*A. Ü. Vet. Fak. Derg.*
**31 (3)** : 508-516. 1984

# CHARACTERIZATION OF AEROMONAS SOBRIA ISOLATED FROM DISEASED CARPS (Cyprinus carpio)

**K. Serdar Diker**[1] **Ömer Akay**[2] **Ayşe Burgu**[3]

**Hasta Sazanlardan (Cyprinus carpio) İzole Edilen Aeromonas sobria'nın Çeşitli Özellikleri Üzerinde İncelemeler**

**Özet:** *"Hareketli aeromonas septisemisi" belirtileri gösteren hasta sazanlardan bir Aeromonas sobria suşu izole edildi. Hasta balıklarda, deride kanamalar ve karın boşluğunda kanlı sıvı birikimi görüldü. Balıkların ekim yapılan tüm dokularından izole edilen mikroorganizma, biyokimyasal testlerle Aeromonas sobria olarak identifiye edildi. A sobria, A hydrophila'dan negatif arabinoz, salisin ve eskülin testleri ile ayrıldı. Mikroorganizma, tavşan ve koyun eritrositlerinin lizisine neden oldu. İzole edilen suş, süt emen fare deneyi ile enterotoksijenik olarak bulundu. Canlı bakteri ve bakterinin hücresiz sıvısı erişkin fareleri de öldürdü. Bakteri kültürüne katıldığında, amonyum sülfatın bakterinin tüm toksijenik özelliklerini artırdığı saptandı . Bu bulgular sonucunda, sazanlardaki septiseminin A. sobria'dan ileri geldiği belirlendi.*

**Summary:** *An Aeromonas sobria strain was isolated from the moribund carps with motile aeromonas septicemia. Gross lesions of diseased fishes were mainly hemorrhagies on skin and the bloody fluid in abdominal cavity. The organism isolated in pure culture from all tissues of fishes was identified as Aeromonas sobria on the basis of biochemical tests. A. sobria was separated from A. hydrophila with negative reactions in arabinose and salicin fermentation and aesculin hydrolysis tests. The organism caused the hemolysis of rabbit and sheep red blood cells. Enterotoxigenicity of A. sobria was tested by suckling mouse assay and the organism was considered as enterotoxigenic. Live bacteria and cell free supernatant of organism were found to be lethal for adult mice. All the toxigenic activities of microorganism were increased by the addition of ammonium*

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*sulfate to culture. In view of results, it is postulated that A. sobria is associated with septicemia found in carps.*

## Introduction

Motile aeromonas septicemia (MAS) is a common bacterial disease syndrome in cultured, farm pond and reservoir fish populations. Diseases caused by aeromonad organisms range from acute, rapidly fatal septicemia to latent infections and have been referred to as "infectious abdominal dropsy", "hemorrhagic septicemia" and "motile aeromonas septicemia"(10). The many names given to these diseases unfortunately were based on some of the symptoms of the particular disease and has resulted in taxonomically distinct bacteria being implicated in the same diseases.

The genus Aeromonas comprises species known to be pathogenic for a wide spectrum of hosts from fish to human (1, 13). In the last edition of Bergeys' Manual, the genus Aeromonas has been divided to three species, *A. hydrophila, A. punctata and A. salmonicida,* with a further dividing in a total of eight subspecies (22). However, Popoff and Veron (21), considered that *A. hydrophila* could not be distinguished from *A. punctata* and suggested a change in the classification of motile aeromonads. This taxonomic study resulted in a proposal for a division *A. hydrophila* (A. punctata syn.) into two biovars and creation of a new species of *A. sobria* (18). The current classification of aeromonads thus identifies three species: *A. hydrophila, A. sobria* and *A. salmonicida.* Former two species contain motile strains whereas *A. salmonicida* comprises nonmotile strains. Both *A. hydrophila* and *A. sobria* cause almost same diseases or closely related disease syndromes.

Unlike most other gram-negative organisms, Aeromonas species produce a range of extracellular enzymes and toxins (16). The extracellular protein profiles of *A. hydrophila* and *A. sobria* are similar. Two products mainly associated with the virulence of strains are hemolysin and enterotoxin (17). Enterotoxin produced by motile aeromonads is immunologically related to cholera toxin (12).

In the present study, an aeromonas strain isolated from the moribund carps with hemorrhagic syndromes was characterized biochemically and examined for possible virulence determinants.

## Materials and Methods

*Diseased fishes:* The diseased wild carps (Cyprinus carpio) on average lenght of 60 cm were taken from the Lake of Gölbaşı-Mogan, Ankara. The fishes were submitted to the laboratory in a moribund stage. Necropsies were performed on these carps. During this procedure, macroscopic lesions were recorded and various tissues were taken for the parasitological and bacteriological examinations. The main gross lesions of affected fishes were exophthalmus, prolapsus ani, focal dermal hemorrhagies particularly on the lateral sides and whole congestion of ventral part of body. Musculature lying under skin lesions was also found hemorrhagic. Fins were oedematous and pale. There were a foul -smelling bloody-watery fluid and a bloody gelatinous mass formed by the clotting of ascitic fluid. Peritoneal-visceral adhesion was also recorded. The liver and the kidney swollen and the color of liver was in pale. The gallbladder was distend and gall was dark in color. Gastrointestinal tract was almost empty and congestive.

*Parasitological examination:* The parasitological examination comprised body surface, gills, abdominal cavity, digestive, tract, blood, swimming bladder, liver, kidney and muscles.

*Bacteriological examination:* Samples taken from skin, musculature, kidney, liver and gills were cultured on % 7 sheep blood agar plates at 37°C and 25°C for 24 hours.

*Morphology and motility:* After incubotion period, the morphology of isolated organism was investigated in gram stained preparations from blood agar plates and nutrient broth. Motility in broth cultures was studied by dark field microscopy at 4°C, 25°C, 37°C and 42°C. This property was furthermore confirmed by using a semisolid medium.

*Growth characteristics:* The growth at different temparatures (5°C and 42°C) was determined by using nutrient broth and blood agar plates. The ability of growth on McConkey agar (Difco) was also determined. Pigment production was detected on TSA plates.

*Biochemical activities:* Procedures used in the determination of biochemical characteristics were taken from Cowan (7), but a few supplementary tests were conducted. In all tests, incubation temparature was always 25°C . In carbonhydrate fermantation tests, nut-

rient broth containing 1% of specific carbonhydrate along with bromt-thymol blue as indicateor was used to determine acid production from different carbonhydrates. Gas production from glucose was detected by inverted Durham tubes. Hydrogen sulfide production was detected by blackening of lead acetate paper strips above nutrient broth cultures. The ability to hydrolyse aesculin was determined by a blackening following growth in nutrient broth containing 0.1 % of aesculin and 0.1 % of ferric chloride. DNA hydrolysis were tested by using DNA ase test agar (Oxoid) and 1 N HC l as described by Pham and Davis (20).

*Preparation of cell-free supernatant:* One colony from blood agar was inoculated into 5 ml of BHI broth (Difco) complemented with 1 % $(NH_4)_2SO_4$ and agitated at 25°C for 24 hours. Five millilitres of broth culture were centrifuged at 7000 rpm for 15 min and passed through a sterile 0.45 millipore filter. The filtrate obtained was used as cell-free supernatant.

*Detection of hemolysin:* Two fold serial dilutions of filtrate in 0.85 % saline containing 0.1 % bovine albumin (pH 7.2) were mixed with an equal volume of a 1 % suspension of washed rabbit red blood cells. The amount of hemolytic activity was expressed as the highest dilution of filtrate showing complete hemolysis after incubation at 37°C for 45 min.

*Detection of enterotoxigenicity:* Enterotoxin activity was tested by suckling mouse assay in which both cell-free supernatant and cell-free supernatant with $(NH_4)_2SO_4$ were used as inoculum. Both inoculums (each 0.1 ml) were tested in the suckling mice followings Dean's (8) procedure except that three mice instead of four were used in each test and oral administration route was used. A ratio of gut to body weight of 0.09 after 5 hours was considered as positive response.

*Detection of pathogenicity:* Adult mice were used as experimental animals. Three group of two mice were inoculated with following routes: 1) 0.1 ml of cell-free supernatant was injected intravenously, 2) 0.5 ml of 18 h broth culture was injected intra-peritonally and 3) 0.5 ml of broth culture was given orally.

*Antibiotic susceptibility test:* The sensitivity of organism to 16 different antibiotics was determined on DST agar (Oxoid) plates by using disc-diffusion method (4).

## Results

In parasitological examination, Dactylogyrus sp., Gyrodactylus sp., Trichodina sp. and Trichodinella sp. were detected on gills and skin. Trypanosoma sp. and Myxosporidia spores were found in blood. There were also development stages of Coccidia in intestine.

An organism was isolated in pure culture from all tissues of diseased fishes cultured bacteriologically. On the basis of following tests, the organism identified as *Aeromonas sobria*. On sheep blood agar, the colonies were 2–3 mm in diameter, round entire, flat raised, semitranslucent, greyish - white and hemolytic with a clear zone. A yellow pigmentation was observed on TSA plates. Microscopic examination of Gram's stain revealed gram-negative straight rods with parallel sides and rounded ends showing some short chains. The organism was able to grow at 5°C and 42°C with a faint turbidity or a few colonies after 5 days of incubation. The organism was motile at 5°C, 25°C and 37°C but not at 42°C. Growth was observed on McConkey agar plates (Table 1).

Table 1. Several characteristics of *A. sobria*

| Examination | 5°C | 25°C | 37°C | 42°C |
|---|---|---|---|---|
| Growth | +[a] | + | + | +[a] |
| Hemolysis on sheep blood agar | — | + | + | — |
| Motility | + | + | + | — |
| Pigment production | ND | + | ND | ND |
| Growth on McConkey Agar | ND | + | + | ND |

(a) = with a few colonies or a faint turbidity
ND = Not Done

The organism was O/F, oxidase, catalase, VP and DNA ase positive. It reduced nitrate to nitrite. Negative reactions were found in indol, urea, $H_2S$, aesculin and salicin tests. The organism attacked some carbonhydrates fermentatively and produce gas from glucose (Table 2). Cell-free supernatant caused the complete hemolysis of rabbit red blood cells at 1/16 titre.

The results of suckling mouse test are shown in Table 3. In groups given cell-free supernatant with $(NH_4)_2SO_4$, cell-free supernatant alone and 0.85 % saline as control, the ratios of gut to body weight were 0.1, 0.09 and 0.06, respectively. In the view of this result, the organism was considered as enterotoxigenic.

Table 2. Biochemical characteristics of A. sobria

| | | | |
|---|---|---|---|
| O/F | +/+ | Lactose | — |
| Oxidase | + | Raffinose | — |
| Catalase | + | Arabinose | — |
| Nitrate | + | Galactose | + |
| Urea | — | Maltose | + |
| $H_2S$ | — | Trehalose | + |
| Indole | — | Mannose | + |
| VP | + | Fructose | + |
| DNA ase | + | Xylose | — |
| Aesculin | — | Dulcitol | — |
| Salicin | — | Adonitol | — |
| acid | + | Sorbitol | — |
| Glucose | | Inositol | |
| gas | + | | — |

Table 3. The results of suckling mouse test

| Group | Suckling mouse | Gut weight (g) | Body weight (g) | G/B |
|---|---|---|---|---|
| Cell-free supernatant with $(NH_4)_2SO_4$ | 1 | 0.16 | 1.41 | |
| | 2 | 0.17 | 1.69 | |
| | 3 | 0.15 | 1.73 | |
| | Total | 0.48 | 4.73 | 0.101 |
| Cell-free supernatant | 1 | 0.13 | 1.45 | |
| | 2 | 0.14 | 1.55 | |
| | 3 | 0.14 | 1.53 | |
| | Total | 0.41 | 4.53 | 0.090 |
| 0.85 % saline (control) | 1 | 0.11 | 1.72 | |
| | 3 | 0.11 | 1.64 | |
| | 2 | 0.10 | 1.40 | |
| | Total | 0.32 | 4.76 | 0.067 |

Cell-free supernatant injected i.v caused the death of mice after 24 hours of infection. Mice inoculated i.p with broth culture died 12 hours after injection. Broth culture given orally caused the death of mice 36 hours after inoculation.

The organism was found sensitive to tetracyclin, chlorotetracyclin, neomycin, colistin sulfate, chloromphenicol, streptomycin, rifamycin, nitrofurantoin, erythromycin and trimethoprim-sulphamethaxol, but resistant to penicillin, ampicillin, carbenicillin, bacitracin and novobiocin.

## Discussion

Gross lesions on skin and musculature of diseased fishes were similar to septicemia and erythrodermatitis defined by Antrychowicz and Ragulska (2). However, other lesions on internal organs and bloody-ascitic fluid indicated that the disease was hemorrhagic septicemia. Furthermore, the organism was very often found in pure culture from the kidney and liver indicating the presence of septicemia.

Since the initial of the genus Aeromonas, the taxonomic classification of the motile aeromonads has presented a confusing picture, mainly due to the relatively minor biochemical differences which have been found between strains (15, 22). The taxonomy of motile aeromonads is still under dispute but in this study we fallowed Popoff and Veron's (21) simplified classification. The bacteriological methods used in the present study were effective in isolating motile aeromonads from organ samples of affected fishes. The isolated organism showed typical biochemical characteristics of genus Aeromonas, furthermore *A. sobria* . *A. hydrophila* can use arabinose and salicin as source of carbon and energy (22). It can also hydrolyse aesculin. On the other hand, *A. sobria* is negative to all these properties (21). Based on these biochemical tests, the organism was classified as *A. sobria.*

*A. hydrophila* and *A. sobria* both produce many extracellular enzymes and toxins related with their toxigenicity (16). De Figueiredo and Plumb (9) have reported that the differences in biochemical test between the different isolates of motile aeromonads were insufficent to separate the virulent from avirulent strains. Boulanger et al (5) and Olivier et al (19) have reported that *A. sobria* strains were less hemolytic than the *A. hydrophila* strains and the type of hemolysis produced by *A. hydrophila* and *A. sobria* was different. In contrast, *A. sobria* isolate caused the hemolysis of rabbit red blood cells in saline and sheep red blood cells on the sheep blood agar with a zone as clear as *A. hydrophila.* Boulanger et al (5) and Olivier et al (19) have reported that all the *A. sobria* isolates were positive in suckling mouse test. It was determined that addition of ammonium sulfate to culture increased the production of hemolysin by Aeromonas (24). Olivier et al (19) also showed that all the toxigenic activities were increased by the addition of ammonium sulfate. In the present study, the organism was considered as enterotoxigenic by the suckling mouse assay. It was also obtained

a greater ratio (0.1) with ammonium sulfate than without ammonium sulfate (0.09). This work has also shown that isolated organism was very pathogenic for adult mice. In view of these results, it is postulated that *A. sobria* strain could be associated with septicemia found in carps.

Under normal conditions, motile acromonads normally found in water and usually do not cause a problem in fish populations (14). But, when the fishes are under environmental and physiological stress, motile acromonads are potential pathogens. Some parasites may also play a role in the initiation of bacterial diseases together with environmental factors (11). In Turkey, These parasites are not uncommon for wild and cultured carp (6). These parasites were determined in affected carps, but fishes were not heavily infected with them. Teherefore, parasites mentioned above were not found directly related with symptoms and lesions.

Baran et al (3) have reported an outbreak of bacterial hemorrhagic septicemia in rainbow trout caused by *A. hydrophila* in a fish farming station in Turkey. Recently, Timur (23) has described an outbreak of *A. hydrophila* infection among the ell at same station. In the present study., *A. sobria* is isolated from an outbreak of motile acromonas septicemia of diseased carps for the first time in Turkey.

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