of skin lesions has not received much attention. Gill necrosis was not a consistent finding in this experimental model, but skin lesions were readily apparent. Thus, columnaris disease may have skin or gill presentations based on the adherence and virulence of a given strain of *F. columnare* as well as the site of damage of the protective mucus layer. Furthermore, tissue destruction is enhanced by bacterial protease production. [3,5,20]

Based on PCR and DNA ISH studies, experimental columnaris disease is restricted to the skin and fins with only infrequently gill involvement. Only a few specimens of kidney and liver were PCR positive for *F. columnare*. Since *F. columnare* is a ubiquitous bacterium, it is very difficult to avoid surface contamination when clinical or necropsy specimens are obtained for laboratory analysis. In a previous report of experimental infection of salmonids with *F. columnare*, the bacterium was cultured from kidney of infected fish. Significant histologic changes were not observed in the kidney other than deposits of proteinaceous fluid in Bowman's capsule. Similar glomerular deposits were observed in fish with and without saddleback lesions and significance of this finding was unknown.[19,23] Opportunistic bacteria were frequently cultured during the isolation of *F. columnare* from the internal organs, suggesting that rare systemic infection with *F. columnare* had occurred or that the tissue specimens were contaminated. [19,23]

Other reports of *F. columnare* infection also have documented an absence of significant lesions in internal organs. [8,19,23]

PCR is very reliable diagnostic technique for most infectious diseases and can readily amplify a few copies of bacterial DNA to give a positive PCR reaction. Since *F. columnare* is ubiquitous and usually of very low pathogenicity, detecting a few bacteria in a lesion may not be diagnostic of clinical disease. In addition, PCR has more potential problems with sample contamination resulting in a false-positive test result. The PCR test-positive liver and kidney samples probably were contaminated during necropsy because it is very difficult to sterilize the skin surface (especially in scaled fish). Furthermore, DNA ISH failed to detect bacteria in these tissue specimens. DNA from live or dead bacteria can easily contaminate the tissue specimens during collection and give a positive PCR reaction.

Cytology is preferred for the presumptive diagnosis of *F. columnare* infection because this technique is rapid and economical. A relatively homogeneous population of long, thin bacilli was visualized microscopically in wet mounts or in Romanowsky-stained preparations. Histopathology also can be used for tentative diagnosis of columnaris disease but is more labor intensive, expensive, and has a longer turnaround time. Bacteria are frequently observed on the surface of infected skin but not in the internal organs. However, organisms may be washed from the surface of some lesions during routine tissue processing.

Definitive diagnosis of columnaris diseases is difficult because *F. columnare* does not grow in blood agar or other commonly used media. Its isolation requires special media supplemented with antibiotics to prevent the overgrowth of contaminating

bacteria.[10] In the present experiments, problems also were frequently encountered in retrieving the bacteria from frozen stock. A new, modified medium containing a higher concentration of nutrients and lacking antibiotics was helpful to retrieve the bacterium from frozen (-80°C) stock.[27] Subsequently, the bacteria could be propagated in Shieh medium and used for infectivity studies. Pure culture of *F. columnare* is based on the presence of characteristic colony morphology on Shieh agar and of innumerable, long, thin bacilli on Romanowsky-stained cytologic smears.

Traditionally, bacterial isolation, culture, and biochemical characterization have been used as the "gold standard" in the definitive identification of *F. columnare*. More recently, PCR analysis has been developed as an alternative method for specific identification of this bacterium. This diagnostic test is based upon amplification of the 16S rRNA gene using species-specific primers to detect *F. columnare* in culture and tissue samples.[1,2,28]

Fluorescent antibody staining, using monoclonal antibodies, has been used to detect *F. columnare* infection in salmonids.[14,25] However, this technique requires fluorescence microscopy. DNA ISH can also provide specific detection of *F. columnare* and has the advantage of being able to localize the bacterium in tissues and organs and only requires a standard light microscope. Foci of positive DNA ISH reactivity were observed in the skin of experimentally infected koi; however, the bacterium was not detected in the internal organs of infected fish or in the skin and internal organs from the uninfected controls. These observations also suggest that only cutaneous *F. columnare* infection was produced by the present experimental model of columnaris disease.

The hematologic changes in *F. columnare*-infected koi included the development of a microcytic, normochromic, nonregenerative anemia. However, mild regeneration was observed in a few blood smears. In koi, microcytosis may reflect an impending regenerative response because erythrocyte precursors in fish are smaller in size than mature erythrocytes. Environmental stress, such as increased population density may also cause microcytic, normochromic anemia.[6,16] The WBC count usually indicated a leukopenia characterized by lymphopenia, mild neutrophilia, and monocytosis. Hematologic changes were more pronounced with extensive skin ulcers. Because lymphocytes are the predominant circulating leukocyte in koi, redistribution of these cells in response to stress or antigenic stimulation can promote leukopenia. In fish, leukopenia associated with lymphopenia and neutrophilia is a classical leukocyte response to stress, which is similar response to that in mammals.[22] Exogenous or endogenous corticosteroid, produced in response to long-term disease, also may cause lymphopenia.[6] The exact mechanism of lymphopenia is not clear but it may be similar to corticosteroid-induced redistribution of lymphocytes as occurs in mammals.[18] The mild neutrophilia and monocytosis probably occur in response to tissue demand for these cells as observed in histologic sections. Leukopenia with lymphopenia neutrophilia and occasional monocytosis is observed frequently with various infectious diseases of fish, including viral infections such as spring viremia of carp[24] and Gram-negative bacterial infection with *Aeromonas, Edwardsiella*, and *Pseudomonas* spp.[16,22]

Biochemical testing indicated markedly decreased sodium and chloride concentrations. Minimal decreases were observed in calcium and magnesium concentrations. Gill perfusion is increased in fish under stressful conditions and allows

passive diffusion of sodium and chloride ions from the gills out of the fish's body. In addition, more water also diffuses into the fish's body during stress and may further contribute to the severity of the hyponatremia and hypochloridemia.[16] Finally, the loss of sodium and chloride ions probably occurs via a disrupted skin barrier secondary to ulcerative dermatitis. Decreased total calcium and magnesium concentrations may be associated with hypoproteinemia because a considerable portion of these ions is bound to serum proteins. [16] Total serum protein and albumin concentrations were moderately decreased but the A:G ratio was within the reference interval. This observation suggested that plasma protein was being lost via skin ulcers or that excess water was being absorbed by infected fish resulting in slight hemodilution. Marked hyperglycemia usually is caused by a glycogenolytic effect of catecholamines in acute stress. Corticosteroids maintain this effect in long-term stress by stimulation of hepatic gluconeogenesis or by suppression of glycogen assimilation.[16] Freshwater fish have a hyperosmolar environment inside their body compared to their aquatic environment. Therefore, any damage to the skin barrier allows massive diffusion of water into the fish's body, potentially resulting in disturbed osmotic regulation and electrolyte homeostasis.[16] ALP, AST, CK, and LDH activities were increased markedly, while ALT and SDH activities were relatively unaffected. Previous studies have shown that the increased activity of AST, CK, and LDH are associated with venipuncture, which is done through the musculature of the caudal peduncle. SDH and ALT appear to be present in low concentration in skeletal muscle and may be better indicators of hepatocellular damage. [29] These data generally indicate that there was no substantial damage to internal organs such as liver.

Tricide-Neo treatment moderately reduced the mortality rate of koi with experimentally-induced columnaris disease. However, treatment was most effective when started within the first 24 hours of *F. columnare* infection when cutaneous lesions were first visible. Treatment was not very effective in advanced disease when extensive skin ulcers or gill necrosis had developed. The minimal mortality observed in the uninfected control group probably was the result of stress caused by excessive handling and anesthesia.

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Sources and manufacturers

a. US Filter Inc. Lowell, MA.

b. Chlorine test kit, Lamotte Chemical, Chestertown, MD.

c. Ammonia test kit. Lamotte Chemical, Chestertown, MD.

d. Game Fish Chow, Melick Aquafeed Inc., Catawissa, PA.

e. Tricaine methanesulfonate, MS-222, Aquavet, Hayward, CA.

f. Kimwipes EX-L, Kimberly-Clark Corp., Roswell, GA.

g. Extran 300, VWR Scientific Products, West Chester, PA.

h. 0.22 μm vacuum filter unit, Corning, Fisher Scientific, Atlanta, GA.

i. SpeedVac, Savant Instrument Inc., Farmingdale, NY.

j. LIVE/DEAD Baclight, Molecular Probes, Eugene, OR.

k. Axiovert 35, Zeiss, West Germany.

l. Spot 2.2, Diagnostic Instruments Inc., Sterling Heights, MI.

m. DNeasy, Qiagen Inc. Valencia, CA.

n. BD Vacutainer, Lithium heparin, Becton Dickinson, NJ.

References

1. Bader JA, Shotts EB: 1998, Identification of *Flavobacterium* and *Flexibacter* species by species-specific polymerase chain reaction primers to the 16S ribosomal RNA gene. J Aquat Anim Health 10:311-319.

2. Bader JA, Shoemaker CA, Klesius PH: 2003, Rapid detection of columnaris disease in channel catfish (Ictalurus punctatus) with a new species-specific 16-S rRNA gene-based PCR primer for *Flavobacterium columnare*. J Microbiol Methods 52:209-220.

3. Bader JA, Vinitnantharat S, Klesius PH: 1999, An inducible protease in *Flavobacterium columnare*: a possible role in virulence? 24th Annual Eastern Fish Health Workshop, Atlantic Beach, NC. March 9-11.

4. Bernardet JF, Segers P, Vancanneyt M, et al. 1996, Cutting a Gordian knot: emended classification and description of genus *Flavobacterium*, emended description of the family Flavobacteriaceae and proposal of *Flavobacterium hydatis* nom. nov. (basonym, Cytophaga aquatilis Strohl and Tait 1978). Interntl J Syst Bact 46:128-148.

5. Bertolini JM, Rohovec JS: 1992, Electrophoretic detection of proteases from different *Flexibacter columnaris* strains and assessment of their variability. Dis Aquat Org 12:121-128.

6. Campbell T, Murru F: 1990, An introduction to fish hematology. Comp Cont Edu Pract Vet 12:525-533.

7. Davis HS: 1922, A new bacterial disease of fresh water fishes. Bulletin U. S. Bureau of the Fisheries (Doc. 924), 262-280.

8. Decostere A, Ducatelle R, Haesebrouck F: 2002, *Flavobacterium columnare* (*Flexibacter columnaris*) associated with severe gill necrosis in koi carp (*Cyprinus carpio* L). Vet Rec 150:694-695.

9. Decostere A, Haesebrouck F: 1999, Outbreak of columnaris disease in tropical aquarium fish. Vet Rec 144:23-24.

10. Decostere A, Haesebrouck F, Devriese LA: 1997, Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare (Flexibacter columnaris)* from diseased fish. J Clin Microbiol 35:322-324.

11. Decostere A, Haesebrouck F, Driessche EV, et al: 1999, Characterization of the adhesion of *Flavobacterium columnare (Flexibacter columnaris)* to gill tissue. J Fish Dis 22:465-474.

12. Durborow RM, Thune RL, Hawke JP, Camus AC: 1998, Columnaris disease: a bacterial infection caused by *Flavobacterium columnare*. Southern Regional Aquaculture Center publication No. 479.

13. Ebran N, Julien S, Orange N, et al: 1999, Pore-forming properties and antibacterial activity of proteins extracted from epidermal mucus of fish. Comp Biochem Physiol 122: 181-189.

14. Griffin BR: 1987, Columnaris disease: Recent advances in research. Aquacult Magazine May/ June pp 48-50.

15. Griffin BR: 1991, Characteristics of a chondroitin AC lyase produced by *Cytophaga columnaris*. Tran Am Fish Soc 120:391-395.

16. Groff JM, Zinkl JG: 1999, Hematology and clinical chemistry of cyprinid fish. Vet Clin North Am: Exotic Animal Pract 2:741-776.

17. Jeney Zs, Jeney G: 1995, Recent achievements in studies on diseases of common carp (*Cyprinus carpio L.*). Aquacult 129:397-420.

18. Latimer KS, Prasse KW: Leukocytes. *In:* Latimer KS, Mahaffey EA, Prasse KW: Duncan & Prasse's Veterinary Laboratory Medicine: Clinical pathology, 4th ed. Ames, Iowa State Press, 2003, pp. 46-79.

19. Morrison C, Cornick J, Shum G, Zwicker B: 1981, Microbiology and histopathology of 'saddle back disease' of underlying Atlantic Salmon, *Salmo salar L.* J Fish Dis 4:243-258.

20. Newton JC, Wood TM, Hartley MM: 1997, Isolation and partial characterization of extracellular proteases produced by isolates of *Flavobacterium columnare* derived from channel cat fish. J Aquat Anim Health 9:75-85.

21. Noga EJ: 1996, Fish Disease, Diagnosis and Treatment. Mosby-Year book, Inc., St. Louis, MO, pp.123-126.

22. Noga EJ: 2000, Fish leukocyte responses. *In*: Feldman BF, Zinkl JG, Jain NC: Schalm's Veterinary Hematology, 5th ed. Lippincott Williams & Wilkins, Baltimore, MD, pp. 433-439.

23. Pacha RE , Ordal EJ: 1967, Histopathology of experimental columnaris disease in young Salmon. J Comp Pathol 77:419-423.

24. Rehulka J: 1996, Blood parameters in common carp with spontaneous spring viremia (SVC). Aquacult International 4:175-182.

25. Speare DJ, Markham RJ, Despres B et al: 1995, Examination of gill from salmonids with bacterial gill disease using monoclonal antibody probes for *Flavobacterium branchiophilum* and *Cytophaga columnaris*. J Vet Diagn Invest 7:500-505.

26. Speare DJ, Mirsalimi SM: 1992, Pathology of the mucus coat of trout skin during an erosive bacterial dermatitis: a technical advance in mucus coat stabilization for ultrastructural examination. J Comp Pathol 106:201-211.

27. Starliper CE, Cooper RK: 2002, An examination of diversity of *Flavobacterium psychrophilum* isolates from different origins and a note on a medium used to recover isolates from long-term frozen storage. 27th Annual Eastern Fish Health Workshop, 18 - 22 March, Mount Pleasant, SC.

28. Tiirola M, Valtonen ET, Kinnunen PR, Kulomaa MS: 2002, Diagnosis of Flavobacteriosis by direct amplification of rRNA genes. Dis Aquat Org 51:93-100.

29. Tripathi NK, Latimer KS, Lewis LL, Burnley VV. Biochemical reference intervals for koi (*Cyprinus carpio*) (submitted to Comparative Clinical Pathology).

Figure 6.1 A young koi, experimentally infected with *F. columnare*, has severe skin ulceration with necrosis of underlying muscle. Necrosis of the caudal peduncle and caudal fin also is present.

Figure 6.2 A. Cotton-like growths on the right side (wiped with detergent) of a koi with experimental columnaris disease. B. Left side (unwiped) of the same koi lacks cutaneous lesions (reprinted with permission of the Compendium on Continuing Education for the Practicing Veterinarian).

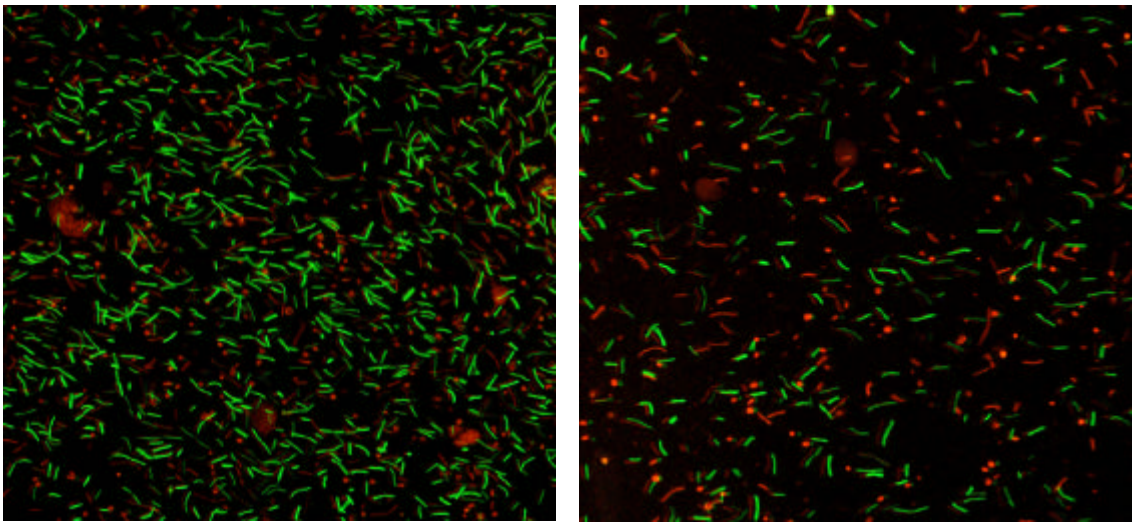

Figure 6.3 *F.columnare* stained with a bacterial viability kit (LIVE/DEAD Baclight). When examined by fluoresence microscopy light (triple filter), live bacteria stain with florescein and appear green whereas dead bacteria stain with propidium iodide and appear bright red. A. Bacterial culture without mucus (negative control) contains large numbers of live bacteria (high live:dead ratio). B. Bacterial culture with mucus contains fewer bacteria, most of which are dead (low live:dead ratio).

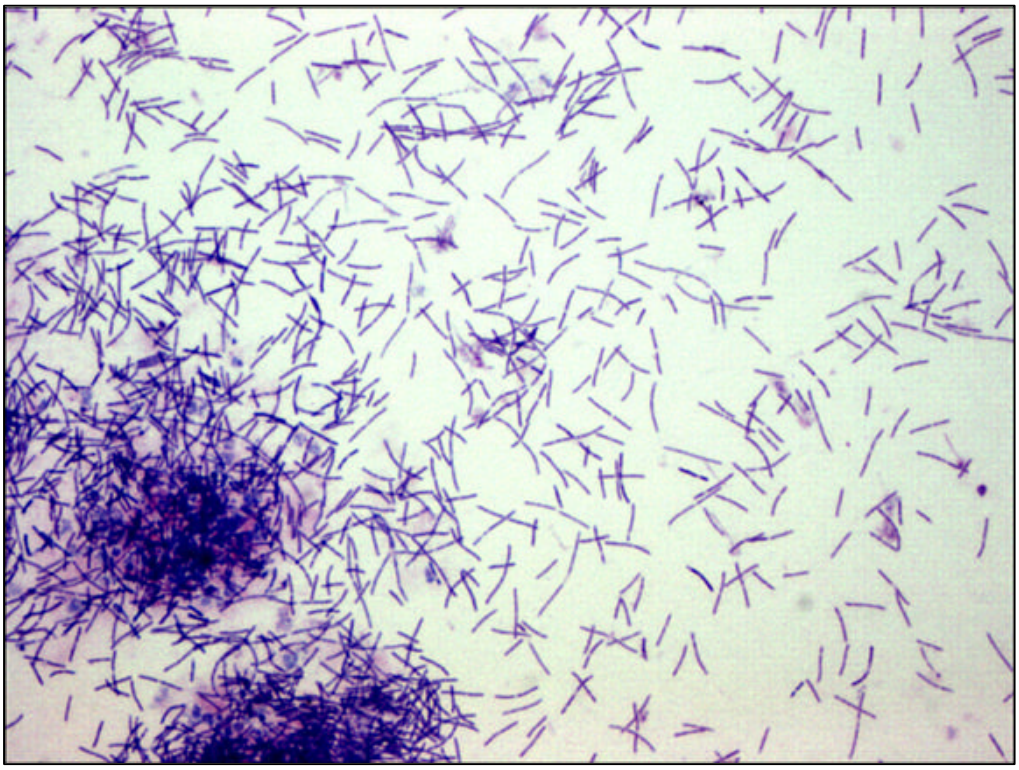

Figure 6.4 *F. Columnare* appear as a uniform population of long, thin bacilli (0.5-10mm) in a Wright-Leishman-stained cytologic preparation from a cutaneous ulcer.

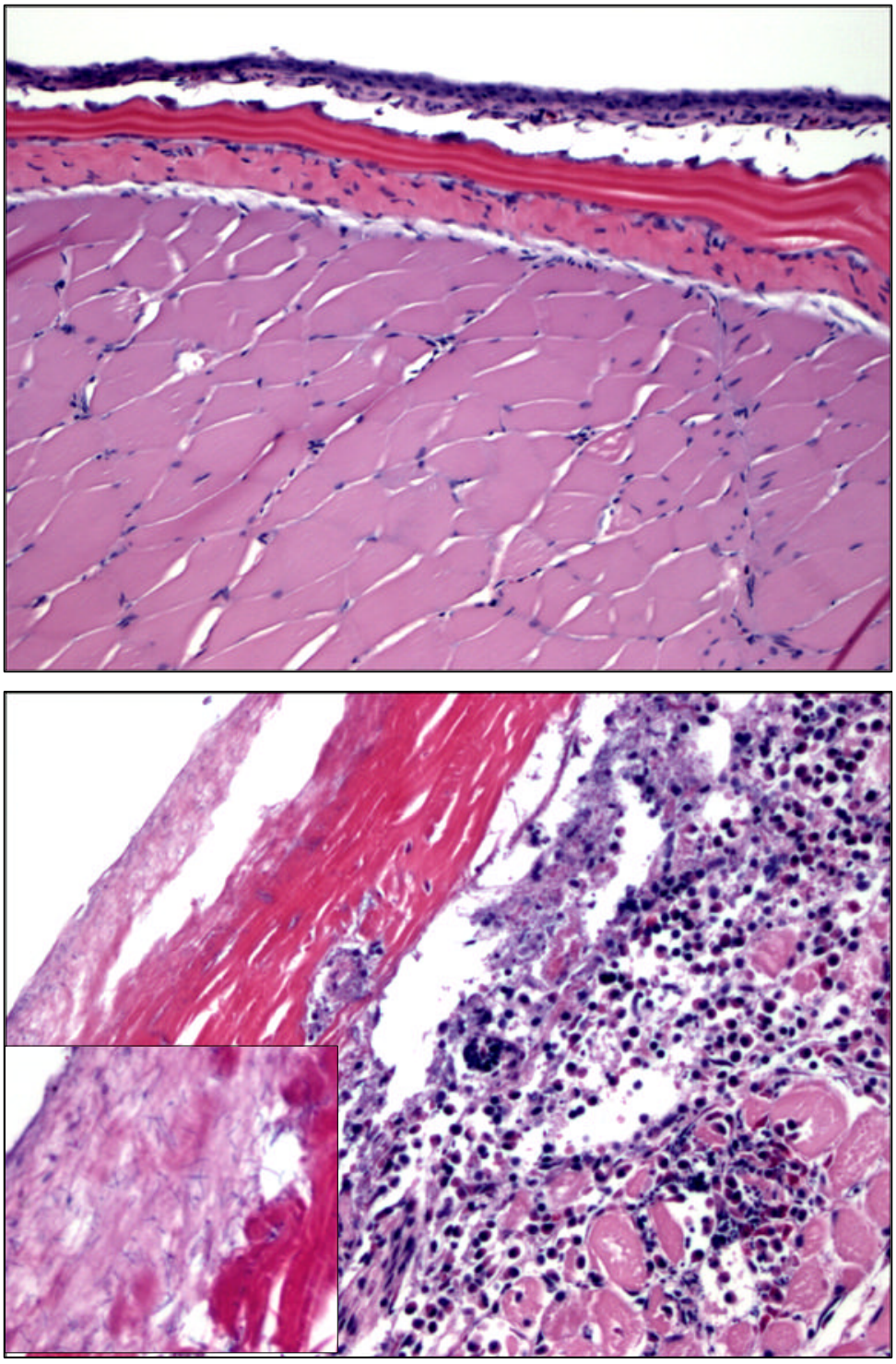

Figure 6.5 A. Normal skin (left) showing the epidermis, scale, dermis, and underlying skeletal muscle. B. Skin from a koi with experimental *F.columnare* infection has epidermal ulceration and necrosis with scale loss and a severe dermal infiltrate of neutrophils. The surface of this cutaneous lesion is invaded by numerous, thin, elongate bacilli characteristic of *F. columnare* (inset) (hematoxylin and eosin stain).

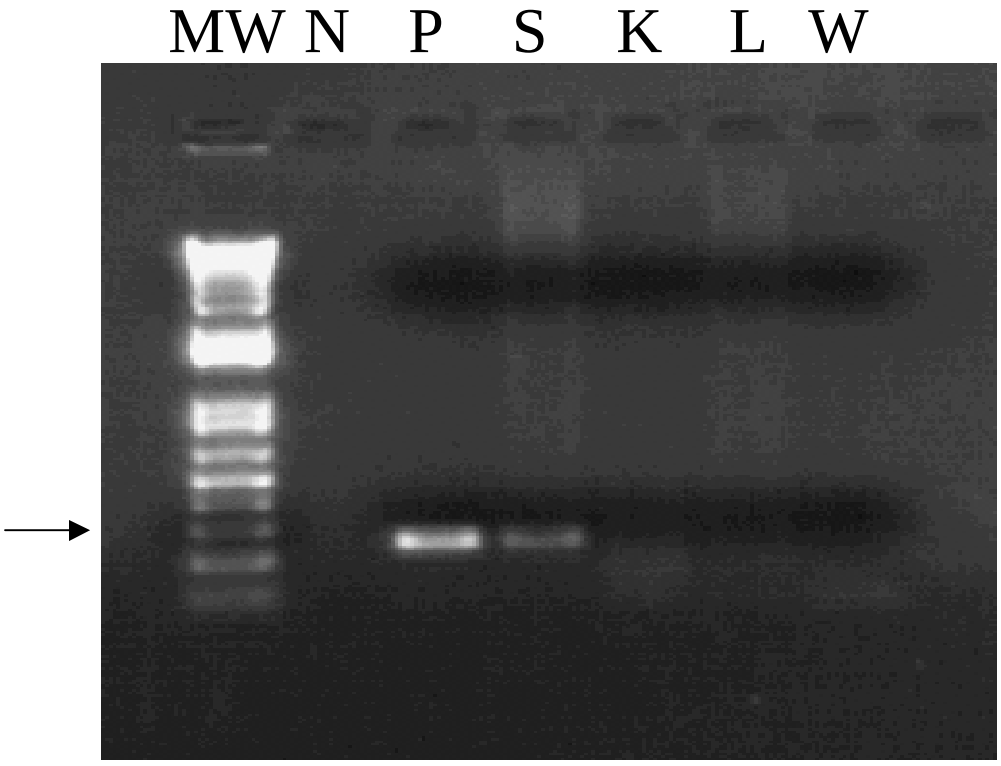

Figure 6.6 A 250 bp PCR product (arrow) is present lanes containing the positive *F. columnare* control (P) and infected extracts of skin (S). Amplicons are not present in the lanes that contain the water control (W), negative control (N), kidney (K), and liver (L). A 1 kb molecular weight ladder (MW) is present on the left side of the gel.

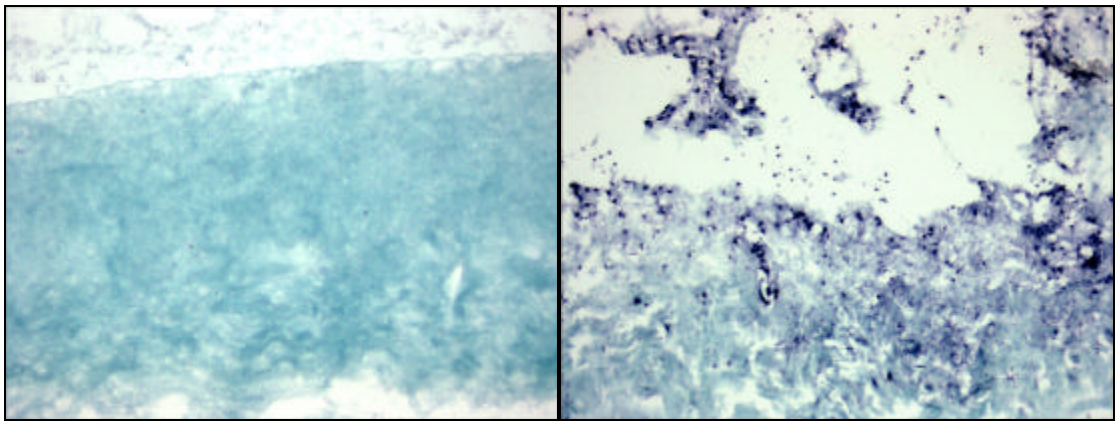

Figure 6.7 Specific identification of *F. columnare* in replicate sections of skin using DNA *in situ* hybridization. A. Negative control section of a skin ulcer that lacks *F. columnare* colonization. B. Cutaneous ulcer in a koi with experimental columnaris disease. The presence of F. columnare is indicated by the deposition of insoluble, blue-black, formazan pigment. DNA *in situ* hybridization using digoxigenin 3' end labled oligonucleotide probes, alkaline phosphatase indicator system, nitroblue tetrazolium dye chromagen solution, and fast green FCF counterstain.

Table 6.1 Total mortality of koi experimentally infected with a virulent strain (12/99) of *F. columnare* compared to uninfected control groups. Data are presented as the percentage of total mortality. The numbers in parentheses indicate the number of fish that died related to the number of fish in the group. Three separate trials were conducted and mortality was recorded over a 7-day period.

Group	Mortality Trial #1	Mortality Trial #2	Mortality Trial #3
Infected	80% (8/10)	100% (20/20)	90% (9/10)
Uninfected Control	0% (0/10)	20% (2/10)	10% (1/10)

Table 6.2 Changes observed in hematologic parameters of koi following experimental infection with a virulent strain (12/99) of *F. columnare.*

Parameter	N=	Mean		Std Error	Prob<t
Group		Healthy	Post Infection		
PCV %	16	34.94	23.56	2.2	0.0001
Hb (g/dl)	16	7.98	5.31	0.67	0.0004
RBC (10^3/ul)	16	1.93	1.51	0.16	0.014
MCV (fl)	16	183.4	165.1	11.93	0.13
MCH (pg)	16	41.73	35.42	2.62	0.02
MCHC (g/dl)	16	22.71	22.16	1.23	0.65
WBC (10^3/ul)	16	29.5	18.73	7.16	0.03
Lymphocyte %	16	70.6	39.5	7.21	0.0002
Lymphocyte (10^3/ul)	16	20.8	7.39	5.72	0.003
Monocyte %	16	4.3	12.43	4.1	0.023
Monocyte (10^3/ul)	16	1.2	2.32	0.98	0.137
Neutrophil %	16	22.5	39.75	6.73	0.031
Neutrophil (10^3/ul)	16	6.63	7.45	2.78	0.692
Basophil %	16	2.5	5.56	1.06	0.0098
Basophil (10^3/ul)	16	1.32	1.4	0.53	0.687

Table 6.3 Changes observed in biochemical parameters of koi following experimental infection with a virulent strain (12/99) of *F. columnare*.

Parameter	N=	Mean		Std Error	Prob>t
Group		Healthy	Post-Infection		
A:G ratio	16	0.82	0.91	0.06	0.16
Alanine aminotransferase (U/L)	16	48.81	77.56	14.57	0.05
Albumin (g/dl)	16	1.22	1.08	0.06	0.04
Alkaline phosphatase (U/L)	16	8.13	47.3	13.54	0.01
Anion gap	16	19.94	24.56	1.68	0.01
Aspartate aminotransferase (U/L)	16	150.6	966.2	227.9	0.002
Bile acids (µmol/L)	16	21.7	18.9	10.5	0.79
Bilirubin, total (mg/dl)	16	0.1	0.1	0	1
Calcium, total (mg/dl)	16	10.95	8.45	0.57	0.0002
Chloride (mmol/L)	16	114.6	55.9	8.1	0.0001
Cholesterol (mg/dl)	16	237.9	206.6	42.3	0.46
Creatine kinase (U/L)	16	10625	49516	8643	0.0004
Creatinine (mg/dl)	16	0.14	0.24	0.03	0.002
Gamma glutamyltransferase (U/L)	16	3.06	3	0.06	0.33
Globulin (g/dl)	16	1.51	1.22	0.087	0.002
Glucose (mg/dl)	16	71.43	225.3	32.6	0.0002
Lactate dehydrogenase (U/L)	16	376.3	1602.1	383.9	0.005
Magnesium (mg/dl)	16	3	1.91	0.19	0.0001
Phosphorus (mg/dl)	16	5.65	6.11	0.74	0.54
Potassium (mmol/L)	16	2.25	3.5	0.46	0.01
Sodium (mmol/L)	16	140.9	92.5	6.97	0.0001
Sorbitol dehydrogenase (U/L)	16	0.41	3	1.2	0.04
Total CO2 (mmol/L)	16	7.68	15.31	1.74	0.0004
Total protein (g/dl)	16	2.73	2.3	0.13	0.002
Urea nitrogen (mg/dl)	16	4	6.06	0.704	0.006

Table 6.4 Various hematologic parameters of a control group of koi. These fish were treated with placebo (0.3% saline) and not exposed to *F. columnare*. Significant changes are not present for any parameters evaluated.

Parameter	N=	Mean		Std Error	Prob<t
Group		Healthy	Placebo		
PCV %	5	33	29.2	1.85	0.07
Hb (g/dl)	5	8.14	6.18	0.86	0.06
RBC (10^3/ul)	5	1.98	1.74	0.13	0.12
MCV (fl)	5	161.5	160.7	15.5	0.96
MCH (pg)	5	37.5	38.8	4.3	0.77
MCHC (g/dl)	5	23.2	24.1	1.36	0.53
WBC (10^3/ul)	5	26.3	23.6	7.7	0.73
Lymphocyte %	5	61.4	59	11	0.83
Lymphocyte (10^3/ul)	5	20.4	15.8	9.6	0.64
Monocyte %	5	1.2	2.2	0.53	0.11
Monocyte (10^3/ul)	5	0.29	0.59	0.18	0.15
Neutrophil %	5	30.6	32.4	11.2	0.98
Neutrophil (10^3/ul)	5	5.65	8.33	2.4	0.3
Basophil %	5	6	8.4	4.6	0.61
Basophil (10^3/ul)	5	1.52	2.93	1.9	0.48

Table 6.5 Various biochemical parameters of a control group of koi. These fish were treated with placebo (0.3% saline) and were not exposed to *F. columnare*. Significant changes are not present for any parameters evaluated.

Parameter	N=	Mean		Std Error	Prob>t
Group		Healthy	Placebo		
A:G ratio	5	0.98	1.12	0.68	0.41
Alanine aminotransferase (U/L)	5	28.8	27.8	6.4	0.88
Albumin (g/dl)	5	1.36	1.26	0.07	0.22
Alkaline phosphatase (U/L)	5	12.2	13	7.4	0.92
Anion gap	5	21.6	21.8	1.1	0.85
Aspartate aminotransferase (U/L)	5	204.2	273	59.8	0.3
Bile acids (µmol/L)	5	27.2	20.8	5.4	0.35
Bilirubin, total (mg/dl)	5	0.1	0.1	0	1
Calcium, total (mg/dl)	5	10.7	11.2	0.69	0.49
Chloride (mmol/L)	5	117.2	125.4	4.8	0.15
Cholesterol (mg/dl)	5	211.8	182.4	16.6	0.11
Creatine kinase (U/L)	5	11841	24718	8426	0.18
Creatinine (mg/dl)	5	0.22	0.12	0.07	0.25
Gamma glutamyltransferase (U/L)	5	3	3	0	1
Globulin (g/dl)	5	1.4	1.23	0.26	0.3
Glucose (mg/dl)	5	72.2	125	30.3	0.15
Lactate dehydrogenase (U/L)	5	372	656.4	294	0.37
Magnesium (mg/dl)	5	3.24	2.88	0.36	0.36
Phosphorus (mg/dl)	5	3.82	4.76	0.48	0.08
Potassium (mmol/L)	5	2.4	2.46	0.43	0.89
Sodium (mmol/L)	5	143	150	5.2	0.24
Sorbitol dehydrogenase (U/L)	5	0	0.22	0.14	0.19
Total CO2 (mmol/L)	5	6.8	5.2	0.76	0.07
Total protein (g/dl)	5	2.72	2.57	0.11	0.21
Urea nitrogen (mg/dl)	5	2.4	3	0.5	0.28

Table 6.6 Total mortality of koi experimentally infected with a virulent strain (12/99) of *F. columnare* compared to an uninfected control group. Koi in the infected group were not treated. Koi in the treatment group were dipped in Tricide-Neo beginning 24 hours after exposure to *F. columnare*. Data are presented as the percentage of total mortality. The numbers in parentheses indicate the number of fish that died related to the number of fish in the group. Three separate trials were conducted.

Group	Mortality Trial #1	Mortality Trial #2	Mortality Trial #3
Infected and Untreated	100% (10/10)	90% (18/20)	80% (8/10)
Infected and Treated	60% (6/10)	50% (10/20)	30% (3/10)
Uninfected Control	10% (1/10)	20% (2/10)	0% (0/5)

CHAPTER 7

CONCLUSIONS

A major accomplishment of this research was to develop a reproducible animal model of *Flavobacterium columnare*-induced dermatitis (columnaris disease) in koi. Historically, it has been difficult to experimentally infect healthy fish with this bacterium. Detailed disease research cannot rely exclusively upon natural outbreaks of columnaris disease. With a virulent strain of *F. columnare* (strain 12/99), significantly higher mortality was observed in infected groups (80% to 100% mortality) as compared the uninfected control groups (0% to 20% mortality). Clinical signs of cutaneous columnaris disease included excess mucus production, visible bacterial growth, cutaneous erosions and ulcers, and fin necrosis. These lesions were apparent on the right side of infected fish where the mucus layer had been removed with detergent solution and a Kimwipe. In contrast, the unwiped left side of the fish had a normal appearance.

This model of disease indicates that the surface mucus layer of the skin and fins is protective against *F. columnare* infection in healthy fish. Furthermore, the antibacterial properties of this mucus also were demonstrated by two different techniques. A viable plate count method determined that fewer colonies of *F. columnare* were present on Shieh agar plates after a 24-hour incubation of the bacterium with isolated, filter-sterilized mucus. The second technique, using a bacterial viability staining kit, also demonstrated that fewer live bacteria were present following incubation of log growth

phase *F. columnare* with mucus. These observations support the protective role of the cutaneous mucus layer against the development of columnaris disease.

Experimental infection of koi with *F. columnare* had a slightly different clinical presentation than natural disease outbreaks. In the experimental model of disease, the mucus was removed from one side of the body, but the gills were not disturbed. This resulted in skin and fin lesions where the mucus was removed. In natural disease outbreaks, the gills may be colonized by the bacterium resulting in necrosis, excessive mucus production, severe hypoxia, and death. Gill necrosis was observed infrequently in the experimental model of disease.

This experimental model of columnaris disease was used to study the pathogenesis of cutaneous infection in koi. During these investigations, long, thin, bacilli (measuring 5 to 12 μm in length by 0.5 μm in width) characteristic of *F. columnare* were frequently observed in Wright-Leishman and Diff Quik-stained cytologic smears of skin, gill, and fins of infected koi. Similar bacteria also were observed in hematoxylin and eosin-stained histologic sections of infected skin. *F. columnare* stains faintly with hematoxylin and eosin and is better visualized microscopically following Giemsa staining. However, bacteria may be lost from the surface of the skin, fins, and gills during routine tissue processing. Therefore, cytology provides an easier, more economical, and rapid technique to make a presumptive diagnosis of *F. columnare* infection. Ultimate identification of the bacterium requires microbiological culture or other diagnostic techniques that specifically detect *F. columnare*.

The isolation and culture of *F. columnare* on agar medium is difficult. Antibiotics must be added to the medium to prevent secondary bacterial overgrowth.

Although *F. columnare* produces typical yellow colonies with rhizoid edges, colony morphology and characteristics alone do not provide definitive identification of the organism. Standard biological characteristics have been used to identify *F. columnare*, but these techniques are time-consuming. During the course of this research, two additional diagnostic techniques were developed to identify *F. columnare*. The polymerase chain reaction (PCR) was used to identify *F. columnare* nucleic acid in DNA extracts of fresh and paraffin embedded tissues. A DNA *in-situ* hybridization (ISH) technique was developed to specifically identify and localize *F. columnare* in formalin fixed, paraffin embedded tissue sections. Using these two molecular diagnostic techniques and the experimental model of columnaris disease, *F. columnare* infection in koi was found to be restricted to surface tissues including skin, fins, and, rarely, gills. Systemic infection with *F. columnare* could not be confirmed.

The experimental model of disease also was used to evaluate the hematologic and biochemical changes following cutaneous *F. columnare* infection in koi. Hematologic and biochemical reference intervals were established for koi because these baseline values were unknown for this particular species of fish.

Koi leukocytes were identified as lymphocytes, monocytes, neutrophils, basophils, and eosinophils based on their cellular morphology, cytochemical staining patterns, and ultrastructural characteristics. Lymphocytes were the predominant leukocyte in the blood of healthy koi and accounted for up to 80% of the total leukocytes. Neutrophils were the most abundant granulocyte, while eosinophils were rare. Cytochemical staining patterns were not consistent for leukocytes and, thus, could not be used as the sole identifying characteristic for a given leukocyte type.

Biochemical evaluation of koi revealed some interesting findings. The multiple peaks observed in serum electrophoretograms were inconsistent among fish. Furthermore, the albumin concentration determined by serum electrophoresis did not correlate with that obtained via bromcresol green dye binding. The presence of albumin or an albumin-like protein in koi is still questionable. Further research will be necessary to isolate, characterize, and identify these proteins.

In fish, venipuncture of the caudal vein usually is performed by a ventral approach to obtain blood samples. A potential problem with this technique is the penetration of skeletal muscle in the caudal peduncle. Increased serum activity of creatine kinase, lactate dehydrogenase, and aspartate aminotransferase was observed following venipuncture through the musculature. In contrast, the activity of alanine aminotransferase was unaffected. Serum activity of sorbitol dehydrogenase is low in healthy koi. This enzyme may be liver-specific in fish as it is in mammals. Alanine aminotransferase activity also may be more liver specific, but further research should be done to determine its concentration in piscine hepatocytes.

Hematologic and biochemical changes were observed in koi with experimental cutaneous *F. columnare* infection. Mild nonregenerative anemia and mild leukopenia characterized by lymphopenia, neutrophilia, and monocytosis were observed. Biochemical profiles indicated minimal hypoproteinemia and hypoalbuminemia. In addition, decreased sodium and chloride concentrations were observed. The above changes may be caused by hemodilution resulting from the loss of the cutaneous-water barrier secondary to skin erosions and ulcerations. Increased activity of liver-specific enzymes was not observed, which also suggested that systemic infection was unlikely.

A new proprietary treatment, Tricide-Neo, was evaluated for its effectiveness in resolving experimental columnaris disease. This treatment was especially effective in treating cutaneous disease. In three separate experimental trials, Tricide-Neo reduced the mortality of *F. columnare* infection by 40% to 50% compared to untreated, *F. columnare*-infected groups. This treatment provided better clinical results if used early and before deep skin ulcers developed.

Although different clinical presentations of columnaris disease are observed with experimental as opposed to natural infection, this model of disease should be of value in future research studies. These studies could include a comparison of various strains of *F. columnare* to investigate differences in molecular determinants of virulence and to evaluate factors that are important in host defense. Another benefit of this experimental model of disease will be the evaluation of new methods of treatment and assessment of candidate bacterins.