Detection and study of the experimental infection of Aeromonas strain in the common carp (*Cyprinus carpio* L.)

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Summary

The aim of this study was to isolate motile *aeromonad* strain of common carp (*Cyprinus carpio*) which was obtained from farms around Baghdad and identification of the isolate by using Analytical profile index API20E. Experimental infection was carried out by intramusculer injecting LD50 does $0.3\times 10^{8.66}$ cell/fish into common carp *Cyprinus carpio* L. Infected fish after 14 days of injection showed hemorrhage and ulceration on the body surface, eye abnormalities and accumulation of red-colored ascetic fluid . Macroscopically pale liver with hemorrhagic and necrotic spots was seen and kidney showed liquefaction. Microscopically severe necrotic changes in muscular structure and internal organs with intense polymorphonuclear cell. Infiltration characterized by of pyknosis of nuclei with presence of cellular detritus were seen.

Keywords: Aeromonas *hydrophila,* bacteria, common carp, *Cyprinus carpio*.

التحري واحداث اصابة تجريبية لعترة *Aeromonas* في اسماك الكارب الشائع (*Cypninus carpio* L.)

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الخلاصة

هدفت الدراسة التحري عن بكتريا المتحركة عترة *Aeromonad* من اسماك جلبت من حقول لاسماك الكارب حول بغداد وشخصت العزلة باستعمال عدة تشخيصية جاهزة . $API_{20}E$. تم احداث اصابة تجريبية بحقن الجرعة المميتة LD50 $0.3\times10^{8.66}$cfu/ml من العزلة المرضية في داخل العضلة لاسماك الكارب الشائع *Cypnuris carpio* L ظهرعلى الاسماك بعد 14يوم من الحقن نزف على سطح الجسم, تقرح مع بروز العين وتجمع سوائل حمراء في منطقة البطن. عيانيا ظهر وجود بقع نزفية وتنخرية على سطح الكبد وهشاشة الكلية اما نسيجيا ظهر وجود تغيرات تنخرية شديدة بالتركيب العضلي والاعضاء الداخلية مع ارتشاح شديد لخلايا متعددة النوى تمتاز بتضخم النوى ووجود بقايا الخلايا التالفة .

الكلمات المفتاحية : بكتريا, الايروموناس هايدروفيلا, الكارب الشائع .

Introduction

Aeromonas hydrophila has been recovered from a wide range of freshwater aquarium and cultured fish species worldwide (1 and 2) . *Aeromonas hydrophila* , a Gram negative motile rod, a member of the family *Vibrionaceae*, has been widely studied and regarded as the most important bacteria causing "aeromonosis, or hemorrhagic septicemia or motile aeromonas septicemia" in fish (3,4 and 5)and other aquatic animals such as frog (6 and 7), prawn (8), crab (2) and mussel (9). The pathological conditions include tail/fin rot and hemorrhagic septicemias in freshwater fish species and occasionally in marine fish (3 and 10).. *A. hydrophila* has also been described as the dominant infectious agent of 'fish-bacterial-septicemia' in freshwater cultured cyprinid fishes, mainly common carp. Although it has been reported that water temperature and environmental factors such as crowding and handling, as well as low dissolved oxygen and nitrite levels, have to be adverse, the pathogenesis of *aeromonads* is not known (7 and 2).

Aeromonas hydrophila was known to produce toxic substances evoking hemorrhage and necrosis (11). Those in vivo and in vitro studies confirmed the bacterial toxin including enzymes such as protease and elastase. This bacterium also produces βtype hemolysin on blood agar plates (12). An infection of *A. hydrophila* producing strong β-hemolysin was found among common carps, Aerolysin and hemolysin genes are reported to be the putative virulence genes of *A.hydrophila* (13).

Aerolysin, produced by some strains of *A. hydrophila*, is an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties. Aerolysin binds to specific glycoprotein receptors on the surface of eukaryotic cells before inserting into the lipid bilayer and forming holes. Hemolysins are exotoxin protein produced by bacteria and the lytic activities of hemolysins on red blood cells are reported to be important for nutrient acquisition *A. hydrophila* is one of the main causes of its mass mortality in Baghdad fish farms, there was only little knowledge of this bacterium from common carp. This paper describes the LD_{50} of pathogenecity of *A. hydrophila* on common carp.

Materials and Methods

Healthy fish and carp with haemorrhages or dermal ulcers on their bodies were obtained from different farm around Baghdad. The samples of the, gill, kidney, and skin of each fish were collected. The samples were placed in 5% sheep blood agar plates (Oxoid) tryptic soy agar (oxoid) and MacConkey agar (Oxoid) plates and then incubated at 25-30ºC for 1-2 day under aerobic conditions. After incubation, the pure hemolytic yellow colonies were isolated from skin and internal organs of all the carp. The bacteria were identified as *A. hydrophila* on the basis of colony morphology, Gram-staining, and biochemical characteristics were used to identification of *A.hydrophila*. Wet mounts of skin, fin, and gill smears were also examined microscopically.

Analytical profile index for *Enterobacteriaceae* API 20E kits (BioMerieux) were used according to the manufacturer's instructions and comparison of the results was made to the BioMerieux database. This kit provides an easy way to identify members of the Enterobacteriaceae and associated organisms. The kit comprises of plastic strip holding 20 mini-test tubes. The strip is normally inoculated with a saline suspension of a pure bacterial culture (as per manufacturer's instructions). This process also rehydrates the desiccated medium in each tube. A few tubes are completely filled (Citrate utilization, Voges Proskauer and Gelatinase) and some tubes are overlaid with mineral oil such that anaerobic reactions can be carried out (Argininedihydrolase, H_2S production, Lysine decarboxylase, Ornithine decarboxylase and Urease). Incubation is in a humidity chamber for 24 hours at 37°C. After the incubation, the color reactions were noted (some with the aid of added reagents). The reactions and the oxidase reaction done separately, and the data are converted to a seven-digit code, which is entered into the manufacturer's computerized database (BioMerieux, Inc; Hazelwood, MO), identification is usually given to genus and species.

The bacterial isolate send to the collaborating laboratory (central health laboratory/Baghdad /ministry of health) to Confirm the identification of *Aeromonas hydrophila* isolated in fish disease laboratory / Baghdad University /Veterinary Medicine College.

The bacteria isolate was passaged for two times in to healthy *Cyprinus carpio* samples to enhance the virulence of bacteria. The bacteria re-isolated from the moribund fish were used for the infection experiment after growing for 24 h at 25-

30°C in Trytic soy agar. The bacterial concentration was determined by plate count **(**spreading method**).**

The lethal dose 50% (LD_{50}) of the pathogens was determined using *Cyprinus carpio* of average weight 50-60 g. The fish were divided into 6 groups using random sampling method. Each group containing 5 fish were injected intramuscularly (i.m.) with 0.3 ml volumes of 10 fold dilutions of freshly prepared (*A.hydrophila)* bacterial suspensions in saline ranging from 10^1 10^2, 10^3, 10^4, and 10^5 viable cells/fish. The control group was injected with 0.3ml of sterile saline and all groups observed for 14 days. Dead fish were subjected to standard microbiological and pathological examinations. The LD_{50} value was calculated after the method of Reed and Muench (1938) adopted by (15) the inoculated bacterium *(A.hydrophila*) was re-isolated from kidney of dead fish by using tryptic soy agar and identified as *A.hydrophila* by characterization tests.

Biopsy of the liver, kidney spleen, intestine and infected muscle fixed in 10% formalin and were examined for a histopathological. Afterwards, the biopsy was dehydrated in a series of ethanol solutions: 50%, 70%, 80%, 90%, 95% and 100% for 1, 1, 2, 2, 1.5 and 16 hours respectively. Each of the samples was then transferred into a xylene solution for another 30min and then embedded in a solution of xylene and wax for 1h. A 5-mm-thick section was cut using a rotary microtome (A.O. Spencer, model Reic heit-Jurg 820, Leica, Germany) and quickly transferred onto a slide and kept in an oven at 40ºC 50 ºC for 24 h. Each sample was observed under a compound light microscope attached with a camera. According to the method described by Drury *et al*., (16).

Results and Discussion

Streaking of inoculum from affected tissues into agar (TSA and NA)plates led to heavy growth of bacterial colonies with similar morphological characteristics that which was inoculated into the blood agar grew into β-hamolysis colony while into Rimler-Shott media grew into yellow colonies .The isolates was found to be gram negative ,motile ,fermentative ,oxidase positive ,0/129 resistant and novobiocin resistant as *Aeromonads* in the primary characterization tests.

The API 20E rapid identification system enabled identification isolate Table (1). According to the result of API 20E test, the isolates were identical to the reference of Bergey's Manual of Determinative Bacteriology. Characterization (based on their morphological and biochemical reactions using the API 20E test). The isolate was identified as *A. hydrophila* with 99 % confidence, according to the manufacturer's database.

Table (1) Identification of bacterial isolates by the API 20E rapid identification system.

Tests	Active ingredients	QTY (mg/cup.)	Reactions/Enzymes	results
ONPG	2-nitrophenyl-βD-galactopyranoside	0.223	B-galactosidase (Ortho NitroPhenyl-βD-Galactopyranosidase)	+
ADH	L-arginine	1.9	Arginine Dihydrolase	+
LDC	L-lysine	1.9	Lysine DeCarboxylase	+
ODC	L-ornithine	1.9	Ornithine DeCarboxylase	_
CIT	Trisodium citrate	o.756	Citrale utilization	+
H_2S	Sodium thiosulfate	0.075	H_2S production	_
URE	Urea	0.76	UREase	_
TDA	L-typtophane	0.38	Tryptophane DeAminase	_
IND	L-tryptophane	0.19	INDole production	+
VP	Sodium pyruvate	1.9	Acetoin production (Voges Proskauer)	+
GEL	Gelatin(bovine origin)	0.6	GELatinase	+
GLU	D-glucose	1.9	Fermentation/oxidation (INOsitol)(4)	+
MAN	D-mannitol	1.9	Fermentation/oxidation (MANnitiol)(4)	+
INO	Inositol	1.9	Fermentation/oxidation (INOsitol)(4)	_
SOR	D-sorbitol	1.9	Fermentation/oxidation (SORbitol)(4)	_
RHA	L-rhamnose	1.9	Fermentation/oxidation (RHAmnoe)(4)	_
SAC	D-sucrose	1.9	Fermentation/oxidation (SACcharose)(4)	+
MEL	D-mlibiose	1.9	Fermentation/oxidation (MELiliose)(4)	_
AMY	Amygdalin	0.57	Fermentation/oxidation (AMYgdalin)(4)	_
ARA	L-arabinose	1.9	Fermentation/oxidation (ARAbinose)(4)	_
OX	(see oxidase test package insert)		Cytochrome-OXidase	+

+ = positive reaction, – = negative reaction

Result of mortality of *Cyprinus carpio* at different concentration of *A.hydrophila* are presented in table (2).LD_{50} fell exactly at the concentration $0.3\times10^{8.66}$ bacterial /ml at 14 day even when plotted arithmetically.Motality of 100% was observed at the concentration 0.3×10^{10} while no mortality was monitored in the control treatment and at the concentration of 0.3×10^{6} at 14 days. Percent mortalities at 10^{7}, 10^{8} , 10^{9} were 11.1%, 37.5% and 75% respectively.

Table (2).LD_{50} of *A. hydrophila*

Bacterial concentration× 0.3ml	Bacterial dilution	Mortality ratio	Died	Survived	Accumulated values			
					Died (D)	Survived (S)	Mortality ratio	Per cent $\frac{(D)}{(D+S)}\times 100$
10^{10}	10^{-1}	5/5	5	0	11	0	11/11	100
10^{9}	10^{-2}	3/5	3	2	6	2	6/8	75
10^{8}	10^{-3}	2/5	2	3	3	5	3/8	37.5
10^{7}	10^{-4}	1/5	1	4	1	9	1/9	10
10^{6}	10^{-5}	0/5	0	5	0	14	0/14	0
control	saline	0/0	0	5	0	19	0/19	0

The LD_{50}= $10^{2.66}$= $0.3\times10^{8.66}$cfu/ml

Disease signs were frequently accompanied by hemorrhagic skin ,ulcerations ,loss of scales ,eye abnormalities, unilateral or bilateral included hemorrhage in the orbit which degenerated to opacity exophthalmia and bursting that dislodged the eyeball out of the socket. When both eyes were affected, darkening of the body was noted internal gross signs included pale liver occasionally with hemorrhagic spots and necrotic kidneys showing liquefaction.

Aero monad infection causes severe necrotic changes in muscular structure characterized by severe fragmentation and separation of muscle Bandal's that appear more eosinophilia and haylanized appearance as well as mononuclear and polymorph nuclear cell infiltration presents mainly intra muscular interstitial tissue (fig 1 and 2).

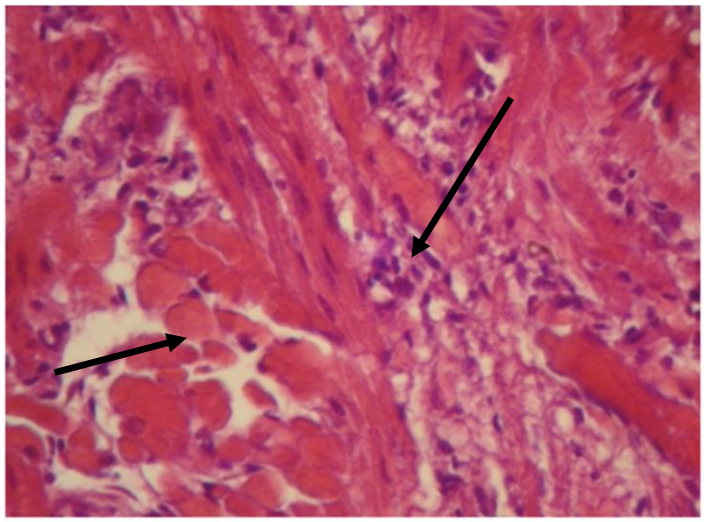

Figure1: Histopathological section of muscle 14day post infection show severe necrosis with haylanized appearance and infiltration PMNCs intra interstitial tissue (H&E40×).

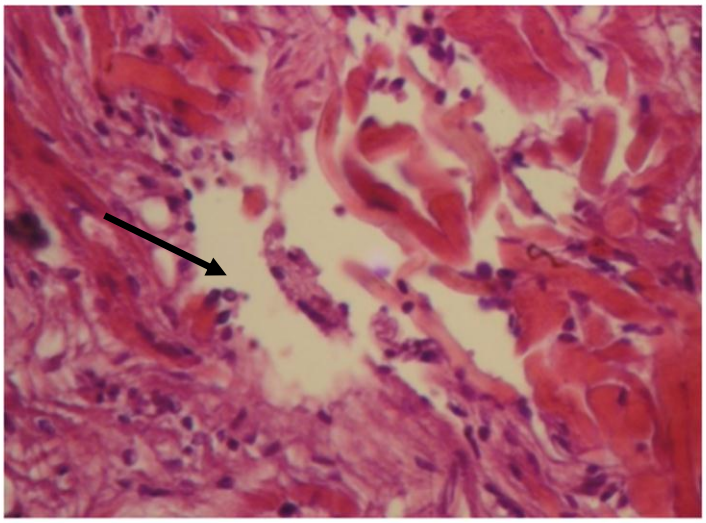

Figure2: Histopathological section of muscle 14day post infection show muscle structure fragmentation with inflammatory odema (H&E40×).

Histopathological finding of liver section **(**fig 2) in the infected fish indicate the presence of severe (diffuse) necrotic in the liver parenchymal accompanied with

intense polymorpho nuclear cells infiltration characterize of pyknosis of nuclei also present of cellular detritus mix with polymorph nuclear cell infiltration in liver parenchymal with atrophy of hepatocyte.

The pathological changed in kidney section (fig 4) showed severe vacuolation and hydropic degeneration in epithelium lining of renal tubules accompanied with mononuclear cells infiltration mainly plasma cell lymphocyte and macrophage in the interstitial tissue that giving feature interstitial nephritis .Also there is instance cellular inflammation with severe tubular necrosis changes that display most renal tubules a companied with sever hemorrhage. The result also showed infiltration melanomacrophag with vascular degeneration changes of blood vessels.

Spleen showed that there was severe congestion with hemosiderin disintegration associated with ellipsoid atrophy and lymphoid depletion (fig 5).

while intestine showing varying degree ranging between sever degeneration changing with sloughing to complete necrosis also the result showed increase and proliferation goblet cells in mucosal intestinal villi with mucinous exudate and proliferation of eosinophilia granular cells in sub mucosal area (fig 6).

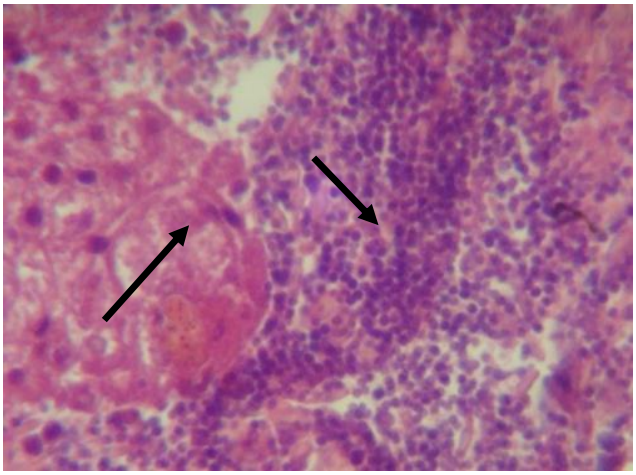

Figure3: Histopathological section of liver 14 - day post infection show inflammatory exudate that consists mainly of PMNCS and fibrinus deposit (H&E40×).

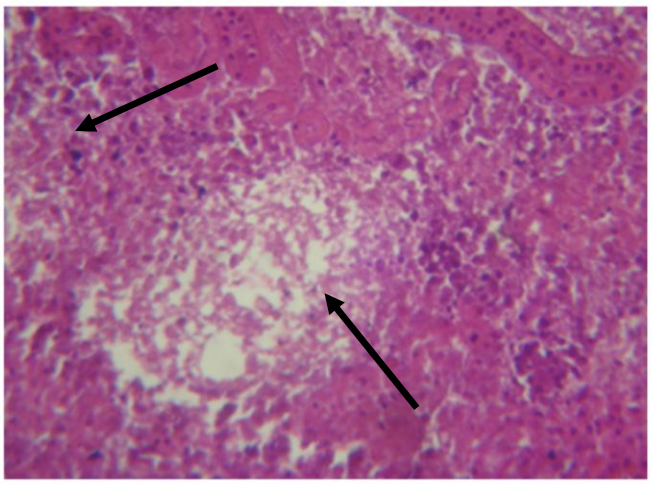

Figure4: Histopathological section of kidney 14day post infection show severe tubules necrosis with focal area of necrosis associated with intense inflammatory reaction (H&E40×).

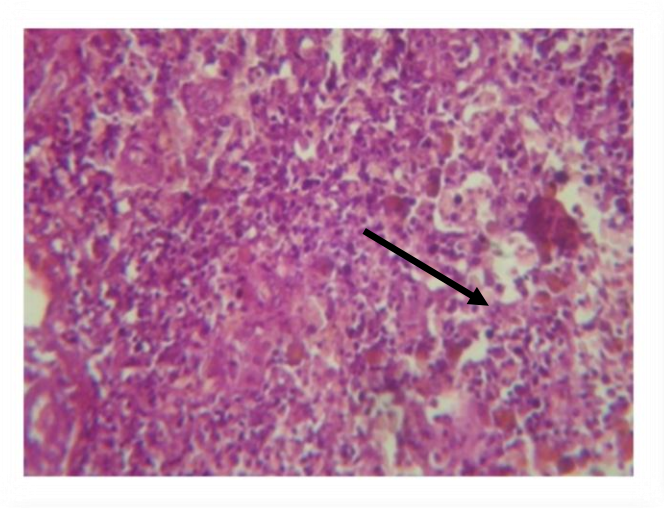

Figure5: Histopathological section of spleen 14day post infection show congestion with hemosiderin dipposites (H&E40×).

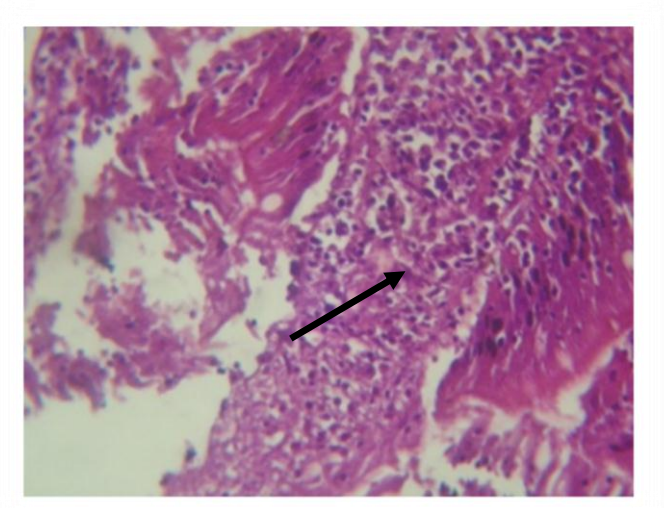

Figure6: Histopathological section of intestine 14-day post infection show present of cellular detritous in the lumen with sloughing of enterocyte (H&E40×).

The presence of *Aeromonads* particularly *A.hydrophila* in healthy and diseased fish of the *Cyprinus carpio* obtained from different localities suggests an epizootic. (17) were able to isolate *Aeromona spp*. from healthy tilapia tissues such as kidney, spleen and liver. LD_{50} of *A.hydrophila* was 2.94 $\times 10^7$cfu/ml in common carp (*Cyprinus carpio*)(18) (19) used 1.5-0.3 $\times$ 10^6 cfu/ml intraperitonealy for all fingerlings groups while (20), found the LD_{50} of *A.hydrophila* was 6×10^7cfu /ml when injected Cyprinid loaches intraperitoneally.

Although the LD_{50} of *A.hydrophila* is high and *A. hydrophila* is commonly isolated in an aquatic environment, it can be concluded that the urgent mass mortality of *Cyprinus carpio* was caused by acute stress causing factors such as over stocking and poor quality of the water. Those findings are so alarming that fish farmers should realize that opportunistic bacteria (*A. hydrophila)* can contribute high mortality and economic loss to aquaculture.

This study clearly indicated that *A. hydrophila* was the causative agent of the mortality of *Cyprinus carpio*. A number of virulence factors derived from *A. hydrophila* have been proposed in an effort to explain the pathogenesis of infections

(21). Toxins with hemolytic, cytotoxic and enterotoxin activities have been described in many *Aeromonas* spp. (22).pathology of this bacterial disease was characterized by either septicemia (23) or toxemia (24).

The severity of lesion in the experimental fishes attributed to toxic substance produce by *A.hydrophila* which evoking hemorrhage and necrosis(25). Those *in vivo* and *in vitro* studies confirmed the bacterial toxin including enzymes such as protease and elastase. This bacterium also produces β type hemolysin on blood agar plates (26). An infection of *A. hydrophila* producing strong β-hemolysin was found among common carps, Aerolysin and hemolysin genes are reported to be the putative virulence genes of *A.hydrophila* (27). Aerolysin, produced by some strains of *A. hydrophila*, is an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties. Aerolysin binds to specific glycoprotein receptors on the surface of eukaryotic cells before inserting into the lipid bilayer and forming holes. Hemolysins are exotoxin protein produced by bacteria and the lytic activities of hemolysins on red blood cells are reported to be important for nutrient acquisition or for causing certain conditions such as anemia (28).

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