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Antimicrobial Resistance of *Aeromonas salmonicida* Isolated From Common carp (*Cyprinus carpio*) Fishes in Erbil City/ Iraq

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Abstract:

Aeromonas salmonicida is a fish pathogen and recognized to cause a variety of diseases in humans. There are a few information about *A.salmonicida* in Iraq and there is no any previous molecular study on it. During the period of December 2017 to May 2018; Sixteen isolates of the *A. salmonicida* were isolated and identified from 300 common carp (Cyprinus carpio) fishes stomach in aquarium of Erbil city/ Iraq by using manual, automated Vitek 2 compact system, and confirmed by PCR using gene TonB-dependent siderophore (364bp). Antimicrobial susceptibility was determined by disk diffusion method and the results found that all isolates 100% susceptible to imipenem, 100% resistant to nalidixic acid and variable resistance to other studied antibiotics. The antibacterial effect of aqueous and alcohol extracts of *Eminium spiculatum* was studied by MIC and Molecular studies. The results found that aqueous and alcohol extracts of *E. spiculatum* have inhibitory effect and the MIC were 1400, 1800 µg/ml respectively. The sub MIC was used for both plant extracts, in plasmid profile the number of plasmid DNA was differ when treated with plant extracts. The inhibitory effect of the plant extracts against total proteins was studied by SDS- polyacrylamide gel electrophoresis and the results showed that there were variations in the protein bands in studied isolates and induction of new bands after treating with plant extracts. It was concluded that the leaves extracts of *E.spiculatum* could be used as antimicrobial for treatment of *A. salmonicida* infections and the results were confirmed by molecular studies.

Key words: *A. salmonicida,* Antimicrobial resistant, *E. spiculatum*, TonB-dependent siderophore gene.

Introduction:

Aeromonas salmonicida is a gram-negative pathogen attribute to fish and human (1, 2). *A. salmonicida* causes furunculosis and septicemia in many types of fishes that associated with an important economic loss in aquacultures worldwide (3), while its pathogenicity in human is not directly related. Moreover, they have been reported in many cases such as gastroenteritis, wound infections, respiratory and urinary tract infections, peritonitis, septicemia and other extra intestinal diseases (4, 5, 6, 7). Optimal temperature required for its growth is between 22-25ºC and after about a 24 hour of growth period the colonies appeared as a pin point, while after 48-72 hours the colonies have a brown pigmented color. *A. salmonicida* is facultative anaerobe, non sporated rods that wide spread in water and soil, most strains of bacteria are non-motile, ferments and oxidises glucose, positive for catalase and oxidase test (8).

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Using of antibiotic treatment is necessary at critical points of the outbreaks, but the continous use of antibiotics has some associated problems such as the increase of asymptomatic carriers and also the emergence of antibiotic resistant strains of *A. salmonicida* (9). In the past the medical plants and their constituents were used as drugs for treating of many diseases. The plant *Eminium spiculatum* belong to family Araceae is an indigenous plant in Iraq and it is use as food after cooking because it is poisonous if eaten raw and this toxin is easily destroyed thoroughly by cooking (10). On the other hand, it was reported that *E. spiculatum* can be used as antimicrobial, anticancer agent and antiproliferative activities (11). The aims of this study are: isolation of *A. salmonicida* from fishes in Erbil city also studing their resistant to antibiotics and to *E. spiculatum* plant extracts by using different methods.

Material and Method:

Bacterial Isolation and Identification

During the period of December 2017 to May 2018, sixteen isolates of A. salmonicida were

isolated from stomach of 300 common carp (Cyprinus carpio) fishes in aquarium of Erbil province/ Iraq. All samples tested bacteriologically, then sub-cultured and the pure brown rod shaped Gram negative colonies, non spore forming, and oxidase positive (12). All isolates were identified by using Vitek 2 compact system (Biomerieux, France).

Design of Primers

In order to ensure that isolates were *A. salmonicida*, the sequences of the TonB-dependent siderophore gene (accession No AM712656.1) of *A. salmonicida* which available in both gene lengths (partial and full) sequences for above mentioned gene. The specific primers designed up 5'-CAG TCG AGG CCA ATG GAA GT- 3' and dn 5-' GGC CAG TGA CAT GAC CTT CA-3' were synthesized by (Genscript, USA).

Preparation of DNA (Genomic DNA extraction and purification)

The sixteen *A. salmonicida* isolates were grown in Tryptic soy agar(Spain) supplemented with NaCl 1%, then incubated at 25 ºC for 48 hours. Stock cultures were maintained frozen at -20ºC. The total DNA chromosome from *A. salmonicida* was prepared depending on Wizard Genomic DNA purification kit as described by manufacture (Promega, USA).

Target DNA Amplification (PCR analysis)

The TonB sidophore gene of the 16 isolates was amplified using PCR in Eppendrof Master Cycler Thermocycler machine (Hamburg, Germany). Specific primer up 5'-CAG TCG AGG CCA ATG GAA GT-3' and dn 5-'GGC CAG TGA CAT GAC CTT CA-3' were used. In brief, PCR was performed in a 50µl reaction mixture containing 0.5µl of Taq DNA polymerase (5 U/µl; Promega), 5µl of 10× NH4 buffer, 2µl of 10mM dNTP mix, 10µl of 10mM MgCl2, 2µl of 10 µM forward and reverse primer, 2µl of bacterial genomic DNA (100ng/µl), and 26.5µl of sterile H_2O. The cycles were a single initial cycle at 95ºC for five minutes, for melting 25 cycles at 95ºC for one minute, for annealing 30 seconds at 59 ºC for , for elongation 1 minute at 72ºC and finally one cycle at 70ºC for five minutes.

Post-PCR Analysis

The PCR product of *A. salmonicida* isolates were analyzed on 1.5% agarose gel with TAE electrophoresis buffer, then stained with ethidium bromid and visualized with UV transilluminator (13). The ladder was 1kb (Fermentas, Germany).

Antibiotic Resistant Test

Antibiotic resistant assay was studied by using disk diffusion method as reported by CLSI (14). The antimicrobial were included; chloramphenicol (30 µg), gentamycin (30 µg), ampicillin (10µg), amikacin (30 µg), amoxicillin (25 µg), erythromycin (30 µg), nalidixic acid (30 µg), cotrimoxazole (25 µg), ciprofloxacin (10 µg), novobiocin (5 µg) imipenem (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), tetracycline (30 µg), trimethoprim (5 µg), and levofloxacin (5 µg).

Bacterial Inoculums

In current study, twenty four hours bacterial cultures grown in Mueller Hinton Broth (MHB) at 22°C , adjusted at $2x10^6$ CFU mL-1.

***E. spiculatum* Extracts**

The leaves of *E. spiculatum* were collected from Erbil province. They were identified and classified in the Education Salahaddin University Herbarium (ESUH), Erbil-Iraq. The samples were washed, dried, powdered, then soaked in aqueous and ethanol separately (ratio of ratio of *E. spiculatum* powder to solvent was 1:10 m/V) for 24 hours at 25°C and shaking at 150rpm. Both extracts were filtered by filter paper whatman no. 1, then the crude of each extract was obtained and stored at 4°C.

Antimicrobial Assay of Aqueous and Ethanol Extracts

The most resistant isolate of *A. salmonicida* was chosen to investigate the antibacterial activity of the *E. spiculatum* extracts on it using broth dilution method (Minimum inhibitory concentration, MIC). Concentrations ranged between 100 - 2000 µg/ml separately for each of ethanol and aqueous plant extract, in addition to control samples (14).

Extraction, Purification of Plasmids

Plasmids were extracted using GeneJET Plasmid Miniprep Kit (Thermo fisher, USA) with some modification. Plasmids were separated using agarose gel electrophoresis at 45 V for 1h. Bands were visualized and photographed by ChemiDoc-it 2 ImagerChemiDoc-it2 Transillminator UV (USA).

Electrophoresis of *E. spiculatum* Plasmids

To run electrophoresis 5 µl of loading dye (Fermentas, Germany) mixed with 10 µl of extracted plasmid was loaded on agarose gel contained ethidium bromide. Plasmids were separated by agarose gel electrophoresis at 45 V for 1 h. Bands were visualized and photographed by ChemiDoc-it 2 ImagerChemiDoc-it^2 Transillminator UV (USA).

Total Protein Extraction

The total protein of *A. salmonicida* was extracted by using Minute™ Bacterial Total Protein Extraction Kits (Invent Biotechnology, USA). Then total proteins were analyzed by using 10% SDS-PAGE in which performed with 5% stacking and 10% separating gel after the sample preparation was solubilized at 100 °C for 5 minutes in 0.05 M of Tris-HC1 buffer (2.5% SDS, 5% 2-mercaptoethand,

25% glycerol, and 0.03% bromophenol blue). The bands were formed after 1-2 hours in a constant 35 mA per gel with 120 volts using 1x electrophoresis buffer of staining 0.25% Coomassie brilliant blue R250 (Sigma, Germany) using pre-stained protein markers (ROTH, Germany). After separation, proteins were visualized and gels were photographed. All molecular techniques used in this current study depended on (13).

Results and Discussion:

Three important objectives were studied in present work. The first one was to isolate and identify *A. salmonicida* from Iraqi common carp (Cyprinus carpio) fishes for first time, sixteen isolates (5.33%) of the *A. salmonicida* were isolated from stomach of 300 common carp fishes in aquarium of Erbil province/ Iraq. All samples tested bacteriologically, then sub-cultured and the pure brown rod shaped Gram negative colonies, non spore forming, and oxidase positive bacteria were identified (8) and confirmed by Vitek 2 compact system. All isolates were further identified and differentiated from *A. hydrophila* by using specific designed primer for *A. salmonicida* TonB-dependent siderophore (accession No AM712656.1) which is available for above gene retrieved from the EnBank and the results of current study showed that all isolated bacteria and the standard *A. salmonicida* ATCC 33658 have the mentioned gen with expected size 364bp (Fig. 1). It is worth to mention that the bacteria *A. salmonicida* is isolated for first time from fish in Iraq. Detection of above mentioned bacteria has mainly been achieved by bacterial culture, but more rapid and sensitive methods are needed. The application of PCR assay for detection and identification of *A. salmonicida* bacteria is now well established (15, 16). The application of this technology is particularly useful for further confirming when an organism was isolated for first time proves difficult to culture by normal bacteriological techniques.

A. salmonicida is found in aqueous environments and it infects animals that live at low temperature especially fishes and it is considered to be a fish pathogen that is associated with an important economic loss in aquacultures worldwide (3, 17). Fishes are an important component of human food in many countries, therefore it may cause infection to human. However, the gastrointestinal infections by this bacteria is due to ingestion of contaminated food. The outbreaks occur in aquaculture because *A. salmonicida* is widely distributed in freshwater and causes many diseases such as ulcerative and hemorrhagic skin ulcer (5, 18).

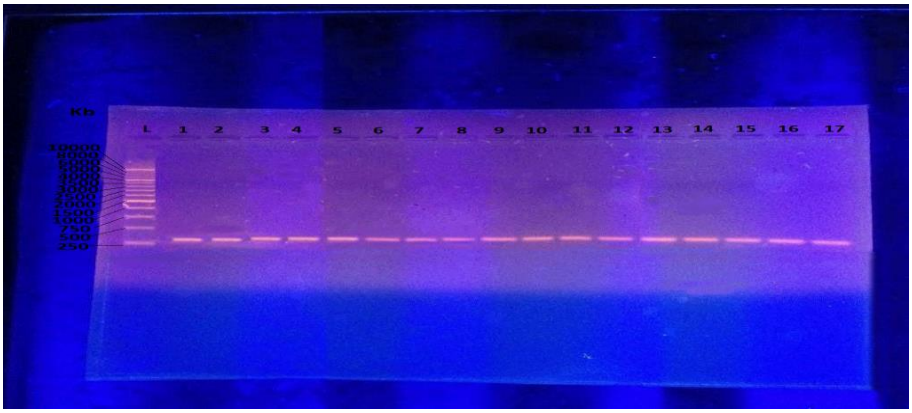

Figure 1. PCR products presenting TonB-dependent siderophore amplified from *A. salmonicida* genomic DNA with expected size 364bp. Lane L: 1000 kb DNA marker; Lane 1 : Amplified PCR product of TonB-dependent siderophore gene *(*364 bp) for *A. salmonicida* ATCC 33658 ; L 2-17: : Amplified PCR product of TonB-dependent siderophore genes *(*364 bp) for local isolates of *A. salmonicida* .

The second aim was to find the antimicrobial resistance of *A. salmonicida* isolates to sixteen antibiotics which are used in aquaculture and in treatment of human infections caused by *A. salmonicida* during the last years in Erbil province.

Indeed, the results found that all isolated bacteria were 100% susceptible to imipenem and 100% resistant to nalidixic acid. Furthermore, the other isolated *A. salmonicida* showed different resistance to other studied antibiotics such as chloramphenicol, Trimethoprim, Tetracycline, Novobiocin, Gentamycin, Levofloxacin, Erythromycin, Ampicillin, Ceftriaxone, Amoxicillin, Ceftazidime, Amikacin, Cotrimoxazole, Ciprofloxacin with percentage 87.5%, 81.25%, 75%,75%, 68.75%, 62.5%, 56.25%, 50%, 43.75%, 37.5%, 37.5%, 31.25%, 25%, 18.75% respectively (Table 1), these results were similar with those results obtained by (19,

20), which found that *A. salmonicida* isolates are resistant to most above antimicrobials.

Table 1. Antimicrobial resistant pattern of *A. salmonicida* isolates.

Antimicrobials	Number of resistant isolates n=16	Resistance (%)
Amikacin	5	31.25
Amoxicillin	6	37.5
Ampicillin	8	50
Ceftazidime	6	37.5
Ceftriaxone	7	43.75
Ciprofloxacin	3	18.75
Chloramphenicol	14	87.5
Cotrimoxazole	4	25
Erythromycin	9	56.25
Gentamycin	11	68.75
Imipenem	0	0
Levofloxacin	10	62.5
Nalidixic acid	16	100
Novobiocin	12	75
Tetracycline	12	75
Trimethoprim	13	81.25

The antimicrobials are used usually against fish pathogens, like *A.salmonicida*, (21).Indeed, the continuous and repeated use of antibiotics with large doses use has led to the emergence of resistant, in addition to many side effects (22). In the intestinal contents of the farmed fish included the three major resistance mechanisms: antibiotic deactivation, cellular protection, and efflux pumps (23) and to solve these problems, other antibiotic molecules have been studied such as imipenem which is a good antibiotic to be use in future programs to control furunculosis outbreaks if good administration protocols for fishes in farm are designed and legal conditions for its use are adopted in our country.

The third important objectives of present study was to solve the resistant and side effects of antibiotics by using medicinal plants such as *E. spiculatum* which is considered as a good inhibitor for bacterial growth (24). Different concentrations of ethanol and aqueous plant extracts of *E. spiculatum* against the most resistant isolate of *A. salmonicida* by using different methods such as MIC, plasmid profile, and protein profile was studied. However, the results of MIC method showed that the MIC for ethanol and aqueous plant extracts were 1400, 1800 µg/ml respectively (Fig. 2). These mentioned results are in agreement with that reported by (25) which found that the studied plant extracts have potent activity against some Gram positive and Gram negative bacteria. These inhibitory effects were attributed to the toxic effect of the calcium oxalate crystals, proteolytic enzymes and triterpenoid of the *E. spiculatum* (26, 27, 28).

On the other hand, molecular study was used to ensure the results of antibacterial activity of both plant extracts. The sub MIC 1200, 1400 µg/ml of aqueous and ethanolic alcohol extracts against bacterial plasmid DNA profile was studied and it was found in this study that the *E. spiculatum* extracts have inhibitory activity against plasmid DNA profile of *A. salmonicida* by reducing the bands from 3 to 2 bands (Fig. 3; Lanes 3,4), these results are close to (29).

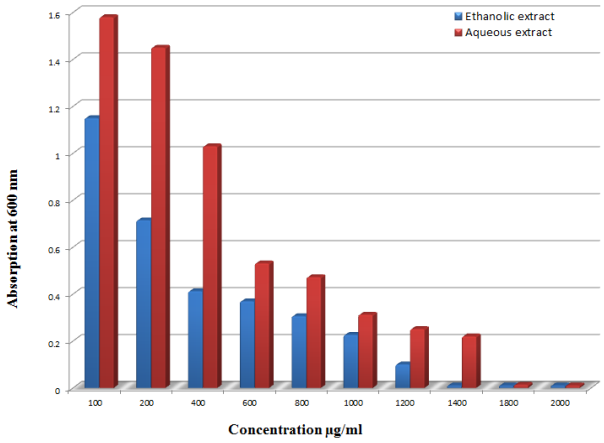

Figure 2. The antibacterial activity of *E. spiculatum* plant extracts against *A. salmonicida*.

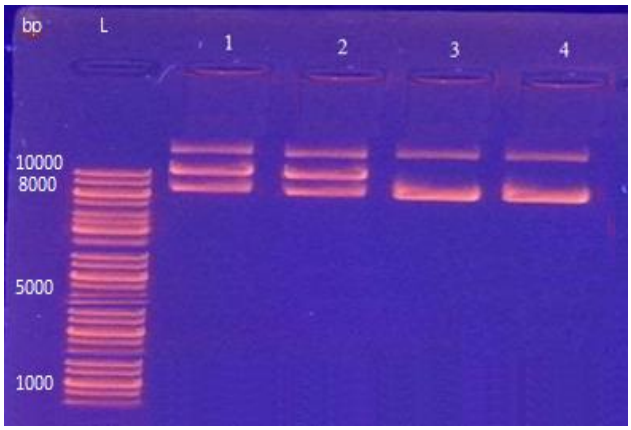

Figure 3. Plasmid profile of *A. salmonicida*. Lane L: 1kb DNA lader;Lane 1:purified plasmid from purified plasmid from *A. salmonicida* before treating with plant. Lane 2: purified plasmid from standard *A. salmonicida* ATCC 33658 before treating with plant extract.; Lane 3:purified plasmid from *A. salmonicida* treated with aqueous extract (1400 µg/ml). Lane 4: purified plasmid from *A. salmonicida* treated with ethanol extract (1200 µg/ml).

Moreover, the other part of the present study was testing the antibacterial activity of the *E. spiculatum* plant extracts against protein profile of *A. salmonicida* isolate by using SDS-PAGE to known that the SubMIC effect on bacterial protein or not. From fig. (4 lanes 3, 4) the results found that

there were variation in protein banding of *A. salmonicida* and formation of new protein bands was detected when treated with Sub MICs of plant extracts and results similar to results reported by (29, 30), this may explaine the ability of *E. spiculatum* extract to apply a stress and under this stress the treated isolates of *A. salmonicida* could respond with increasing of expression level of proteins and induction of others. Therefore, *E. spiculatum* constituents might inactivate the proteins found in membranes and causing decreasing the permeability, as well as destroyed of cytoplasmic membrane and finally causing cell death.

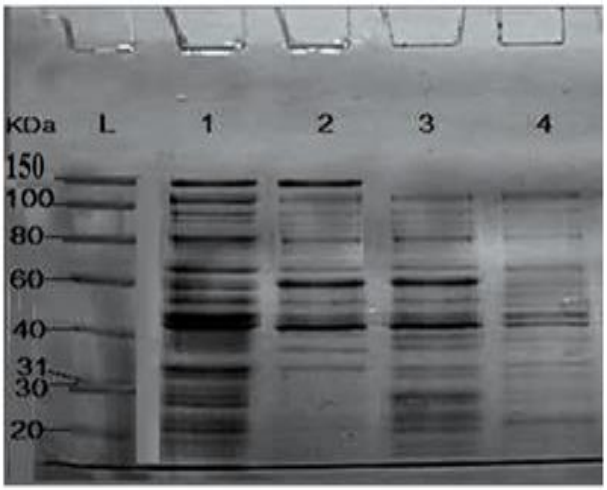

Figure 4. SDS-PAGE electrophoresis of *A. salmonicida* protein. Lane L: protein marker. Lane 1: *A. salmonicida* after treated with 1200 µg/ml ethanol extract. Lane 2: Cell lysate after treating with 1400 µg/ml aqueous extract. Lane 3: cell lysate of local isolate of *A. salmonicida* before treating with plant extract. Lane 4: standard *A. salmonicida* ATCC 33658 before treating with plant.

Conclusion:

The above results showed that the isolated *A. salmonicida* were resistant to most used antimicrobials and it was concluded that the leaves extracts of *E.spiculatum* could be used as antimicrobial for treatment of *A. salmonicida* infections and results confirmed depending on molecular studies.

Conflicts of Interest: None.

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المقاومة الضد جرثومية لبكتريا *Aeromonas salmonicida* المعزولة من الاسماك في مدينة اربيل/ العراق

خديجة خليل مصطفى سازان قادر مولود بشتيوان احمد حمد

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الخلاصة:

A. salmonicida مرضية للاسماك وتسبب امراض مختلفة للانسان. كما ان المعلومات قليلة عن هذه الجرثومة وليس هناك اي دراسة جزيئية عنها.عزلت *A. salmonicida* وشخصت 16 (5.33%) عزلة من امعاء 300 اسماك في احواض التربية في مدينة اربيل /العراق معتمدا على الفحوصات التقليديةوجهاز vitek 2 compact system ولقد تم تاكيدها باستخدام PCR وجين TonB-dependent siderophore (364bp), حددت حساسيتها للمضادات الجرثومية باستخدام طريقة الانتشار القرصي. اظهرت النتائج بان جميع العزلات كانت100% حساسة لـ imipenem, 100% ومقاومة 100% لـ nalidixic acidواعطت مقاومة مختلفة للمضادات كما درست تاثير المستخلص المائي والكحولي لنبات اللاعية *Eminium spiculatum* وباستخدام التركيز المثبط الادنى MIC ودراستها جزيئيا. اظهرت الدراسة بان للمستخلص الكحولي والمائي تاثير مثبط وكانت ال MIC 1400و 1800 ميكروغرام/مل لكلا المستخلصين على التوالي. ايضا استخدم Sub MIC لكلا المستخلصات النباتية في الدراسة الجزيئية. حيث وجد في دراسة بروفايل بلازميد الدنا بان اختلفت عدد حزمها عندما عملت مع كلا المستخلصات النباتية، كما درس تاثير هذه المستخلصات على البروتين الكلي وباستخدام DS- polyacrylamide gel electrophoresis واظهرت النتائج بان كانت هناك اختلاف في عدد الحزم البروتينية في العزلات التي هي تحت الدراسة ولحظت ظهور حزم جديدة للبروتين عندما عملت مع الستخلصات المذكورة اعلاه.

الكلمات المفتاحية: *A. salmonicida*، المقاومة للمضادات الحيوية، نبات اللاعية، مورث TonB-dependent siderophore.