

### **CITROBACTER FREUNDII AS A CAUSE OF DISEASE IN FISH**

JEREMIĆ SVETLANA\*, JAKIĆ-DIMIĆ DOBRILA\* and VELJOVIĆ LJ\*\*

\*Serbian Institute of Veterinary Science, Belgrade, \*\*Veterinary Institute, Zemun

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*The paper describes an illness of one-year rainbow trout fry that was characterized by gastroenteritis and progressively high mortality, but which did not indicate a typical bacterial infection; and a clinical illness of cyprinids that indicated typical acute bacterial septicemia caused by Gram-negative bacteria. These diseases of rainbow trout and cyprinids were caused by the Gram-negative motile bacterium *Citrobacter freundii*.*

*Cultures of *Citrobacter freundii* were isolated and identified on the basis of key phenotypic characters and with the aid of the Api 20 E system.*

*Pathohistological examination confirmed inflammatory changes in the intestine of rainbow trout; and inflammatory and necrotic changes in the internal organs of cyprinids.*

*We were able to reproduce the illness by means of artificial infection with a pure culture of *Citrobacter freundii*.*

*This is the first published report confirming *Citrobacter freundii* as a cause of fish disease in Serbia.*

*Key words: *Citrobacter freundii*, isolation, identification, fish, biological experiment*

### INTRODUCTION

Intensive fish farming is among the most profitable means of producing animal protein. The main condition for accomplishing this task is that vital and productive functions of the fish be maintained within physiological limits. However, every change of abiotic environmental factors (decrease of oxygen percentage, frequent changes of water temperature, changes of pH value, increased concentration of ammonia and carbon dioxide), high nursery density, and nonobservance of necessary measures prior to overwintering of carp all facilitate the spread of many diseases, including bacterioses. The incidence of a bacterial disease of fish new for Serbia is examined herein.

Mass mortality of cyprinid fish occurred in a reservoir at the end of March and beginning of April. The percentage of deaths among fry of carp (*Cyprinus carpio*) and crucian carp (*Carassius carassius*) here was significantly greater than in fry of amur (*Ctenopharyngodon idella*) and roach (*Rutilus rutilus*). The disease was characterized by typical hemorrhagic septicemia on the skin and internal organs.

On a fish farm during the summer period, gastroenteritis leading to progressively high mortality appeared in one-year fry of rainbow trout with average weight of 130 g. The gastroenteritis of rainbow trout and hemorrhagic septicemia of cyprinids were caused by the Gram-negative motile bacterium *Citrobacter freundii*.

The first publication describing pathology and isolation of this species of bacteria from aquarium fish was the communication of Sato *et al.* (1982). *Citrobacter freundii* was subsequently isolated from Atlantic salmonids in Spain and the USA (Bava *et al.*, 1990); and from carp in India (Karunasager *et al.*, 1991).

The present work describes an illness of one-year rainbow trout fry characterized by gastroenteritis and a disease of cyprinids characterized by hemorrhagic septicemia, both of which are caused by *Citrobacter freundii*. Also given are morphological and biochemical characteristics of the isolated bacteria, which are consistent with the those of *Citrobacter freundii* as described by other authors.

We were able to reproduce the disease by means of artificial infection.

#### MATERIAL AND METHODS

For laboratory examination, we took 39 specimens of cyprinids with darkening of the body, pronounced exophthalmus, bleeding of the eyes and fins, and locomotor ataxia; and 50 specimens of rainbow trout with gastroenteritis.

A part of the altered organs of cyprinids (liver, kidney, spleen) and intestine of rainbow trout were fixed in 10% Formalin, embedded in paraffin, and used to make tissue sections 6 : thick, which were stained with hematoxylin eosin.

Chemical and microbiological analyses of water from the trout farm were performed using standard methods.

As material for isolation of agents, we used gills, parenchymatous organs (liver, kidney, spleen), and intestine of sick and clinically healthy fish. Seeding was conducted directly from the kidney, liver, spleen, and intestine of sick and healthy fish by coating the surface of nutritive substrates: nutritive agar, blood agar, tryptose soya agar containing 10% FTS, and endoagar. The seeded substrates were incubated at 30EC (for material originating from cyprinids) and 20EC (for material from trout) for a period of 5 days. Suspected colonies from tryptose soya agar and endoagar were reseeded on 2% nutritive agar to obtain pure cultures, which were then identified on the basis of key phenotypic characters and with the aid of the API 20 E system for enterobacteria. In addition to this, we tested oxidase and catalase.

Isolates (01-870 and 4138) were tested for pathogenicity. Carp and rainbow trout fry were used for the biological experiment. The first group consisted of 20 specimens of carp fry with average weight of 10-13 g, the second of 15 specimens of trout fry with average weight of 7 g. Both groups were kept in aquaria with aerated static water at 11EC. The fish were injected with 0.2 ml of a bacterial suspension of isolate 01-870 and isolate 4138 containing about  $10^7$  c.f.u./ml. All infected fish were examined daily, and dead and moribund fish were examined bacteriologically.

## RESULTS AND DISCUSSION

Before the 80's of the last century, there were indications that *Citrobacter freundii* can cause disease in fish. However, definite data were not obtained until the papers of Sato *et al.* in 1982, when the organism was proved to be pathogenic for aquarium fish, and in the 90's for farmed fish. *Citrobacter freundii* was isolated from diseased Atlantic salmonids in Spain (Bava *et al.*, 1990) and the USA (Sans, 1991), and from carp in India (Karunasager *et al.*, 1992).

*Pathoanatomical Examination:* The first deaths of cyprinids started at a water temperature of 11EC during the period of March-April. Diseased fish are calm, execute uncoordinated movements, and float on the surface of the water. They do not respond to external stimulation and are darkly pigmented.

External examination encompassing the skin, gills, and fins, and examination of natural openings revealed an increase in the quantity of mucous mass on the surface of the skin and gills. Erosion and dropping off of scales were established on the skin. Diffuse bleeding was recorded on the skin and fins. Bilateral exophthalmus and bleeding in the eyes were recorded in all specimens. Diffuse bleeding was established on the ventral part of the abdomen. The anal orifice was bloody. The gills were pale due to anemia, exhibited petechial hemorrhages, and were edematous and with necrosis of the tips of the gill filaments in some specimens.



Figure 1. Erosion of the skin and dropping off of scales, beeleeding in jugularis region



Figure 2. Diffuse bleeding on the skin, exophthalmus and beeleeding in the eyes



Figure 3. Patchial hemorrhages on the gills associated. With necrosis of the tips of the gills

All internal organs and the wall of the swim bladder were edematous in section. A small quantity of reddish fluid was present in the abdominal cavity. Bleeding was recorded on the internal organs, primarily on the inner wall of the swim

bladder and on the gonads, intestine, muscles, kidneys, and liver. The spleen was enlarged and unevenly colored. The wall of the intestine was edematous, the lumen expanded, lacking food, and filled with a bloody fluid. Petechial and diffuse hemorrhaging was present in the wall.



Figure 4. Bleeding on internal organs (Liver, kidneys, gonads and muscles)



Figure 5. Edematous and congestive intestinal mucosa as sign of inflammatory process

Our results of pathoanatomical examination were identical with those obtained by Karunasager *et al.* (1991), who established erosion and hemorrhaging on the skin in carp, focal nodules in the kidneys, and other lesions typical of hemorrhagic septicemia.

The first deaths of rainbow trout were recorded at a water temperature of 10EC during the period of June-July. Sick trout with an average weight of 130 g kept to the rim of the basin, adhered to the grill, and dropped to the bottom. Mortality increased progressively every day and at the end of a week's time comprised 150 to 200 dead trout per basin. Other than dark pigmentation, no characteristic disease symptoms were evident externally. Sections showed the digestive tract to be without food and filled with watery mucoid contents that was bloody in certain specimens. The other internal organs (liver, kidney, spleen, and swim bladder) had a normal appearance. Our results of pathoanatomical examination were identical with those obtained by Austin *et al.* (1992) on rainbow trout.

*Pathohistological Examination:* We established pathohistological changes on the liver, kidneys, and intestine of cyprinids. Fatty degeneration of the liver, i.e., accumulation of fatty cells, was recorded in a large number of cases. In other specimens, inflammatory and necrotic changes and weaker bleeding were established in tissue of the liver.

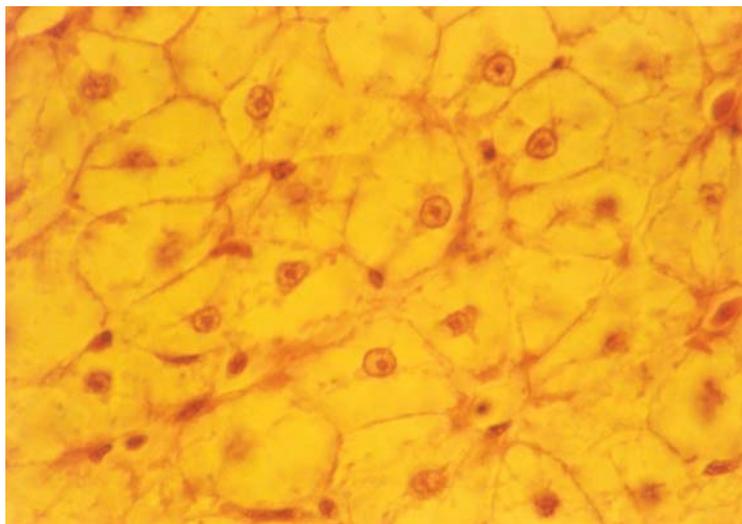


Figure 6. Lipoid liver metamorphosis

Microscopic examination of renal tissue showed epithelium of the renal canals to be intact. The lumen of the renal tubules was visible and completely empty. A mononuclear cellular infiltrate was detected in the interstitia of the kidneys, i.e., intertubularly and perivascularly. The infiltrate in places exhibited a tendency

toward mutual confluence, encompassing greater areas of renal tissue in this way. Weak bleeding was detected here and there.

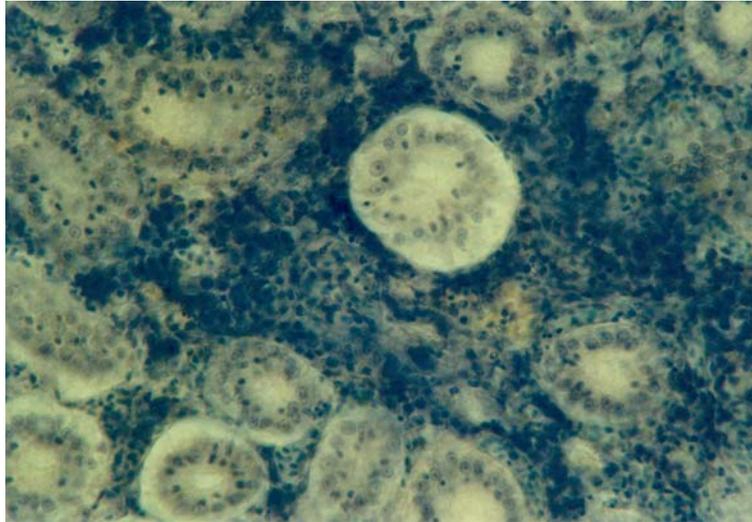


Figure 7. Interstitial nephritis

Microscope slides of the intestine showed the mucosal propria to be infiltrated with inflammatory cellular elements of medium intensity. Enlarged cup-

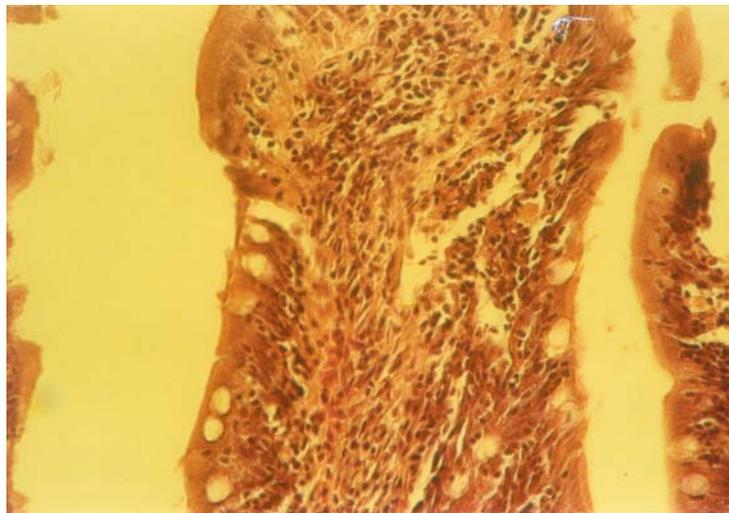


Figure 8. Propria mucosa is infiltrated with inflammatory cell elements. Along the intestinal epithelium, multiplied epithelial cell are visible

shaped cells were visible in places along the intestinal epithelium. The intestinal villi were hypertrophied with a tendency to fuse in certain preparations.

Total destruction of mucosal epithelium was evident on pathohistological preparations of the intestine of rainbow trout, so that only remnants of villus architecture were discernible, or else it was completely unorganized. The mucosal tunica was infiltrated with mononuclear cellular elements.

**Bacteriological Tests:** Seeding on TSA gave an apparently pure transparent bacterial growth from the liver, kidneys, and intestine of all sick fish. Colonies measuring 2-4 mm in diameter were formed on nutritive agar. These colonies were round, smooth, and convex. They were not pigmented and did not induce hemolysis on blood agar. Bacteria were not isolated from 10 clinically healthy cyprinids. On endoagar, medium-sized colonies were formed that were transparent and colorless. These colonies resembled colonies of *Salmonella* and *Shigella*. After incubation for more than 48 h, such colonies became pinkish. They acquired a red color after 3-5 days, since they decomposed lactose slowly.

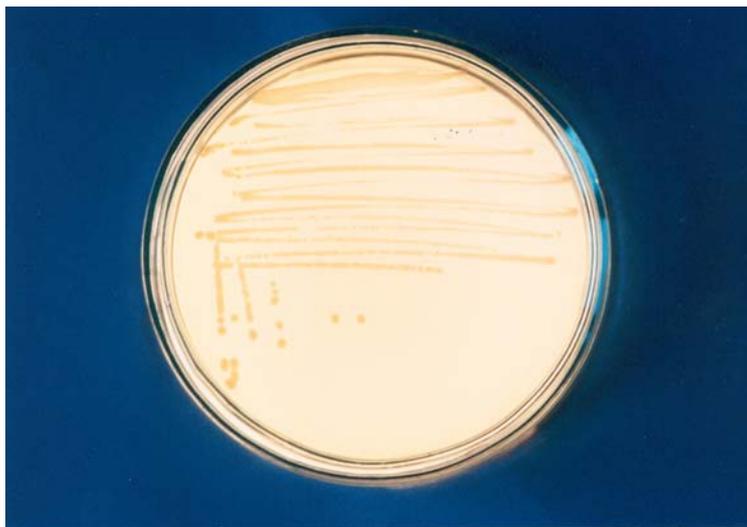


Figure 9. *Citrobacter freundii* - growing on the TSA

The cultures contained Gram-negative asporogenic rods.

As can be seen in Table 1, the tested isolates from carp, crucian carp, and trout- and that of Austin *et al.* (1992) -formed catalase,  $\beta$ -galactosidase, and  $H_2S$ , but not arginine dehydrolase, ornithine lysine, decarboxylase, tryptophan deaminase, or indole. These isolates differed in that the isolate from trout also formed ornithine decarboxylase. Urea was not degraded.

Table 1. Biochemical Characteristics of Isolates of *Citrobacter freundii* from trout and cyprinids

Reaction	Isolate from carp	Isolate from crucian carp	Isolate from trout	Austian isolate
ONPG	+	+	+	+
ADH	-	-	-	-
LDC	-	-	-	-
ODC	-	-	+	-
CIT	-	-	-	-
H <sub>2</sub> S	+	+	+	+
URE	-	-	-	-
TDA	-	-	-	-
IND	-	-	-	-
VP	-	-	-	-
GEL	-	-	-	+
GLU	+	+	+	+
MAN	+	+	+	+
INO	-	-	-	-
SOR	+	+	+	+
RHA	+	+	+	-
SAC	+	+	-	-
MEL	-	+	-	-
AMY	+	+	+	+
ARA	+	+	+	+
OX	-	-	-	-
CAT.	+	+	+	+

The obtained results indicate that our isolates from crucian carp, carp, and trout did not degrade gelatin, whereas the isolate of Austin *et al.* did so. Sodium citrate was not used.

The isolate from crucian carp formed acid from glucose, mannose, sorbitol, rhamnose, saccharose, melebiose, amygdalin, and arabinose. The isolate from carp decomposed all of the foregoing carbohydrates except for melebiose, whereas that from trout decomposed a significantly smaller number of carbohydrates (it did not form acid from saccharose and melebiose). Comparing the biochemical characteristics of our isolates with those of the isolate described by Austin *et al.* (1992), we conclude that there are strains of *Citrobacter freundii* which weakly decompose carbohydrates and ones that do so more strongly.

From the obtained results, the cultures were identified as *Citrobacter freundii*. These results also enabled us to conclude that there are strains of *Citrobacter*

*freundii* which weakly decompose carbohydrates and ones that do so more strongly.

The isolates were sensitive to flumequin, nalidixic acid, OTC, and strengthened sulfonamides.

Disease symptoms and 60% mortality in the biological experiment with trout set in on the 5<sup>th</sup> day after i/p infection. Bleeding in the eyes, gastroenteritis, and the presence of ascitic fluid in the peritoneal cavity were recorded in moribund and dead trout.

In carp, disease symptoms and 50% mortality set in on the 8<sup>th</sup> day after i/p infection. Petechial and diffuse hemorrhaging was detected on the skin in moribund and dead carp. The gills were anemic with petechial hemorrhages and swelling of the gill filaments. Sections revealed peritonitis and a bloody transudate in the abdominal cavity. The liver was light-pinkish in color, with petechial hemorrhages. The spleen was enlarged, with rounded edges. The kidney was grayish in color. Inflammation of the intestine was recorded, together with the presence of slimy contents.

*Citrobacter freundii* was isolated from all dead and moribund fish. Moreover, the pathogen was reisolated from the kidneys and liver of fish surviving at the end of the experiment.

Chemical and microbiological testing of water from the canal supplying the trout farm showed it to be turbid, pale-yellow in color, and with a significantly reduced percentage of oxygen (6.20 mg/l). Microbiological testing of the water indicated that the number of coliform bacteria was significantly greater than that permissible for water of quality class I.

Although *Citrobacter freundii* is commonly present in eutrophic waters (Allen *et al.*, 1983), we feel that the sickness of trout was caused by poor quality of the water, which was turbid, pale-yellow in color, and with reduced O<sub>2</sub> concentration and an increased number of coliform bacteria. For processes of their growth and development, trout require cold, clean, and clear water, with an adequate flow rate and sufficient oxygen. With the least bit of muddying, uncleanness, or pollution of the water supplying trout farms, it becomes extremely difficult to satisfy the requirements of farmed rainbow trout fry. The amount of dissolved oxygen plays a significant role because it ensures metabolism in the organism of the fish. An O<sub>2</sub> content of 9-11 mg/l in the water is considered ideal. The degree of saturation of the water with O<sub>2</sub> largely depends on water temperature, and oxygen content at 10EC should be 11.25 mg/l, but in our case it was only 6.20 mg/l for longer periods of time.

We note that sickness in cyprinids occurred following a cold winter, with many freezing days and frequent snows. Fish are poikilothermal organisms, i.e., they take on the temperature of the water surrounding them, and every change of temperature has a great effect on the course of vital processes. The fry of cyprinids are more sensitive than adults, and the lowest permissible temperature for them on farms is 0,1-0,2EC. During long cold spells at such temperatures, resistance of the organism of the fish declines, disease sets in, and mass death of fry occurs.

The present paper represents the first published report confirming *Citrobacter freundii* as a cause of fish disease in Serbia.

Address for correspondence:  
Dr Jeremić Svetlana  
Scientific Veterinary Institute of Serbia,  
Vojvode Toze 14,  
11 000 Belgrade, Serbia & Montenegro

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#### CITROBACTER FREUNDII UZROČNIK OBOLJENJA RIBA

JEREMIĆ SVETLANA\*, JAKIĆ-DIMIĆ DOBRILA\* i VELJOVIĆ LJ\*\*

#### SADRŽAJ

U radu je opisano oboljenje jednogodišnje mlađi kalifornijske pastrmke koje se karakterisalo gastroenteritisom i progresivno visokim mortalitetom, kao i kliničko oboljenje šaranskih vrsta riba koje je ukazivalo na tipičnu akutnu bakterijsku septikemiju izazvanu Gram negativnim bakterijama. Ova oboljenja su bila izazvana Gram negativnom pokretnom bakterijom *Citrobacter freundii*.

Bakteriološkim pregledom 39 uzoraka obolelih šaranskih vrsta riba i 50 uzoraka mlađi kalifornijske pastrmke iz promenjenih parenhimatoznih organa i creva obolelih riba izolovana su 3 soja *Citrobacter freundii*.

Dijagnostički materijal uzet od riba, bakteriološki je obrađen, tako što je zasejan na hranjive bakterijske podloge: hranjivi agar, TSA sa 10% FTS, krvni agar i endo agar. Inkubiranje podloga je vršeno na temperaturi od 30°C sa materijalom koji potiče od šaranskih vrsta riba i na temperaturi od 20°C sa materijalom

koji potiče od pastrmki, tokom 3-5 dana. Karakteristične kolonije koje su se odlikovale okruglim izgledom, prozirne, bezbojne i bez hemolize prenete su na nove podloge radi dobijanja "čistih" kultura u cilju daljeg postupka determinacije.

Izolati su bili ispitani radi određivanja biohemijske aktivnosti bakterija na Api 20E sistemu za enterobakterije. Svi ispitani izolati stvarali su katalazu,  $\beta$ -galaktozidazu,  $H_2S$  i razlagali kiselinu iz glukoze, manoze, sorbitola, ramnoze, amigdalina i arabinoze. Pojedini izolati su slabije ili više razgrađivali ostale ugljene hidrate.

Veštačkom infekcijom mlađi riba je *i/p* inficirana sa 0,2 ml  $10^7$  c.f.u./ml čistom kulturom *Citrobacter freundii* nakon čega smo uspeli da reprodukujemo oboljenje.

Na osnovu ukupnih rezultata ispitivanih sojeva koji su izolovani iz promenjenih unutrašnjih organa riba ustanovljeno je da pripadaju vrsti bakterije *Citrobacter freundii*. To je ujedno i prvi objavljeni izveštaj o utvrđivanju *Citrobacter freundii* kao uzročnika oboljenja riba u Srbiji.