

Investigation of *gcat* gene and antibiotic resistance pattern of *Aeromonas hydrophila* isolated from hemorrhagic septicemia's cases in fish farms

Z.M. Taha¹, S.T. Sadiq¹, W.A. Khalil², K.Y. Ali², H.S. Yosif² and H.N. Shamil²

¹Department of Pathology and Microbiology, ²Department of Medicine and Surgery, College of Veterinary Medicine, Duhok University, Duhok, Iraq

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Correspondence:

Z.M. Taha
zananvete@yahoo.com

Abstract

The significance of *Aeromonas hydrophila* concerning hemorrhagic septicemia in aquaculture farms production in Duhok province, Iraq was investigated. Antibiotic-resistant profiles of isolates were also investigated with 8 antibiotics. Bacterial isolates were identified by using morphological and biochemical tests and confirmed molecularly by amplification of *gcat* gene. Out of 25 examined fish, only 19 fish were harbored *A. hydrophila*. Twenty-four *A. hydrophila* strains were isolates from 100 organ samples. Ninety-six percentages of the isolates were resistant to each of the imipenem and gentamicin, followed by doxycycline 92%, ciprofloxacin and trimethoprim-sulfamethoxazole 88%, norfloxacin 58% and ceftriaxone 33%. None were resistant to levofloxacin. Eighty-eight percentages were multiple antibiotics resistant. The high isolation rate of *A. hydrophila* in our study indicates that this species was the major cause of the outbreak in hemorrhagic septicemia's cases in our area affecting carp farms and the high rate of resistance should be considered as these isolate can serve as a resistance source for human being during food series and make great challenge for their therapeutic opportunity.

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Introduction

The largest and most dangerous form of diseases affecting fish production is the bacterial infections, which account for 80% of fish mortality (1). In turn, this would adversely affect aquaculture (2). *Aeromonas hydrophila* is considered the primary cause of septicemia disease, including carp, tilapia, perch, catfish, salmon, and many other freshwaters and marine species (3). The marked clinical signs that seen on the abdominal wall and at the base of fins are congestion and hemorrhage as well as scale erosion at all body surfaces. While the prominent post mortem lesions are highly congested internal organs, ascitic fluid build-up in the abdominal cavity and swollen kidney

and spleen (2). In addition, a variety of human illnesses have involved by *A. hydrophila*, it causes a broad spectrum of infections (gastroenteritis, septicemia, meningitis, endocarditis) in humans, often in immune-compromised hosts (4). *A. hydrophila* is easy to grow on standard laboratory enteric media and produces β -hemolysis on blood agar with grey, flat, round and shiny colonies about 2.0-3.0 mm in diameter, ability to grow on MacConkey agar with non-lactose fermentation and they are oxidase-positive (5,6). Different reports have revealed that *A. hydrophila* is widely antibiotic-resistant. Considerations, including the indiscriminate overuse of antibiotics in fisheries are responsible for this. This contributes to high selection pressure in bacteria to develop resistance through a variety

of mechanisms such as genetic mutation and horizontal gene transfer. This is a public and marine health hazard (7).

Little information is available on the fact that is it *A. hydrophila* regarded as the primary cause of hemorrhagic septicemia in fish as well as antibiotic resistance rate of *A. hydrophila* from clinical cases of fish suffering from hemorrhagic lesions in all around the world. The present study was intended to assess the accurate rate of *A. hydrophila* incidence as a causal agent of septicemias in different fish farms in Duhok city and also to assess their resistant rate against different antibiotics that most commonly used for therapeutic purposes against this bacterium in fish farms and human being.

Materials and methods

Sample collection

The sample collection process and the choosing of sample types, was carried out according to Aboyadak, (2) and Hassan *et al.* (8). Samples were taken from 5 carp fish farms (Agricultural 1, 2 and 3, Mosul dam and one pond in Xhanke Village) with symptoms of septicemia, which include skin hemorrhage with hemorrhagic skin ulcers and de-pigmented zones in the skin, and necropsy for internal organ lesions including extreme congestion and internal organ hemorrhage, enlarged liver and gall bladder. Each dead fish was picked in a separate sterile labeled plastic bag and transported to the laboratory in icebox. From each fish, four different samples were taken from heart blood, liver, kidney tissues, and hemorrhagic or ulcerative area on the skin. The sampling from each part of the fish lesion was carried out under strict sterile conditions to prevent contamination with normal flora, in which bacteria were isolated with a sterile loop from each organ of each fish. Four samples from each fish organ lesions were pooled to make one sample for testing. From 25 collected fish, a total of 100 organ lesion samples were taken.

Isolation and identification of *Aeromonas hydrophila* by phenotypic methods

Each loop sample at the collection time was directly put into tubes containing 10 ml of brain heart infusion broth (HiMedia, India) or tryptic soy broth (Lab M, UK) and incubated at 37°C for 20-24 hrs., as a pre-enrichment step (8). One to two loopful of pre-enrichment broth was inoculated on to blood agar supplemented with 7% sheep blood and incubated as previous. Beta-haemolytic 2-3 mm colonies were directly sub-cultured onto MacConkey agar and incubated as previously. Pale (non lactose fermenter) colonies of *Aeromonas hydrophila* were presumptively identified by Grams stain, indole production test, oxidase test, catalase test and urease test (5,6). Colonies from positive samples were directly preserved at -20°C as stock culture in tubes containing brain heart infusion broth with 25% glycerol (9). The final confirmation was carried

out by PCR amplification of *gcat* (glycerophospholipid-cholesterolacyltransferase) gene which is common for *Aeromonas hydrophila* (10).

DNA extraction and detection of *gcat* gene by PCR amplification

DNA samples were extracted by thermal extraction method according to Taha and Yassin (11). From stock culture 100 µl was inoculated onto MacConkey agar and incubated as previous. From MacConkey agar 2-3 pure (similar morphology) colonies were chosen and mixed with 200 µl of sterile double distilled water in a 1.5 ml tube. For at least 15 s, the mixture was vortexed and directly heated at 95°C for 10 min; the samples then cooled directly by ice, the cooled suspension was centrifuged. One hundred fifty µl supernatant was used as a template DNA for PCR. The purity and concentration of extracted DNA were examined using a nano drop (Thermo Scientific, USA) (12). The extracted DNA samples were stored under freezing (-20) until used as DNA templates for PCR analysis (13). To identify the *A. hydrophila*, *gcat* gene was amplified as potential markers of detection (10). The *gcat*-PCR amplification was made in a total volume of 25 µl containing 12.5 µl of hot start premix (Genedirex, Taiwan), 1 µl of each of reverse and forward primer (F:5'-CTCCTGGAATCCCAAGTATCAG-3' and R: 5'-GGCAGGTTGAACAGCAGTATCT-3'), which amplify a 237 bp fragment (concentration: 10 pmol), 2 µl of sample DNA (50-150ng/µl), the remainder was filled with 8.5 µl nuclease-free water (Qiagen, Germany). The process of amplification was performed in thermocycler (GeneAmp® PCR System 9700, Singapore, Applied Biosystems) according to Latif-Eugenin *et al.* (14). PCR program was 95°C for 3 min followed by 35 amplification cycles of denaturation, annealing and elongation, were 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, respectively and final elongation of 72°C for 5 min. Amplification of PCR products was confirmed in 1.5% agarose gel prepared with 1× Tris-acetate-EDTA (TAE) buffer and stained by red safe DNA staining solution (GeNetBio, Korea). Successful *gcat* gene amplification is considered when bands in agarose gel at the anticipated size of 237 bp seen.

Antibiotic susceptibility test

The identified isolates by PCR assay were tested against 8 antibiotics as described by Stratev and Odeyemi (7) and Li *et al.* (15), using the disk diffusion method on Mueller-Hinton agar (Lab M, UK). The concentration of each antibiotic per disc (Bio-analyze, Turkey) was as follow: doxycycline 30 µg, trimethoprim-sulfamethoxazole 1.25 and 23.75 µg, gentamicin (10 µg), ciprofloxacin 5 µg, ceftriaxone 30 µg, imipenem 10 µg, levofloxacin 5 µg and norfloxacin 10 µg. The protocol and interpretations of results breakpoints were carried out according to clinical and laboratory standards institute (CLSI) (16). Isolates were marked as either susceptible or resistant isolates that were

intermediately susceptible to specific antibiotic were classified as resistant. Any isolate that was resistant to three or more antibiotics (≥ 3) was known as multiple antibiotics resistance (MAR).

Results

Phenotypic methods for the detection of *Aeromonas hydrophila*

The cultural characteristics of all isolates of *Aeromonas hydrophila* on blood agar were found as β -hemolytic, with grey, flat, round and shiny colonies about 2-3 mm in diameter. On MacConkey agar, their colonies were showed as pale non lactose fermenter. With Grams stain all isolates were showed as medium-sized, straight, Gram-negative rods under oil immersion objective lens (Figure 1). While the results of biochemical tests, all isolates were indole positive characteristic red ring above fluid media in tryptophan broth, oxidase test positive (the color was changed of cotton swab with a loopful of bacterial culture to dark purple within 10 seconds after adding of 1% solution of N, N-dimethyl-p-phenylenediamine hydrochloride, catalase test positive bubble formation after adding of one drop of hydrogen peroxide on a loopful of bacterial culture on sterile microscopic slide and urease test negativity the color of Christensen's urea agar containing 40% urea solution was changed to yellowish as a result of acidic byproduct from glucose utilization (Figure 2).

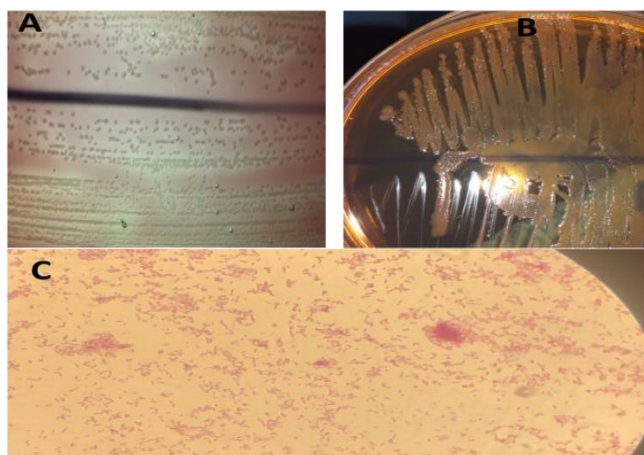


Figure 1: Characteristic β -hemolytic, round colonies of *A. hydrophila* on blood agar (A); pale (non lactose fermenter) colonies on MacConkey agar (B); Medium-sized, straight, Gram-negative rods (1000x) (C).

Genotypic method for the detection of *Aeromonas hydrophila*

According to the PCR amplification of *gcat* gene, all identified *Aeromonas hydrophila* isolates by both morphological and biochemical tests, were showed a band

size of about 237 bp on agarose gel after staining with red safe DNA staining solution and after electrophoresis process (Figure 3). These were indicated that all isolates were *A. hydrophila*.

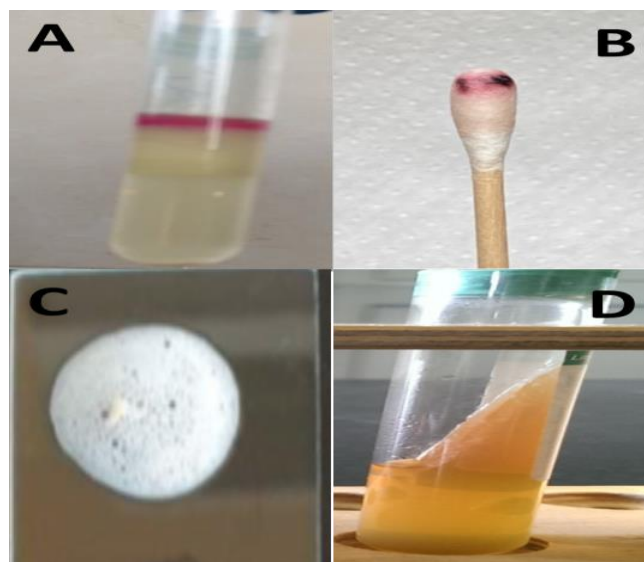


Figure 2: Indole positive (characteristic red ring above fluid media in tryptophan broth) (A); dark purple color formation indicative of oxidase test positive (B); bubble formation indicates of catalase test positivity (C); yellow color of Christensen's urea agar containing 40% urea indicate urease test negativity (D).

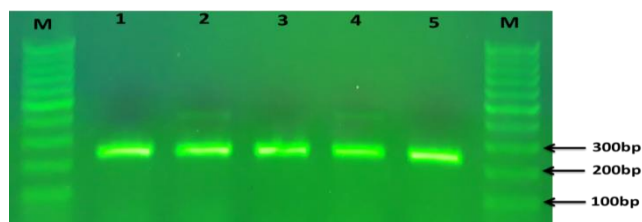


Figure 3: Agarose gel electrophoresis of PCR products. M: 100 bp DNA ladder, lines (1-5) positive result at 237 bp for *gcat* gene of *A. hydrophila* isolates.

Out of 25 carp examined, only 19 fish were found to be infected with *A. hydrophila*. While out of 100 collected organ samples (25 livers, 25 kidneys, 25 hearts, and 25 skins) from a total of 25 examined carps, only 24 strains of *A. hydrophila* were identified. These were in accordance with phenotypic methods (Figure 1 and 2) and genotypic methods (PCR amplification of *gcat* gene) (Figure 3). The liver was the predominated organ to have *A. hydrophila* (9 isolates) followed by kidney and heart each of (6 isolates) and skin of about (3 isolates) (Table1). Regarding the geographical region, fishes from Khanke area and agricultural pond 3 were

showed a higher isolation rate (100%) followed by Mosul dam (80%), Agricultural 2 (75%), and Agricultural 1 (43%) (Table 1).

Antibiotic susceptibility test

All isolates were resistant to at least one tested antibiotic. Ninety-six percent of the isolates were found to be resistant to each of imipenem and gentamicin, followed by

doxycycline 92%, 88% by each of ciprofloxacin and trimethoprim-sulfamethoxazole, norfloxacin 58% and ceftriaxone 33%. Neither of the isolates was levofloxacin resistance (all were susceptible) (Figure 4), (Table 2). Twenty-one isolates 88% were multiple antibiotics resistant (resistant to ≥ 3 antibiotics) (Figure 4). The MAR was high in agricultural 2 and 3 farms 100% (Table 2).

Table 1: Isolation rate of *A. hydrophila* in different fish farms

Geographical region	No of sampled fish	No and % of positive fish	No of isolates	Liver	Kidney	Heart	Skin
Agricultural 1	6	2 (43%)	3 ^a	2	-	-	1
Agricultural 2	4	3 (75%)	6	2	1	1	2
Agricultural 3	4	4 (100%)	5	3	1	1	-
Khanke	6	6 (100%)	6	2	2	2	-
Mosul dam	5	4 (80%)	4	-	2	2	-
Total	25	19 (76%)	24 ^a	9	6	6	3

No: number; ^a: represent the number of *A. hydrophila* isolates but not the exact percentage of positive fish.

Table 2: Antibiotic-resistant profile of 24 *A. hydrophila* isolates

Geographical region (No)	N (%)								
	IMI	CFN	CIP	GEN	SXT	NOR	DOX	LEV	MAR
Agricultural 1 (3)	2 (67%)	1 (33%)	2 (67%)	2 (67%)	2 (67%)	1 (33%)	3 (100%)	-	2 (67%)
Agricultural 2 (6)	6 (100%)	2 (33%)	6 (100%)	6 (100%)	5 (83%)	3 (50%)	6 (100%)	-	6 (100%)
Agricultural 3 (5)	5 (100%)	2 (40%)	5 (100%)	5 (100%)	5 (100%)	3 (60%)	5 (100%)	-	5 (100%)
Khanke (6)	6 (100%)	2 (33%)	5 (83%)	6 (100%)	5 (83%)	4 (67%)	5 (83%)	-	5 (83%)
Mosul dam (4)	4 (100%)	1 (25%)	3 (75%)	4 (100%)	4 (100%)	3 (75%)	3 (75%)	-	3 (75%)
24 isolates	23 (96%)	8 (33%)	21 (88%)	23 (96%)	21 (88%)	14 (58%)	22 (92%)	-	21 (88%)

IMI: imipenem, CFN: ceftriaxone, CIP: ciprofloxacin, GEN: gentamicin, SXT: trimethoprim-sulfamethoxazole, NOR: norfloxacin, DOX: doxycycline, LEV: levofloxacin, MAR: Multiple antibiotics resistance. No: number.

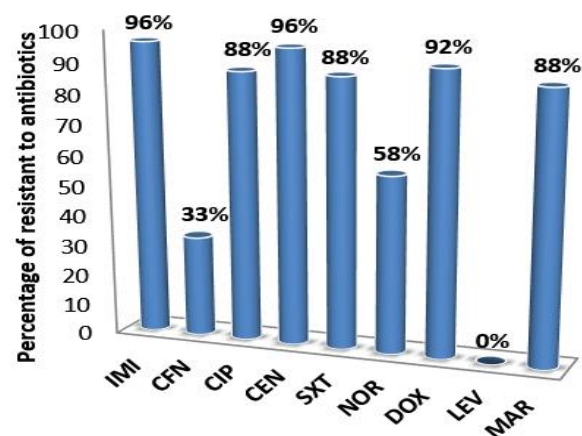


Figure 4: Antibiotic-resistant profile a total of 24 *A. hydrophila* isolates to different tested antibiotics. IMI: imipenem, CFN: ceftriaxone, CIP: ciprofloxacin, GEN: gentamicin, SXT: trimethoprim-sulfamethoxazole, NOR: norfloxacin, DOX: doxycycline LEV: levofloxacin, MAR: Multiple antibiotics resistance.

Discussion

Aeromonas hydrophila is a significant opportunistic pathogen in the aquatic environment of freshwater where the most critical stressors include rough handling, crowding, malnutrition, heavy free ammonia (NH₃) and elevated nitrites (NO₂) are present (1,8). Adding to these, in muddy water with heavy organic content, *A. hydrophila* is abundant (17). Any changes in water parameters can lead to immune stress situations and this will predispose fish from being infected with *A. hydrophila*, and encourage opportunist bacterial infections (1,18). The high isolation rate of *A. hydrophila* seen in this study can all be due to these factors. However, in this study, not all fish showing clinical signs of septicemia were harbored *A. hydrophila*, this mostly due to that a large number of *Aeromonas* spp other than *hydrophila* are responsible for hemorrhagic septicemia in fish. There is strong evidence that many *Aeromonas* spp as a cause of diseases have been found in aquatic environments (wild and farmed fresh water and/or marine species) (19). The causes of epizootic ulceration and hemorrhagic septicemia in fishes have become well established with *Aeromonas veronii* (8).

In another study which was carried in China, proposed that motile *Aeromonas* spp. other than *A. hydrophila* were also found in moribund fish with hemorrhagic septicemia (17).

According to the clinical and laboratory standards institute CLSI (16) isolates that are initially susceptible or intermediately resistant to one antibiotic may become resistant after initiation of therapy. Therefore, in this study any isolate that was intermediately resistant to specific antibiotic was classified as resistant isolate to antibiotics. In this study, all the isolates showed a high degree of resistance to the tested antibiotics with a significant resistance to both imipenem and gentamicin. However, Sreedharan *et al.* (20), reported a total sensitivity of *Aeromonas* spp., to imipenem and gentamicin. As well as, Al-Dabbagh (21) in Mosul, Iraq, also found that *A. hydrophila* isolated from milk samples in bovine mastitis was susceptible to gentamicin. On the other hand, Stratev and Odeyemi (7) reported that 50% of *A. hydrophila* isolated from Tilapia were resistant to imipenem. This finding most likely reflects the presence of selective pressures in these ecosystems mainly because antimicrobial agents are used in both aquatic and human clinical prevention and treatment strategies (15,22). However, imipenem has never been used neither therapeutically nor prophylactically in an aquatic environment but the resistant in these fields is most likely created due to human wastewaters (hospital wastewaters) or rivers containing imipenem resistant genes (carbapenems) or antibiotic residues with subsequent contamination to the aquatic environment which in turn lead to the development of selective pressure of resistant in these environments (23,24). In a study carried out to explore the existence of antibiotic resistance in hospitals wastewater collected in Mumbai, India, identified new resistance genes and novel carbapenemases including NDM, VIM, IMP, KPC, and OXA-48 (25). In comparison, all isolates were levofloxacin susceptible in this study and this is consistent with Li *et al.* (15). This indicates that this antibiotic is not yet used in aquatic environments as a therapeutic or prophylactic purpose.

An alarming level of MAR in this work was observed especially in isolates from Agricultural 2 and 3. This indicates that in these farms different antibiotic types were used as a prophylactic purpose to reduce the probability of the occurrence of infectious diseases or to accelerate aquaculture growth (24,26). The presence of bacteria in overcrowding microbial ecosystems favorable for the transfer of genetic materials such as plasmid, transposons, and integrons carrying multiple antimicrobial-resistant genes is usually regarded a reason for MAR development (27) or that isolates carried genes codes for efflux pumps to multiple antibiotics, resulting to the higher rates of MAR (28). The presence of MAR in *A. hydrophila* has also been reported by others (7,22,29).

Conclusion

A high isolation rate of *A. hydrophila* found in this study, means that this is a common organism associated with disease outbreaks in aquaculture in the Dohuk province, Iraq. This indicates that *A. hydrophila* is a primary invader in farmed fish in our area. The high levels of antibiotic resistance recorded in this study should be carefully considered as these isolates can serve as a human source of infection during food series and pose a major challenge for the therapeutic possibility.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript

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بحث عن جين *gcat* ونمط المقاومة للمضادات الحيوية في ايرومونس هايدروفلا المعزولة من حالات تسمم الدم النزفية في المزارع السمكية

زانان محمد أمين طه¹، شعبان طيار صديق¹، وارهيان عزيز خليل²، كاهين يوسف محمد علي²، هيام صالح يوسف²، و هوزان نزار شامل²

¹قسم الأمراض والأحياء المجهرية، قسم الطب الباطني والجراحة، كلية الطب البيطري، جامعة دهوك

الخلاصة

الهدف من الدراسة هو بحث أهمية ايرومونس هايدروفلا والعلاقة مع تسمم الدم النزفي في مزارع إنتاج الأحياء المائية في محافظة دهوك، العراق. كذلك تم بحث ملامح المقاومة للمضادات الحيوية للعدلات مع ٨ أنواع من المضادات الحيوية. تم تحديد العزلات الجرثومية باستخدام الاختبارات المظهرية والاختبارات الكيميائية الحيوية وقد تم تأكيدها جزئياً من خلال جين *gcat*. أظهرت النتائج انه من بين ٢٥ سمكة تم فحصها، ١٩ منها كانت مضيقة للايرومونس هايدروفلا. تم عزل أربعة وعشرين عترة من الايرومونس هايدروفلا من ١٠٠ عينة من عينات الأعضاء. ٩٦% من العزلات كانت ذات مقاومة لواحدة من ايمبيبينيم والجنتاميسين، ثم ٩٢% سيبروفلوكساسين، التراي مثيريم - سلفاميثوكزال ٨٨%، نورفلوكساسين ٥٨%، وسيفاترايكلوزون ٣٣%. فيما لم تكن أي من العزلات مقاومة الى الليفوفلوكساسين. كما إن ٨٨% من العزلات كانت مقاومة لأكثر من مضاد جرثومي، يشير معدل العزلة المرتفع للايرومونس هايدروفلا في دراستنا إلى أن هذا النوع كان السبب الرئيسي لانتشار حالات تسمم الدم النزفية في منطقتنا والتي تؤثر على مزارع الكارب ومعدل المقاومة المرتفعة، ينبغي النظر إلى أن هذه العزلات قد تكون بمثابة مصدر للعدوى للإنسان أثناء سلسلة الغذاء وتشكل تحدياً كبيراً لفرصهم العلاجية.