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# Clinical, Pathological and Haematological Effects of *Micrococcus luteus* Infections in Rainbow Trout (*Oncorhynchus mykiss* Walbaum)

[1]Seyit Aydin, [2]Abdulkadir Ciltas, [2]Hasan Yetim, [3]İhsan Akyurt
[1]Gazi University, Education Faculty, 37100 Kastamonu, Turkey
[2]Atatürk University, Agriculture College, 25240 Erzurum, Turkey
[3]Mustafa Kemal University, Fisheries Faculty, 31640 Iskenderun, Turkey

**Abstract:** This study investigates chronic and sporadic *Micrococcus luteus* infections of 3 and 4 -year-old broodstock and 2-year- old juvenile cultured rainbow trout (*Oncorhynchus mykiss* Walbaum) during February through April 1993-1996. Infection had not been observed from 1997 to 2003. Bacterial isolates were identified each year and were tested to determine sensitivity to chemotherapeutants. Infections caused lesions on the skin and caudal fin, and internal organs such as muscle, liver and spleen. In 1995, naturally infected 2-year-old fish were examined for haematological variables and compared to healthy fish. Leucocytosis and thrombocytosis occurred as well as a reduction in haemoglobin and haematocrit values of infected fish. In 1996, histopathological examination demonstrated pathological changes in heart, liver, spleen, gill, muscle, kidney and intestinal tissues of juvenile rainbow trout. The minimum bactericidal concentrations (MBCs) of formalin (90 %) + ethyl alcohol (10 %) mixture and enrofloxacin against pathogenic bacterium isolated in 1996 were 3 $\mu$g/ml and 0.025 $\mu$g/ml, respectively. Formalin + ethyl alcohol mixture baths and oral applications of enrofloxacin were successfully used for the treatment of naturally infected fish in 1996. Pathogenicity of the *M. luteus* isolated from crucian carp (*Carassius carassius* L.) was tested by intramuscular injection using healthy rainbow trout.

**Key words:** Disease, rainbow trout, *Micrococcus luteus*, haematology, histopathology, treatment

## Introduction

Treatments of sporadic and chronic infections of fish in Turkey have not received great attention. However, the impacts of these diseases may increase depending on age and species of fish and virulence of pathogens, resulting in high losses of juvenile fish. Moreover, since isolation of aquatic animals from the environment is not possible, the prevention and control of diseases are difficult and may be impossible.

*Micrococcus luteus* exists in the normal microbial flora of intestines of freshwater fish, and is considered a pathogenic bacterium of fish (Austin and Austin, 1999). *M. luteus* infections have been observed, and fish were experimentally infected in the United Kingdom (Austin and Stobie, 1992).

Haematological parameters are widely used to determine systematic relationships and physiological adaptations including the assessment of the general health condition of animals and are more quickly reflected in the poor condition of fish than in other commonly measured variables. Also, alterations of blood components has become in control of pathologies caused by infectious diseases (Eiras and Saraiva, 1986; Cruz and Muroga, 1989; Aydin *et al*., 1997, 2000, 2002 and Rodgers and Richards, 1998).

The objectives of this study were to study haematological and histopathological characteristics, and treatment of natural *M. luteus* infections in rainbow trout (*Oncorhynchus mykiss* Walbaum) raised in hatcheries and research stations between 1993 and 1996 and to test the pathogenicity of *M. luteus* isolated from fish.

## Materials and Methods

**Farm:** At Atatürk University (at an altitude of 1850 m) rainbow trout fry (*Oncorhynchus mykiss* Walbaum) are produced intensively in concrete and earthen ponds. Production water had pH 7.8, 8.9 mg/l dissolved oxygen, 2.11 mg/l $HCO_3$, 2.45 milliequivalent/l $Ca^{++}$ and $Mg^{++}$ , 1.6 SBV (= acid binding capacity) and 11.95 mg/l total hardness. Temperatures were 9 $\pm$ 1 °C and 6.5 $\pm$ 3.7 °C in ponds of broodstock and juvenile fish, respectively. Morbidities, mortalities and gross examination were recorded daily. Gross pathology was noted, and some organs were examined bacteriologically (104 fish) infected fish. Haematological examinations were conducted to compare the blood parameters of 10 fish each from healthy and naturally infected 2-year-old rainbow trout in 1995. Also, some organs of 30 samples of infected fish were examined histopathologically in 1996.

**Isolation and Identification of Bacteria:** Bacteria were isolated from naturally infected rainbow trout, during winter and spring of each year from 1993 through 1996 (*Micrococcus luteus* could not be isolated in production and research station between 1997 and 2003). The infected fish were killed. Inocula were aseptically obtained from

kidney, liver, spleen and muscle of each naturally infected fish and streaked on tryptone soya (TS) agar (Oxoid, UK) and skimmed milk (2 %) agar (Merck, Germany). After incubation at 22 °C for 48 h, individual colonies were enriched in tryptone soya (TS) broth (Oxoid, UK) at 22 °C for 48 h, and restreaked on skimmed milk agar. Individual colonies were used in the identification tests (Plumb and Bowser, 1983; Anonymous, 1996).

***In vitro* efficacy of some disinfectants and antibiotics:** The pure broth cultures of four isolates during the study each year, were added to sterile phosphate buffer solutions their concentration being adjusted with the use of a spectrophotometer until a 30 % transmittance (525 nm) with sterile phosphate buffer was obtained. Aliquots (0.1 ml in volume) were used to test the sensitivity of the bacterium to chemotherapeutic and other antimicrobial substances. The agar disc diffusion method (Bauer et al., 1966) was employed to determine its sensitivity when growth on enriched Antibiotic medium agar (Merck) to 16 chemotherapeutic agents (Table 2) by National Committee for Clinical Laboratory Standards (NCCLS, 1992). *In vitro* assays were conducted to determine the bactericidal concentrations of formalin (90 %) + ethyl alcohol (10 %) mixture, chloramine-T (due to their availability and cost) and enrofloxacin. For the formalin + alcohol mixture and chloramine-T serial arithmetic dilutions of from 0.4 $\mu$g/ml to 325$\mu$g/ml in test tubes containing 5 ml sterile phosphate buffer solution were prepared. Aliquots (0.1 ml) of each standardized bacterial isolate were added to each of the tubes which were left to stand at room temperature for 1 hour after which a loopful material (0.1 ml) from each tube was inoculated onto plates containing skimmed milk agar medium. These were incubated for 3 days at 25 °C and then examined for growth of *M. luteus*. The bactericidal effect of the enrofloxacin (serial dilutions from 5 to 0.012 $\mu$g/ml, being left to standard for 1 h inoculation following) was tested. Control tubes containing sterile phosphate buffer or TS broth were each inoculated with aliquots of the standardized bacterial mixture and then left to stand for (low long) following which a loopful from each was inoculated onto plates containing skimmed milk medium without stopped contact of antimicrobial compounds and bacteria. These were inoculated for 3 days at 25 °C after which they were examined for growth.

**Chemotherapy:** Fish on farms showing evidence of infection in 1996 were captured and placed in a both containing a solution compressing 80 ml of formalin (90 %) + alcohol (10 %) mixture per 1 000 l of water for 1 hour, and then 20 mg/kg fish dosage of enrofloxacin per day was orally used. Both disinfection procedure and application of antibiotic were carried out for 4 days.

**Histopathological examination:** Tissues of naturally infected fish were excised and placed in Bouin's fixative and processed for light microscopy by routine methods (Bullock, 1989), then embedded in paraffin wax and 5$\mu$m sections were cut and the histological sections were prepared and stained with haematoxylin-eosin (H&E), eosin and Gram Brown&Brenn stains.

**Haematology:** The 2-3 ml of blood was drawn from the caudal vein of fish and immediately transferred into tubes containing ethylene di amine tetra acetate (EDTA, 4 mg/ ml). The blood analysis was conducted as outlined by Bullock (1989). The number of leucocytes and thrombocytes in blood were counted by using Dacie's fluid (for leucocytes) and Natt-Herrick solution (for thrombocytes) into a Neubauer counting chamber. Haemoglobin was determined by the cyanmethaemoglobin method (by using Drabkin's reagent) and haematocrit values determined by microhaematocrit method.

**Infection experiment:** Two different 700 l capacity concrete tanks with adequate freshwater circulation (0.5 l/min) were used for the infection. A total of 20 rainbow trout average 68.6 $\pm$ 15.2 g body weight were used for the experiment. After 10 days preliminary adaptation, fish were injected with approximately 5 x$10^5$ live cell of *M. luteus* isolated from diseased rainbow trout into the muscle around the dorsal fin. The remaining 10 fish (non-infected control) were injected with sterile phosphate buffer saline (PBS).

**Statistical analysis:** The data obtained from blood parameters were subjected to non-parametric ANOVA using Minitab-User Guide package program (Anonymous, 1993). A value of $P<0.05$ was considered to be significant.

## Results

**Identification of the bacteria:** All of the 130 bacterial isolates were identified as *Micrococcus luteus* (Table 1). The isolates were isolated from kidney, liver, haemorrhagic muscle and spleen of 104 fish.

**Antibiogram tests and MBCs determination of disinfectants:** Antimicrobial sensitivity of the isolates varied from year to year (Table 2). National Committee for Clinical Laboratory Standards (NCCLS, 1992) was used as a reference in the evaluation of antibiogram tests.

Table 1: Biological and biochemical characteristics of putative *Micrococcus luteus* isolated from diseased rainbow trout

| Characteristic | Response | References | |
|---|---|---|---|
| | Isolate | 1* | 2** |
| Gram stain | + | + | + |
| Morphology of microorganisms | Tetrad cocci | tetrad cocci | Tetrad cocci |
| Motility (at 5°C) | + | + | . |
| Growth at 37°C | + | + | . |
| Growth on inorganic nitrogen agar | + | + | . |
| Nitrate reduction - | - | - | |
| Growth at 7.5 % NaCl | - | | - |
| Growth at 0 % NaCl | | | |
| Oxidase | + | + | + |
| Catalase | + | + | + |
| Coagulase | - | . | - |
| Esterase, Lipase, Alkaline phosphatase | + | . | + |
| Urease - (80 %) | . | | . |
| Arginine dihydrolase | - | - | - |
| Lysine decarboxylase, Ornithine decarboxylase, | | | |
| ß-Galactosidase, Tryptophan deaminase | - | . | - |
| Gelatin hydrolysis | + | + | + |
| Casein and Lecithin hydrolysis | + | . | + |
| Esculin hydrolysis | - | - | . |
| Acid fast staining | - | . | - |
| Blood haemolysis | + | . | . |
| Production of $H_{2S}$ | - | . | - |
| Methyl-Red test | - | . | . |
| Voges-Proskauer test | - | . | - |
| Simmon's citrate | - | - | . |
| Indole production | - | . | - |
| Acid production from (carbonhydrates): | | | |
| Glucose, Lactose | - | - | - |
| Mannose, Glycerole | - | - | . |
| Adonitol, Raffinose | - | . | . |
| Mannitol | - | . | - |
| Fructose | + (54 %) | . | . |
| O/F | O | O | O |

* = Holt et al. (1994) ** = Austin and Stobie (1992) . = not reported

*In vitro* assays indicated that the pathogenic bacteria tested, MBCs were 3 mg/l formalin and 0.025 mg/l enrofloxacin against *M. luteus* isolated in 1996, but chloramines-T was not effective.

**Infection cases:** Combined, morbidities and mortalities of naturally infected fish with *M. luteus* in juvenile and adult fish populations of all years were summarized in Table 3. The average individual weights of naturally infected juvenile and adult fish were 140 ± 35.5 g and 950 ± 400.5 g, respectively. Natural infection was not observed in the earthen ponds while it was always present in fish in concrete ponds. Disease was appeared during winter and spring of each year from 1993 through 1996 however it was not observed during summer and autumn seasons. Infection had not appeared in rainbow trout farm between 1997 and 2003.

**Medical treatment:** There was no mortality in the fish bathed with formalin + alcohol mixture and orally applied enrofloxacin after 5$^{th}$ day following treatment application. The treatment with formalin + alcohol and enrofloxacin provided normal healthy conditions for the naturally infected fish, which was concluded from the following observations, a mortality caused by this infection was not observed, and no clinical abnormality was detected in year of recovery period.

**Clinical observations:** Infected fish had behavioural abnormalities such as slow movements and anorexia. Presence of haemorrhagic surface lesions in broodstock and complete degeneration or fin-rot in juveniles and muscle

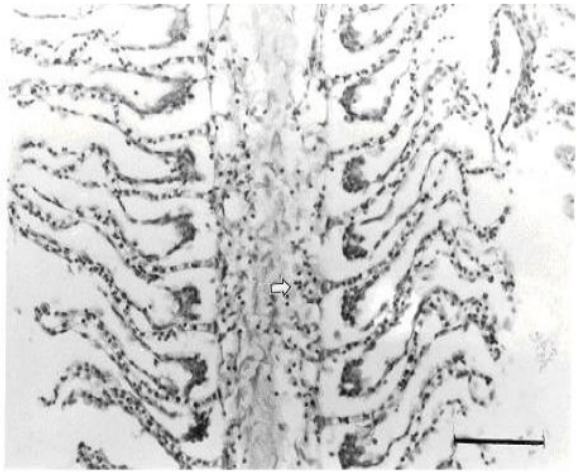

Fig. 1: Infiltration (arrow) of mononuclear cells in the gill tissue of rainbow trout naturally infectd with *M. luteus* (H&E x 200). Bar = 80 μm.

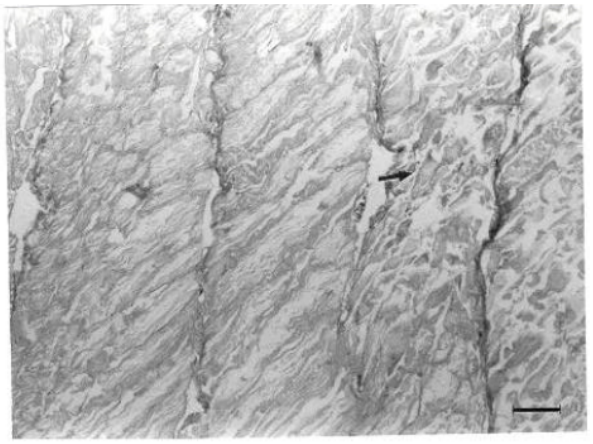

Fig. 2: Necrosis (arrow) of in the muscle of rainbow trout naturally infected with *M. luteus* (H&E x 100) Bar = 80 μm

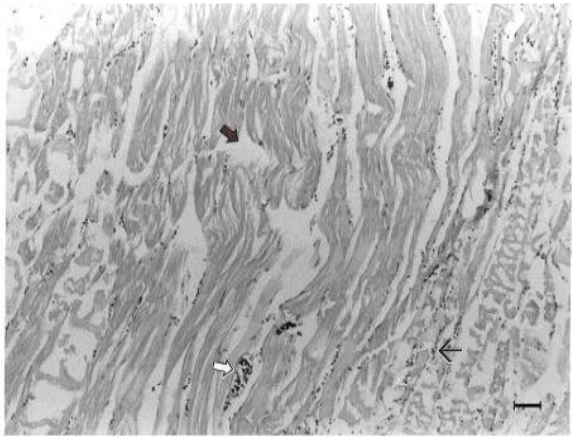

Fig. 3: Mononuclear inflammatory cell infiltration (thin arrow), vascular structures (white arrow) in mature muscle tissue and oedema (dark arrow) and congestion among muscle fibres of rainbow trout naturally infected with M. luteus (H&E x 200). Bar = 80

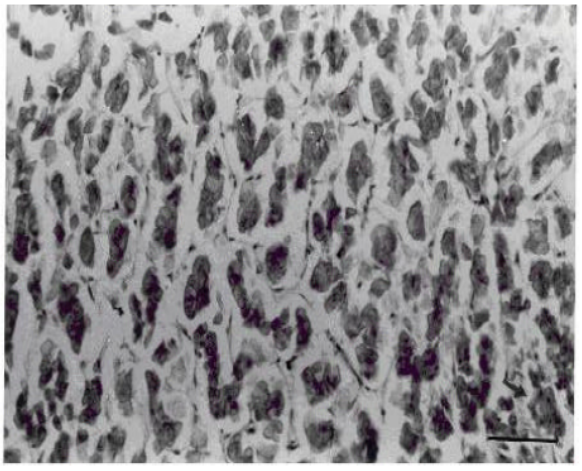

Fig. 4: Necrotic cells (arrow) in the liver of rainbow trout naturally infected with *Micrococcus luteus* (H& E x 200) Bar = 80 μm.

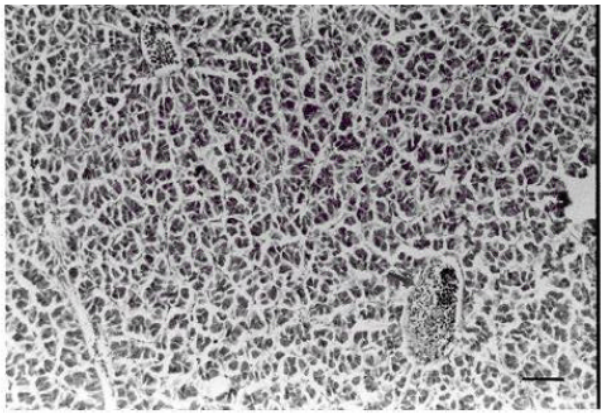

Fig. 5: Intravascular congestion (arrow) in the liver of rainbow trout naturally infected with *M. luteus* (H&E x 100) Bar = 80μm

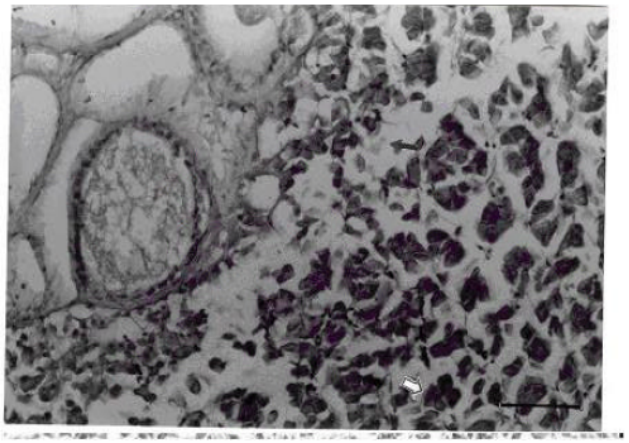

Fig. 6: Oedema, hyperaemia and distention in vascular structures (dark arrow), intracellular vacuolation (white arrow) in the liver of rainbow trout naturally infected with *M. luteus* (H&E x 200). Bar=80 μm

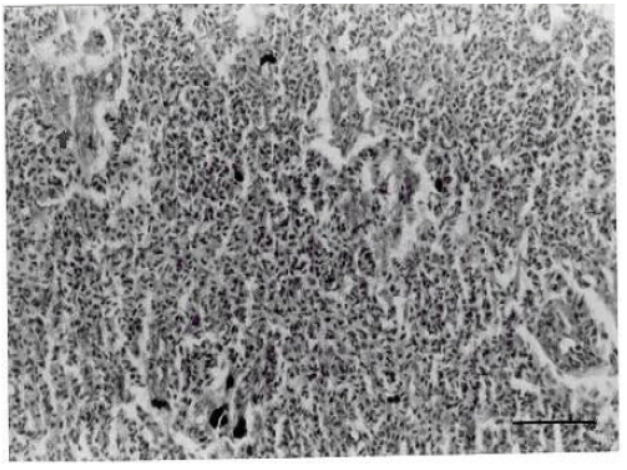

Fig. 7: Fibroblast proliferation, capillary proliferation and mononuclear inflammatory cell infiltration (arrow) in the spleen of rainbow trout naturally infected with *M. luteus* (H& E x 200). Bar = 80 μm

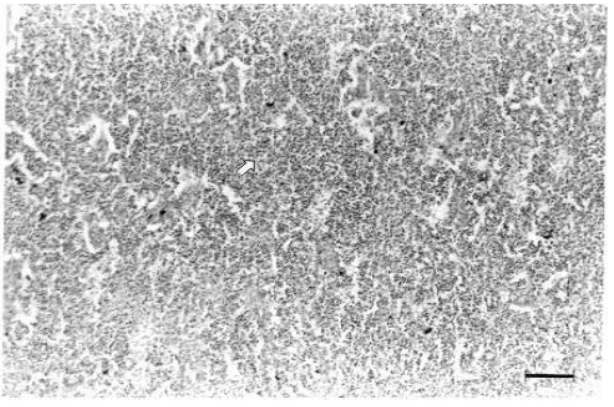

Fig. 8: Haemorrhagic anc congestional stroma (arrow) of spleen of rainbow trout naturally infected with *M. luteus* (H&E x 100). Bar = 80 μm

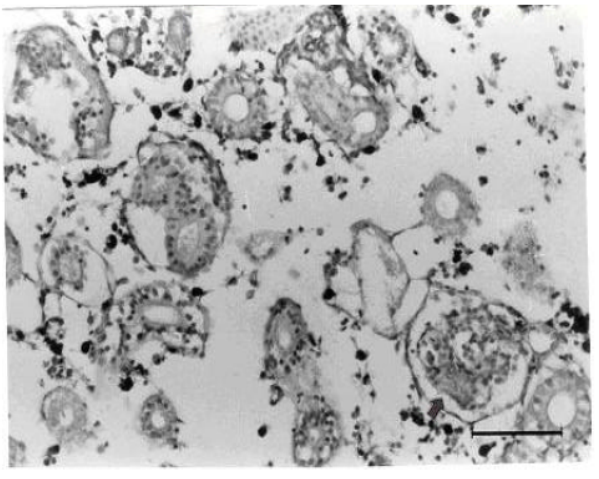

Fig. 9: Hyalinization in the Bowman's capsule of golmeruli in the kidney (arrow) of rainbow trout naturally infected with M. Luteus (H&E x 200) Bar = 80μm

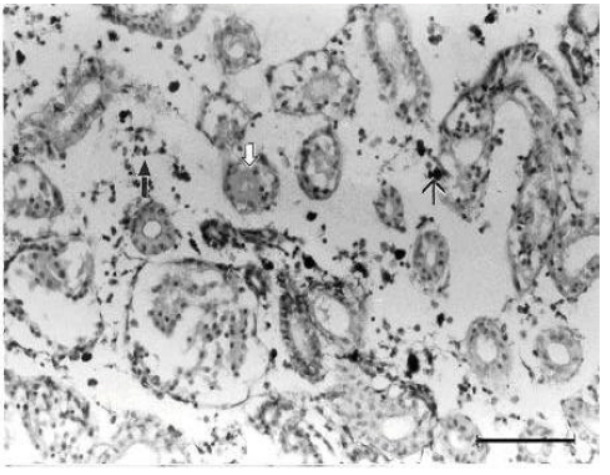

Fig. 10: Mononuclear inflammatory cell infiltratino among tubles (dark arrow), bacterial colonies (think arrow) and foci of homogenous eosinophilic materials (white arrow) in tubules of the kidney tissue of rainbow trout naturally infected with M. luteus (H&E x 200) Bar = 80μm.

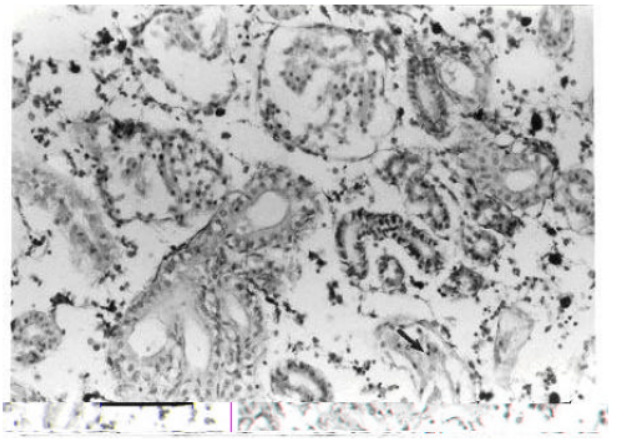

Fig. 11: Degenerated glomeruli and tubules (arrow) in the kidney of rainbow trout naturally infected with M. luteus (H&E x200) Bar=80μm

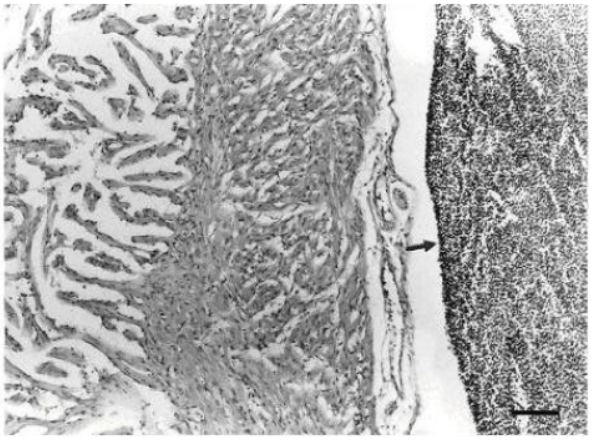

Fig. 12: Mononuclear inflammatory cell infiltration in the pericardial gap and visceral sheet (arrow) of the pericard of rainbow trout naturally infected with M. luteus (H&E x100) Bar=80μm.

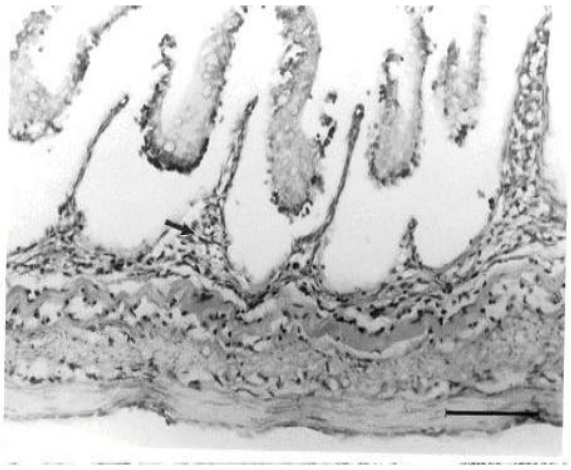

Fig. 13: Vascular congestion, capillary and fibroblast proliferation (arrow) in the stroma beneath mature mucosal glands of intestine of rainbow trout naturally infected with *M. luteus* (H&E x 200) Bar = 80μm.

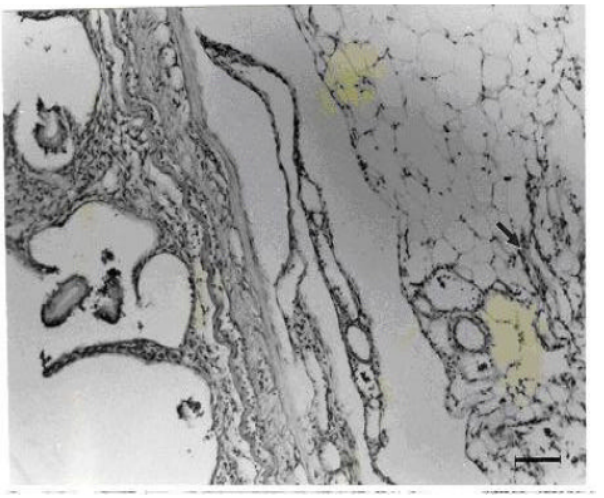

Fig. 14: Mononuclear inflammatory cell infiltration in mature adipose tissue of sersol layer (arrow) of the intestine of rainbow trout naturally infected with *M. luteus* (H&E x100). Ba

degeneration in some fish were observed. Generally, other signs were identical both in juvenile and adult fish that showed haemorrhage in surface of eyes, hyperaemic regions in the liver and pale spleen.

**Histopathological observations:** There was no apparent histopathological change in the gill except the infiltration of mononuclear cells into vascular structures in the cross-section of gill tissue (Fig. 1). Necrosis was present in muscle tissue (Fig. 2). Mononuclear inflammatory cell infiltration into mature muscle tissue and vascular structures were detected (Fig. 3). Oedema and congestion were observed among muscle fibres (Fig. 4), intra-vascular congestion (Fig. 5) and oedema, intracellular vacuolation (Fig. 6) hyperaemia and distention in vascular structures of the liver. Presence of fibrotic regions, fibroblast proliferation, capillary vein proliferation, mononuclear inflammatory cell infiltration (Fig. 7) and haemorrhagic and congestional stroma (Fig. 8) were observed in the spleen. There was hyalinization in the Bowman's capsule of glomeruli in kidney (Fig. 9). Dense mononuclear inflammatory cell infiltrations were observed among tubules (Fig. 10). Bacterial colonies and regions of homogenous eosinophilic materials were also in tubules (Fig. 10). In addition, the presence of degenerated glomeruli and tubules were observed (Fig. 11). Mononuclear inflammatory cell infiltration was detected in the pericardial gap and visceral sheet of the pericard (Fig. 12) and fibrin was present in the pericardium. Also, congestion of capillaries, and regions of fibroblast and capillary proliferation were observed. Vascular congestion, capillary and fibroblast proliferation were present in the stroma beneath mature mucosal glands of intestine (Fig. 13) and mononuclear inflammatory cell infiltration was present in mature adipose tissue of the serosal layer (Fig. 14).

**Haematological examination:** As seen Table 4, the blood leucocyte number of infected juvenile fish was significantly higher ($p < 0.01$) than that of healthy juvenile fish within the normal limits. The number of thrombocytes was also significantly higher ($p < 0.05$) in infected fish compared to healthy fish (Table 4). Both the haematocrit and haemoglobin values of the infected fish were significantly less ($p < 0.05$) than that of healthy fish group (Table 4).

**Pathogenicity of the *M. luteus*:** Moribund state started 7 days after bacterial injection in the some fish. Seven of the fish infected with *M. luteus* died in 11-24 days following the inoculation. Clinical signs observed in these fish were similar to naturally infected fish. There was no mortality in the non-infected group.

## Discussion

Identification test results of *Micrococcus luteus* were in agreement with the literature information on this bacteria species (Austin and Stobie, 1992 and Holt *et al.*, 1994), but results of methyl-red test, urea hydrolysis, haemolysis of blood and acid production from fructose, raffinose and adonitol have not been reported previously. This bacterium is a part of the normal microbial flora of water (Austin and Stobie, 1992; Austin and Austin, 1999), and is a potential disease agent for fish. During the present study, *M. luteus* caused a natural chronic-sporadic infection in juvenile and adult fish. Previous reports had been reported acute infections in rainbow trout fry only (Austin and Stobie, 1992; Austin and Austin, 1999). Therefore, *M. luteus* may also cause chronic infections in juvenile and adult fish. It is suggested that the age of fish and virulence of different bacterial strains may affect the severity and type of infection.

*M. luteus* was resistant to chloramphenicol, potentiated sulphonamides and oxytetracycline which contrast to the findings of Austin and Stobie (1992). Sensitivity of *M.luteus* strains could vary with different geographical areas. The isolate in 1996 to amphicillin/sulbactam, chloramphenicol and the isolate in 1993 to cefixime were sensitive. The isolate 1994 was moderate sensitive to oxytetracycline. *M. luteus* was susceptible against imipenem except the isolate in 1993. According to the results of present study, norfloxacin, enrofloxacin, ofloxacin, tobramycin, gentamycin, ceftriaxone, cephoperazone, cefotaxime and erythromycine observed effective to isolates could be recommended to treat fish infected with *M. luteus*.

Morbidity rates were higher in juvenile fish infected with *M. luteus* when compared to adult fish while the mortality rates of morbid fish of both groups were 100 % except cases in 1996. In 1996, the mortality rates of morbid fish of juvenile and adult groups were 4.3 % and 7.2 %, respectively. It is suggested that the treatment was effective against infection in 1996. In order to treat, dosage of formalin used in the present study was higher than dosages recommended (Scott, 1993; Austin and Austin, 1999), and no toxicity was observed for fish in this application. Earthen ponds could be more reliable than concrete ponds in preventing infection although further studies are needed for evaluating the different environmental conditions. Infections appeared between February and April during the study years possibly due to reduced resistance of fish after spawning activity (broodstock) and winter inappetance (juvenile) periods as previously reported (Ellis, 1989).

In the present study, haemorrhages were observed in the eyes of fish whereas exophthalmia reported by Austin and Stobie (1992). Ascitic fluid was not observed in chronic infections in contrast to the *M. luteus* infection observations previously (Austin and Stobie, 1992; Austin and Austin, 1999). There were hyperaemic regions in liver as previously reported (Austin and Stobie, 1992; Austin and Austin, 1999). The pale spleen and swollen kidney were also similar to findings of previous studies (Austin and Stobie, 1992).

The leucocyte number was in blood of healthy fish within the normal limits. Chronic *M. luteus* infection could cause leucocytosis in rainbow trout, which agrees with observations of several workers (Ellis, 1977; Heath, 1987; Aydin *et al.*, 1997). But contrarily, Studnicka and Siwicki (1986) reported chronic infection causing leucophenia, and Omeregie and Oyebanji (2002) observed significant reductions in leucocyte, erythrocte, thrombocyte, haematocrit and haemoglobin values as a result of used antibiotic (oxytetracycline). In present study, thrombocytes were higher in chronic infected fish than that of healthy fish, which is consistent with the results of Casillas and Smith (1977) although chronic *Ichthyopodo* infestation with bacterial infection causing thrombophenia had been reported by several researchers (Pickering and Pottinger, 1987; Pickering *et al.*, 1987). In any case, both blood thrombocytes of the infected and healthy fish groups were within the normal limits (10 000-40 000), and could show considerable variation (McCarthy et al., 1975; Aydin e*t al.*, 1997). The high haematocrit and haemoglobin levels could be correlated with the effects of disease because both infected and healthy fish groups were grown in identical condition even if values of infected fish were within normal limits in the literature values (Haman and Weber, 1996; Wood *et al.*, 1996a, b; Aydin *et al.*, 1997; Haman *et al.*, 1997). Some researchers described that bacterial infections can reduce both haematocrit (Aydin *et al.*, 2000, 2002) and haemoglobin values (Eiras and Saraiva, 1986; Cruz and Muroga, 1989; Haman *et al.*, 1997; Rodgers and Richards, 1998) although chronic stress of elevated water pH causing an increase of haematocrit values had been previously reported (Wilkie *et al.*, 1996). Besides, Rodger and Richards (1998) stated that EIBS (erythrocytic inclusion body syndrome) did not cause any significant changes in haemoglobin and haematocrit values of fish. On the contrary, the haemoglobin and haematocrit levels were elevated in both diseased and healthy fish, and these could be due to low water temperature (Shimma *et al.*, 1984; Martinez *et al.*, 1994). The blood parameters should also be studied at different locations because the relatively high altitude may have influenced some of the blood parameters.

Histopathological changes would be expected in some organs (gill, kidney, spleen) when the gross clinical and pathological signs of this disease occurred (Austin and Stobie, 1992). In addition, histopathological changes were observed in liver, muscle, heart and intestine in the present study. Oedema and congestion in liver, and fibroblast proliferation in spleen, heart and intestine tissues were typical of chronic infection. The glycogen vacuolations were observed in only a part of liver but it may not be a function of infection. These types of vacuolations may be caused by nutritional factors (Roberts, 1989), but they may also be seen in healthy fish (Roberts, 1989; Grizzle and Kiryu, 1993).

In agreement with Austin and Austin (1999), *Micrococcus luteus* must be considered as a potential bacterial pathogen for fish. Infection caused haematological and histopathological changes in the infected fish examined. The chemotherapy with formalin + alcohol mixture and enrofloxacin provided complete recovery of the naturally infected fish. Generally, the treatments of sporadic infections are not considered an important factor due to the fact that the disease did cause loss. These could play a role for increasing infections, and also changing types of infections depending on the species, age of fish and virulence of bacterial strains. Since epizootics may appear and inflict significant financial losses, the control of sporadic infections should be considered.

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