ISOLATION OF GRAM NEGATIVE OPPORTUNISTIC BACTERIA FROM INTESTINAL CONTENTS OF COMMON CARP *Cyprinus carpio* L.

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ABSTRACT

The aims of the study were isolatetion and identification opportunistic gram negative strains from the intestinal micro-flora of 125 healthy Common carp (*Cyprinus carpio*) range from 400 -1500g in weight from commercial farms in the north of Baghdad, period between September to December 2010 In this study the presence of gram negative bacteria were investigated using API 20E multiple test system and various biochemical tests. The obtained data showed that six strains of *Aeromonas* (A1,A2,A3,A4,A5 andA6) were the dominated bacteria isolated from intestine, two strains of *Serratia rubidaeae* (S1 and S2) and one strain of *Pseudomonas leutola* .The current study is the first report on the isolation of Pseudomonas bacteria from intestine of common carp.

INTRODUCTION

World aquaculture has grown tremendously during the last years becoming an economically important industry (1).

With the increasing intensification and commercialization of aquaculture production, disease is a major problem in the fish farming industry (2) there is evidence that the alimentary tract of fish is a complex ecosystem, containing a large number of microorganisms. Microbial populations in the intestinal contents are much higher than those in the surrounding water

The GI microbiota of fish is characterized by high population density, wide diversity and complexity of interactions، while all major groups of microbes are represented, bacteria predominate، they are the main constituent of the gut microbiota in fish (3). The endogenous microbiota of freshwater fish species tends to be dominated by members of the genera *Aeromonas, Acinetobacter, Bacillus, Flavobacterium, Pseudomonas* representatives of the family *Enterobacteriaceae*, and obligate anaerobic bacteria of the genera *Bacteroides, Clostridium* and *Fusobacterium* [(4)]. Various species of lactic acid bacteria (LAB) (*Lactobacillus, Lactococcus, Streptococcus, Leuconostoc*, and *Carnobacterium* spp.) have been also demonstrated to comprise part of this microbiota [(5,6)]They are not dominant in the normal intestinal microbiota of fish, but some strains can colonize the gut (7) or to inhibit adhesion of several fish pathogens (6).

One of the most important features of GI microbiota in fish is variability، many investigations have demonstrated variation in the microbial flora in different fish species depending of nutrition, intestinal microenvironment, age, geographical location, environmental factors, stress (8). The regulations of bacterial populations in the GI tract of fish are complex processes .

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that are not yet well understood. Few knowledge about the early steps of colonization of the GI tract of fish, the establishment of normal microbiota and its stability. This study aim to isolation and identification opportunistic gram negative strains from the intestinal micro-flora and to compare the $API_{20}E$ multiple test system with Conventional biochemical methods for strains of Gram negative bacteria isolated from fish could be useful for manipulating microbiota as a strategy in prevention pathogenic infection or to improve nutrition.

MATERIALS AND METHODS

125 adults common carp weight range between 400-1500 g were obtained from commercial fish farms in the north of Baghdad from September 2010- to December 2010 and maintained in aquarium filled with tap water at 21 c° .

chemicals and media :

-Analytical grade chemicals and dyes (gram stain,reagent for biochemical test).
-Bacteriological media (oxoide UK) which include blood agar base,Nutreint agar and tryptic soy agar.
-biochemical media from Himedia, India.

isolation of bacterial strain:

Various samples of adult common carp « *Cyprinus carpio*» were brought to the laboratory alive and sacrificed. The abdomen surfaces were thoroughly scrubbed with an alcohol (70% ethanol) and aseptically dissected to remove the intestines. The intestines were macerated with sterile glass rod and homogenized in sterile Normal salin solution (1:10 Wet/Vol) using a vortex mixer. Samples of the thoroughly macerated and homogenized intestines were serially diluted in NSS and aseptically plated by spread plate technique on Nutreint agar and tryptic soy agar then incubated aerobically at 30 c° for 24 h.

Identification based on their morphological and biochemical characteristics (9). Identification of *Aeromonas* strains isolated species depends on Popoff(10) *Serratia* strains isolated species depends on Ewing (11) and *Pseudomonas* strain described by Austin (12) purecultures were kept in semisolid nutrient medium supplemented with 20 % (v/v) glycerol at -20°C. Cultures were routinely grown on TSA or tryptic soya broth (TSB, Oxoid) at 25°C.

api 20e rapid identification system:

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 (Arginine dihydrolase, 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 bacteria isolated in pathological laboratory / Baghdad university /vet. College

Biochemical properties

The bacterial culture was examined Colony morphology which grow on MacConkey agar, catalase production, oxidase production, haemolytic activity, gelatin hydrolysis, MR (methyl red) test ,VP (Vogues Proskauer) test, triple sugar iron (TSI) - & hydrogen sulfide production (H_2S), Reduction of nitrite to nitrate, NaCl require.

RESULTS AND DISCUSSION

Examination of the 125 *Cyprinus carpio* intestinal flora

Six motile *Aeromonas hydrophila*, two *Serritia rubidaea* and one *Psendomonas lacteola* were isolated table (1).

Table 1: Biochemical properties of *A.hydrophila* ,*S.rubidaea* and *Ps. Luteola* isolates

Characteristics	*A.hydrophila*	*Ps. leulola*	*S. rubidaeae*
Colony morphology: Colour on MacConkey	Creamy /yellow	yellow to orang	pink
Size	3-4 mm diameter	4-5mm diameter	1-2mm diameter
Shape	Conical	smooth	circular
Texture	Viscous	Viscous	Viscous
Gram staining reaction	–	-	-
Rods	+	+	+
Motility	+	+	_
Oxidase	+	_	_
Catalase	+	+	_
Reduction of nitrite to nitrate	–	N.D	+
Gas production from gloucose	+	_	+
Gloucose fermentation	+	_	+
NaCl require	_	_	_
Urase	_	+	_
Indol	+	_	_
VP	+	+	+
α haemolysis	+	+	+
β haemolysis	+	+	_

+ = Positive reaction, – = Negative reaction, N.D= not done

Two *Serratia* strains isolated from fish were identified. Gram-negative, rod shaped strains that were oxidase-negative , fermentative glucose non-motile were identified at the species level with biochemical tests two *Serratia rubidaea* were isolated.

The *Pseudomonas* strain isolated from fish were identified. Gram-negative, rod shaped strains that were oxidase-negativ, fermentative glucose motile were identified at the species level with biochemical tests one *Pseudomonas luteola* were isolated table (2,3 and 5).

Table 2: percent of each isolates according to date and weight of fish sample

Sample number	Date	Average weight of sample	*A.hydrophila*	*Ps.Leulola*	*S. rubidaeae*
25	September	750-850	A1,A2	Ps1	S1
25	October	400-500	A3		
25	October	400-550	A4		S2
25	November	1000-1350	A5		
25	December	1100-1500	A6		

Each sample contain 25 fish

On the basis of morphological, physiological and conventional biochemical tests, the six *Aeromonas* strains isolated from fish were identified. Gram-negative, rod shaped oxidase-positive, fermentative glucose motile were identified at species level with biochemical tests six *Aeromonas hydrophila* were isolated according to (10,11 ,12) all the isolated strains were rod gram-negative.

The results between the $API_{20}E$ system and conventional tube test were detected and the test results were similar for both conventional tests and $API_{20}E$ system (Cytochrome Oxidase,Fermentation/oxidationD-glocose,Sodium pyrovate VP, L-tryptophane indol and urea Urease) it was found that the $API_{20}E$ system could be used to identify the gram negation bacteria under certain conditions as shown by (13) while the major rapid identification systems such , API-$_{20}$E, is beginning to recognize the significance of species identification there is one of the systems used in aquaculture for the rapid diagnosis of bacterial fish diseases., the API-$_{20}$E as it is relatively cheap and simple to use. Although this system, as well as other manual systems, was initially designed for the identification of members of the family Enterobacteriaceae, it has now been adapted for identification of bacterial fish pathogens (14).

Motile *Aeromonas* samples were isolated from healthy fish intestinal flora as in previous studies by various authors (15) thus demonstrating that *Aeromonas* are the main members of the normal intestinal flora of fish in both fresh water and Sea water .*Pseudomonas luteola* has been associated with human clinical infections but was not identified in Turkish aquaculture until 2006 *Pseudomonas luteola* infection in fish rainbow trout, *Oncorhynchus mykiss*, was reported by Altinok(16)during the outbreak, 40% of the rainbow trout (10–40 g) died, typical clinical signs were exophthalmia, dark pigmentation, hemorrhage at the base of the pectoral, pelvic, anal fins and around the vent. Internal signs were enlarged spleen, pale liver and intestine filled with yellowish fluid. Liver, kidney and spleen of diseased fish (16).

Table 3: Identification of *Aeromonas* isolates by the API $_{20}$E rapid identification system.

Tests	Active ingredients	REACTIONS/ENZYMES	A1	A2	A3	A4	A5	A6
ONPG	2-nitrophenyl-βD-galactopyranoside	B-galactosidase (Ortho NitroPhenyl-βD-Galactopyranosidase)	+	+	+	+	+	+
ADH	L-arginine	Arginine Dihydrolase	+	_	+	+	+	+
LDC	L-lysine	Lysine DeCarboxylase	+	+	+	+	+	+
ODC	L-ornithine	Ornithine DeCarboxylase	_	_	_	_	_	_
CIT	Trisodium citrate	Citrale utilization	+	_	+	+	+	+
H_2S	Sodium thiosulfate	H_2S production	_	_	_	_	_	_
URE	Urea	UREase	_	+	_	_	_	_
TDA	L-typtophane	Tryptophane DeAminase	_	_	_	_	_	_
IND	L-tryptophane	INDole production	+	+	+	+	_	+
VP	Sodium pyruvate	Acetoin production (Voges Proskauer)	+	+	+	+	_	+
GEL	Gelatin (bovine origin)	GELatinase	+	+	+	+	+	+
GLU	D-glucose	Fermentation/oxidation (INOsitol)(4)	+	+	+	+	+	+
MAN	D-mannitol	Fermentation/oxidation (MANnitiol)(4)	+	+	+	+	+	+
INO	Inositol	Fermentation/oxidation (INOsitol)(4)	_	_	_	_	_	_
SOR	D-sorbitol	Fermentation/oxidation (SORbitol)(4)	_	_	_	_	_	_
RHA	L-rhamnose	Fermentation/oxidation (RHAmnoe)(4)	_	_	_	_	_	_
SAC	D-sucrose	Fermentation/oxidation (SACcharose)(4)	+	+	+	+	+	+
MEL	D-mlibiose	Fermentation/oxidation (MELiliose)(4)	_	_	_	+	+	+
AMY	Amygdalin	Fermentation/oxidation (AMYgdalin)(4)	+	+				
ARA	L-arabinose	Fermentation/oxidation (ARAbinose)(4)	_	_				
OX	Cytochrome-OXidase		+	+				

+ = positive, – = negative reaction

while several *Serratia spp*.,have been described recently as causing infection. Ewing (11) reported 17 strains of *S.rubidaea* isolated from human clinical specimens 10 from the respiratory tract, 4 from blood and 3 from wounds). Subsequently, some strains have occasionally been isolated, mainly from the respiratory tract or from ulcers or wounds (17). because *S.rubidaea* is infrequently isolated from clinical specimens and because there is no clinical information in relation to these isolates its role in human disease has been infectious (18).

According to regulation 91/67/EUaC of European Union council, screening of fish farms reduced the risk that animals carrying opportunistic agents proliferate during shipping, handling, or change of environment and that resistant or tolerant animals transfer a significant pathogen to a population that may be susceptible to infection .

Table 4: Identification of *S.rubidaea and Ps. Luteola* isolates by the API 20E rapid identification system.

Tests	Active ingredients	REACTIONS/ENZYMES	Ps1	S1	S2
ONPG	2-nitrophenyl-βD-galactopyranoside	B-galactosidase (Ortho NitroPhenyl-βD-Galactopyranosidase)	_	+	+
ADH	L-arginine	Arginine Dihydrolase	+	_	_
LDC	L-lysine	Lysine DeCarboxylase	_	_	_
ODC	L-ornithine	Ornithine DeCarboxylase	+	_	_
CIT	Trisodium citrate	Citrale utilization	+	+	+
H_2S	Sodium thiosulfate	H_2S production	_	_	_
URE	Urea	UREase	+	_	_
TDA	L-typtophane	Tryptophane DeAminase	_	_	_
IND	L-tryptophane	INDole production	_	_	_
VP	Sodium pyruvate	Acetoin production (Voges Proskauer)	+	_	_
GEL	Gelatin (bovine origin)	GELatinase	+	+	+
GLU	D-glucose	Fermentation/oxidation (INOsitol)(4)	+	+	+
MAN	D-mannitol	Fermentation/oxidation (MANnitiol)(4)	+	+	+
INO	Inositol	Fermentation/oxidation (INOsitol)(4)	+	_	_
SOR	D-sorbitol	Fermentation/oxidation (SORbitol)(4)	_	_	_
RHA	L-rhamnose	Fermentation/oxidation (RHAmnoe)(4)	_	_	_
SAC	D-sucrose	Fermentation/oxidation (SACcharose)(4)	_	+	+
MEL	D-mlibiose	Fermentation/oxidation (MELiliose)(4)	+	_	_
AMY	Amygdalin	Fermentation/oxidation (AMYgdalin)(4)	_	+	+
ARA	L-arabinose	Fermentation/oxidation (ARAbinose)(4)	+	+	+
OX	Cytochrome-OXidase		–	–	–

+ = positive, – = negative reactione

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عزل وتوصيف البكتريا الانتهازية والسالبة لصبغة كرام من محتويات الامعاء لاسماك الكاربب الشائع (*Cyprinus carpio* L.)

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الملخص

هدفت الدراسة الى عزل وتوصيف البكتريا السالبة لصبغة كرام من الفلورا الطبيعية في 125 سمكة من اسماك الكارب الشائع والسليمة من الامراض بوزن من 400 –-1500 غرام من ألحقول في شمال بغداد للمدة من شهر آب الى كانون أول 2010 .استعمل في هذه الدراسة الفحص بنظام متعدد الفحوص $API_{20}E$ وعدد من الفحوص البايوكيميائية التقليدية. اظهرت الدراسة سيادة العزلات الستة من بكتريا الايرومونس هايدروفيلا (A1،A2،A3،A4،A5وA6) ،يتبعها عزلتين من بكتريا سريشيا روبيديا (S1وS2) وعزلة واحدة من بكتريا السيدومونس .هذه الدراسة سجلت اول عزلة لبكتريا السيدومونس من امعاء الكارب الشائع.

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