Acta Veterinaria Hungarica 54 (2), pp. 191–199 (2006)
DOI: 10.1556/AVet.54.2006.2.6

PATHOLOGICAL EFFECTS OF *ARCOBACTER CRYAEROPHILUS* INFECTION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS* WALBAUM)

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(Received 28 July 2005; accepted 20 October 2005)

Arcobacter cryaerophilus was isolated from naturally infected rainbow trout (*Oncorhynchus mykiss* Walbaum), and its pathogenicity was tested by intramuscular injection into 40 healthy 1-year-old rainbow trout at 16 °C. The lethal dosage of 50% end point (LD_{50}) for *A. cryaerophilus* was calculated 2.25×10^4 viable cells. Experimental infection caused deaths with gross clinical abnormalities such as degenerated opercula and gills, liver damage, haemorrhagic kidney and serous fluid in swollen intestines. The counts of *A. cryaerophilus* in kidney, liver and gills of experimentally infected fish ranged from 1.59×10^{10} colony forming units (cfu)/g to 7.41×10^{12} cfu/g. The means of erythrocyte (RBC) count, haematocrit level, serum cholesterol, triglyceride, albumin and total protein concentrations in the blood of the experimentally infected rainbow trout group were significantly lower than in the healthy fish. Leukocyte (WBC) counts of the experimentally infected rainbow trout were significantly higher than those of healthy fish. The present work shows that the selected blood characteristics may be good indicators of response to infections in rainbow trout.

Key words: *Arcobacter cryaerophilus*, rainbow trout, LD_{50}, leukocyte, haematocrit, cholesterol, triglyceride, total protein, albumin

Species of the genus *Campylobacter* can be found in the reproductive organs, intestinal tract, and oral cavity of humans and animals, with some being pathogenic (Holt et al., 1994). Neill et al. (1985) placed *Campylobacter cryaero-*

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phila as a new species in the genus *Campylobacter*, and it was included in the subsequent publication of Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The taxonomic status of this bacterium was later revised to the newly designated genus and species *Arcobacter cryaerophilus* (Vandamme et al., 1991). *A. cryaerophilus* exists in the normal microbial flora of freshwater fish (Jones, 2001), and it is considered a pathogenic bacterium for rainbow trout (Aydin et al., 2000, 2002). *A. cryaerophilus* infections must be taken into consideration in the aquaculture industry due to the fact that the disease can cause economic losses, however low the number of infection cases is (Aydin et al., 2000, 2002). This requires information on the severity of the problem in aquaculture.

In this study we used the blood parameters of fish as an indicator of their physiological status. In fish, however, the predictive value is compromised by the lack of reliable normal databases and available reference laboratories to properly analyse these samples (Chen et al., 2004). Moreover, the relationship between certain diseases or pathological changes to changes in blood parameters has not been fully established. Those blood characteristics, for which a relationship with a disease state has been established, have been primarily for major cultured fish species. Researchers indicated that the study of some haematological and biochemical parameters in fish blood has been associated with infectious diseases (Aydin et al., 1997, 2000, 2001, 2002; Chen et al., 2004), nutritional deficits (Chen et al., 2004), physiological activity (Chen et al., 2004), toxicity (Chen et al., 2002; Yang and Chen, 2003), anoxic conditions and the other environmental factors in fish farming. However, an experimental approach to reveal the link between disease state and clinical pathology is still urgently needed for many teleost species. The present study was, therefore, designed to test the pathogenicity of *A. cryaerophilus* and to investigate the effects of infection on blood parameters of experimentally infected rainbow trout.

Materials and methods

Isolation and identification of bacteria

The bacteria were originally isolated from naturally infected rainbow trout (*Oncorhynchus mykiss* Walbaum) at various times during the spring and summer in 1997, 1998 and 1999 at five fish farms in the vicinity of Balıkesir (Gönen) and Çanakkale in the Marmara region in western Turkey. The infected fish were killed and gross clinical signs recorded during necropsy.

In the isolation of bacteria from naturally infected fish, inocula were aseptically obtained from kidney, liver, spleen, gills and bloody fluid of naturally infected fish and streaked on enriched tryptic soya (TS) agar, Baird-Parker agar, *Yersinia*-selective agar, thiosulphate citrate bile salt (TCBS) agar, *Salmonella-Shigella* (SS) agar, *Pseudomonas-Aeromonas* selective (GSP) agar, Kligler (KG)

agar, MacConkey agar and *Campylobacter*-selective agar. Cultures were incubated at 25 °C for 1–7 days.

Organ samples passaged on *Campylobacter*-selective agar were incubated at 25 °C and 30 °C for 48 h in an anaerobic jar with the aid of Anaerocult® C mini (Anonymous, 1996). After incubation, the individual colonies grown on *Campylobacter*-selective agar were enriched on *Campylobacter*-selective agar at 25 °C for 48 h, and then inocula were used in the identification tests in both aerobic jars and anaerobic jars with the aid of Anaerocult C mini condition (Plumb and Bowser, 1983; Bullock, 1989; Austin and Austin, 1999). All of the bacteriological media used in this research were from Merck (Merck, Darmstadt, Germany).

Infection experiments

A total of 40 rainbow trout, 1-year-old with an average body weight of 82.62 ± 11.26 g, were used for the infection experiment. Five different 1000 L fibreglass tanks supplied with circulating freshwater (16 °C) under continuous aeration were used in these studies. Ten rainbow trout in each of Group 1, Group 2, Group 3 and Group 4 were experimentally infected with an isolate of *A. cryaerophilus* from a diseased rainbow trout obtained from one of the farms mentioned above. A pure culture of the bacterial isolate was added to sterile phosphate buffer saline (PBS) solution and its concentration was adjusted to 30% spectrophotometric transmittance (at 525 nm) with sterile PBS. After an adaptation period of 15 days, the fish were injected with 1×10^6, 5×10^5, 10^5 and 5×10^4 live cells into the muscle proximal to the dorsal fin to each of Groups 1, 2, 3 and 4, respectively. The remaining 10 fish were inoculated with sterile phosphate buffered saline (PBS), and served as non-infected control. During the experimental infection period, behaviour of the diseased fish as well as their gross external and internal signs were recorded, and then the 10 infected fish becoming moribund were used to measure haematological and biochemical parameters. The LD_{50} (50% mean lethal dose) value was calculated after 18 days by the method of Karber, cited by Plumb and Bowser (1983).

The inoculated bacterium *(A. cryaerophilus)* was re-isolated from the kidney, liver and gills of dead fish quantitatively (colony forming units/g) using *Campylobacter* selective agar and identified as *A. cryaerophilus* by characterisation tests.

Sampling and analytical procedure

Blood analysis was conducted on 10 healthy (non-infected) rainbow trout and 10 infected rainbow trout. As the fish became moribund (except healthy fish), they were weighed, and 4 mL of blood was drawn from each by puncture of the caudal vein or artery and immediately transferred into individual silicone-coated Vacutainer Tubes (Becton Dickinson; not containing EDTA) and 2-mL

tubes containing EDTA (ethylenediamine tetra-acetic acid). Blood in the coated Vacutainer Tubes was centrifuged promptly at 3,100 rpm for 10 min, and the serum was removed with a disposable transfer pipette. The concentrations of cholesterol and triglyceride were determined by colorimetry using Spinreact kits and measuring absorbance at 492 nm with an AWARNES-Stat Fax 1904 spectrophotometer. The concentrations of albumin and total protein were determined kinetimetrically using Spinreact kits at 540 nm wavelength.

The numbers of leukocytes (white blood cell, WBC) and erythrocytes (red blood cell, RBC) in the blood samples in EDTA-containing tubes were counted with Natt-Herrick solution on a Neubauer counting chamber (Bullock, 1989). Haematocrit values of blood samples in EDTA-containing tubes were determined by the microhaematocrit method outlined by Bullock (1989).

Statistical analysis

The data obtained from blood analyses were subjected to nonparametric analysis of variance (ANOVA) using the Minitab User Guide program (Statistically Analytical System, 1993). A value of $P < 0.05$ was considered significant.

Results

Identification of the bacteria

After analysis of the characteristics listed in Table 1, all the isolates obtained from the affected farms were identified as *A. cryaerophilus* on the basis of the revised taxonomic status of this bacterium as a newly designated genus and species.

Pathogenicity of the isolate

At a concentration of 10^6 live cells of *A. cryaerophilus* the mortality was 100%, at 5×10^5 live cells the mortality was 90% and at both 10^5 and 5×10^4 live cells it was 60% after an incubation period of 18 days following inoculation. Hence, 2.25×10^4 cells, the LD_{50} calculated over a period of 18 days at 16 °C, was chosen since it was found to be optimal and ensured 50% survival. No clinical signs or deaths were observed in the group of healthy fish injected with sterile PBS for 1 month after the inoculation.

Clinical observations for the experimentally infected fish were as follow: fin rot, ulcerated lesions on skin, degeneration of opercula, pale and hyperaemic foci in the gill filaments, fallen scales, pale and yellow colour with hyperaemic, haemorrhagic, and necrotic areas in the liver, darkened spleen, bloody and watery inflammation and swelling of the kidney, haemorrhages in the muscle, haemorrhages and bloody fluid in the intestine.

Table 1

Biological and biochemical characteristics of bacteria *(Arcobacter cryaerophilus)* isolated from diseased rainbow trout (plus sign = positive, minus sign = negative, NR = not reported)

Characteristic	Response	
	Isolates	Reference*
Gram stain	–	–
Motility (at 25 °C)	+	+
Sheathed flagella	–	–
Growth on *Yersinia*-selective agar	–	NR
Growth on GSP agar	–	NR
Growth on Baird-Parker agar	–	NR
Growth on TCBS agar	–	NR
Growth on KG agar	–	NR
Growth on MacConkey agar	–	NR
Growth on SS agar	–	NR
Aerobic growth on complex solid media (TS agar)	+	+
Morphology of colonies	Small, white	Small, white
Morphology of cell	Rod	Rod
Oxidase	+	+
Catalase	+	+
Growth at 25 °C	+	+
Growth at 42 °C	+	+
Growth at 15 °C	+	+
Growth in 6% NaCl and 3% NaCl	–	–
Urease, alkaline phosphatase, arginine dihydrolase	–	–
Gelatin, esculin and starch hydrolysis	–	–
Degradation of Tween 20 and Tween 80	–	NR
In Kligler iron agar and triple sugar agar:		
Acid/gas from glucose	–/–	NR
Acid from lactose	–	NR
Production of H_2S	–	NR
Methyl Red test	–	–
Voges-Proskauer test	–	–
Simmon's citrate	–	NR
Valine utilisation	–	NR
NO_3 reduced to NO_2	+	+
NO_2 reduction	–	–
Growth in KCN	+	NR
Indole production	–	–
Acid/gas production from (glucose, arabinose, inositol, maltose, melibiose, mannitol, erythritol, fructose, rhamnose, sucrose, sorbitol, sorbose, mannose, adonitol, galactose, dulcitol, salicin, trehalose, xylose, dextrin, inulin, glycogen, raffinose)	–	NR

*Holt et al. (1994)

The counts of *A. cryaerophilus* in kidney from experimentally infected fish ranged from 3.72×10^{11} cfu/g to 1.13×10^{13} cfu/g (SD = ± 4.72×10^{2}), and Koch's postulates were satisfied. Also, the counts of *A. cryaerophilus* in liver and gills from experimentally infected fish ranged from 1.54×10^{12} cfu/g to 7.86×10^{12} cfu/g (SD = ± 7.19×10^{2}), and from 1.78×10^{11} cfu/g to 8.29×10^{12} cfu/g (SD = ± 8.83×10^{3}), respectively.

Haematological and biochemical parameters

Blood analysis was conducted on 10 healthy (non-infected) rainbow trout and 10 infected rainbow trout. The purpose of this examination was to determine the possible characteristic alterations of blood parameters in response to experimental infection with this pathogen.

The blood leukocyte counts of the infected rainbow trout were significantly higher ($P < 0.05$) than those of the non-infected rainbow trout (Table 2). The mean RBC count and haematocrit levels in the blood of the experimentally infected rainbow trout group were significantly less ($P < 0.05$) than in the healthy rainbow trout group (Table 2). Cholesterol, triglyceride, total protein and albumin concentrations in the serum of the infected rainbow trout were significantly lower ($P < 0.01$) than those of the healthy rainbow trout (Table 2).

Table 2

Nonparametric analysis of variance of blood characteristics of healthy and experimentally infected fish groups (minimum and maximum values in parentheses)

Test[1]	Infected rainbow trout[2] (n = 10)	Healthy rainbow trout (n = 10)
WBC (10^4/mm^3)	6.65* (2.4–20)	2.2 (1.76–3.69)
RBC (10^6/mm^3)	0.23* (0.11–0.38)	0.37 (0.25–0.48)
Ht (%)	18.50** (5–29)	47.00 (31–56)
CHOL (mg/dL)	72.10** (17–103)	201.00 (137–235)
TG (mg/dL)	200** (112–253)	352 (228–605)
TP (g/dL)	0.70** (0.50–1.60)	3.80 (3.40–4.40)
ALB (g/dL)	0.45** (0.20–0.60)	1.80 (1.60–2.10)

[1]WBC = leukocyte, RBC = erythrocyte, Ht = haematocrit, CHOL = cholesterol, TG = triglyceride, TP = total protein, ALB = albumin; [2]= in the moribund stage; n = number of samples examined; $^{*}p < 0.05$; $^{**}p < 0.01$

Discussion

The characteristics of isolates were almost identical those of *A. cryaerophilus* isolates from humans or other animals (Vandamme et al., 1991). Holt et al. (1994) reported that acidic and neutral products of carbohydrates were not produced by *Campylobacter* species just as was seen in the present study. Bacte-

ria were additionally passaged on selective media and the isolates only grew on *Campylobacter*-selective agar and TS agar.

The results of the virulence test demonstrated that the isolate fell in highly virulent category as defined by Nieto et al. (1985) and Santos et al. (1991).

The clinical signs of experimental infection such as fin rot, ulcerative lesions, degeneration of gills, liver damage, bloody kidney and swollen intestine were similar to signs found in natural and experimental *A. cryaerophilus* infections (Aydin et al., 2000, 2002; Bulut and Aydin, 2004). Degeneration of jaw, exophthalmus, darkened or faded surface pigment, haemorrhagic hearts, watery and elongated spleens, observed in previous reports (Aydin et al., 2000, 2002), were not observed in these experimentally infected fish as has been reported by Bulut and Aydin (2004).

The elevated leukocyte count could be expected in infected fish as indicated by several workers (Blaxhall and Daisley, 1973; Kocabatmaz and Ekingen, 1987; Aydin et al., 1997; Harikrishnan et al., 2003). In almost all infected fishes, the homeostatic processes are extended beyond the normal limits due to stress (Harikrishnan et al., 2003). In the present work, the blood leukocyte counts of healthy fish seemed to be lower than the normal limits indicated for rainbow trout (Aydin et al., 1997). A wide range of variation in the blood WBC counts of rainbow trout is commonly observed (Kocabatmaz and Ekingen, 1987).

In the infected fish, the significant decreases in blood RBC and Ht may be a consequence of bacterially mediated haemolysis, and may appear as haemolytic anaemia. The blood RBC counts and haematocrit rates in fish may decrease in *A. cryaerophilus* infections (Aydin et al., 2000, 2002), other bacterial infections (Eiras and Saraiva, 1986; Aydin et al., 1997; Rodger and Richards, 1998; Harikrishnan et al., 2003; Chen et al., 2004), fungal infections (Pathiratne and Rajapakshe, 1998), parasitic infections (Yokoyama et al., 1996) and acute anaemia (Gilmour and MacNeill, 2003; Leedom et al., 2003). In the present study, wide ranges of variation in the blood Ht values as well as RBC and WBC counts of the experimentally infected fish group may also originate from the effect of infection.

A. cryaerophilus infection could cause a decrease in serum cholesterol and triglyceride values of rainbow trout. Although serum cholesterol and triglyceride concentrations may significantly increase with *Serratia liquefaciens* infection (Aydin et al., 2001), *Vibrio vulnificus* infection (Chen et al., 2004) and nephrocalcinosis (Chen et al., 2002), they can also decrease with natural and experimental infections of *A. cryaerophilus* in rainbow trout (Aydin et al., 2000) and albino crosses of rainbow trout (Bulut and Aydin, 2004), *A. hydrophila* infection in carp (Harikrishnan et al., 2003) and *Streptococcus iniae* infection in tilapia (Chen et al., 2004) as was the case in our study. In this work a correlation was observed between changes of serum triglyceride and cholesterol concentrations. It is known that cholesterol, triglyceride and lipoprotein values are correlated with one another, and these are connected with the metabolism of lipids and functions of the liver and

kidney (Aydin et al., 2000, 2001; Yang and Chen, 2003; Bulut and Aydin, 2004), but further studies are needed to explain these biochemical characteristics in fish.

The decreased total protein and albumin values in the serum of infected rainbow trout may be a result of the infection. Also, low albumin concentration may be considered as a marker for degeneration of plasma osmolarity. Total protein is used to evaluate protein metabolism; low concentrations may occur with *A. cryaerophilus* infections (Aydin et al., 2000, 2002), yersiniosis (Altun and Diler, 1999), streptococcosis (Chen et al., 2004), and nephritic syndrome (Chen et al., 2002). Although the serum albumin concentrations may significantly increase with *Serratia liquefaciens* infection (Aydin et al., 2001), they can also decrease in natural and experimental infections with *A. cryaerophila* (Aydin et al., 2000, 2002), yersiniosis (Altun and Diler, 1999) and nephrocalcinosis (Chen et al., 2002).

In conclusion, these results demonstrated that *A. cryaerophilus* could be a significant pathogen for rainbow trout. The most discernible signs of experimental infections were ulcerative skin lesions, degenerated opercula and gills, pale and haemorrhagic liver, bloody kidney, darkened spleen and swollen intestine. Another point of this study is that *A. cryaerophilus* infection could cause decreases in RBC count and in Ht, CHOL, TG, TP and ALB levels, and increases in WBC counts in the blood of rainbow trout as compared to healthy fish. The present results showed that, under experimental conditions, these blood parameters were sensitive to show different aspects of infection. Physiological stress as indicated by some haematological and biochemical parameters could be useful for evaluating the effects of bacterial infections in fish, but the application of these findings for biomonitoring of rainbow trout under farm conditions will need more detailed investigations and a careful consideration of the environmental parameters.

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