REDVET - Revista electrónica de Veterinaria - ISSN 1695-7504
Vol 23, No. 3 (2022)
http://www.veterinaria.org
Article Received: 25 March 2022; Revised: 15 April 2022; Accepted: 17 May 2022; Publication: 02 July 2022

Revealing of Aerolysin and Cytotoxic enterotoxin genes in *Aeromonas hydrophila* isolated from (*Cyprinus carpio*) fish in Mosul, Iraq

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Abstract

The research aimed on recovering *Aeromonas hydrophila* bacteria from the common carp fish (*Cyprinus carpio*) in Mosul city and study of its virulence by Molecular detection of Aerolysin (aer) and Cytotoxic Enterotoxin (alt) genes. A total of 100 fish samples with obvious signs of illness examined. Of the 134 isolates obtained from gills, air sac, skin and intestine 45 (34%) were identified bacteriologically as *Aeromonas hydrophila*. The ferocity of the isolates was investigated by using particular primers for each of the virulence (aer) and (alt) genes, the results revealed at least 2 of these genes in 36 (80 %) isolate. It is clarified that the major of the strains under exam possessed one or more genes coding virulence. These consequences confirm that *A. hydrophila* obtained from carp fish possess virulence factors and may be a source of infections threating such important industry.

Keywords: *Cyprinus carpio, Aeromonas hydrophila*, PCR, Aerolysin, Cytotoxic enterotoxin.

Introduction

The widely distributed Gram negative Aeromonas bacteria in aquatic environments may be an opportunistic pathogen for fish, amphibians, reptiles, and humans (1). Aeromoniasis mainly affects young fish, so represent economically important and accountable for a huge financially loss for fish farmers around the world (2). *A. caviae, A. hydrophila, A. veronii* and *A. sobria* were reported as a fish pathogen especially at stress conditions causing Aeromonas septicaemia (acute – chronic) and dermal ulcer causes and many serous infections such as fin rot, dermal ulceration, red sore disease, hemorrhagic septicemia, exophthalmia, scale protrusion and erythrodermatitis especially for (*Cyprinus carpio*) common carp (3), the ability to survive and multiply at wide range temperature (15–42) with pH values about 5-9 characterize these bacteria (4).

The ferocity of pathogenic strains for *A. hydrophila* achieved by several factors responsible of virulence as cytotoxic cell enterotoxin, haemolysin, aerolysin , S-layer, adhesins exoprotease, lipase, flagellum, elastase, lipopolysaccharides and Dam (DNA Adenine Methyltransferase) (5,6).

Virulent bacterial strains of *A. hydrophila* produce Aerolysin which is an extracellular secreted protein have qualities of cytolytic and hemolytic hydrophilic properties. Detecting of cytotoxic enterotoxin (alt) recommended as a virulence factor used by pathogenic bacteria to

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enter different cells producing many pore after ulcer, they associated with gastroenteritis and diarrheal syndromes (7,8).

Various define and classify methods were used for Aeromonas spp. either supplement or alternate from biochemical tests, recently the most optimum methods include techniques based upon detecting special genes such as Polymerase chain reaction (9).Using of molecular methods conducted to identify the pathogenicity of Aeromonas spp. (10,11). The presence of this pathogen naturally in fish, poses a constant threat, and their possession of virulence factors reflects their ability to become pathogen especially when exposed to stress. This research deal with molecular identification after morphological observation of Aeromonas bacterium from randomly collected *Cyprinus carpio* fish samples from Mosul governorate basing on some important virulence factors such as aer and alt, as well as dam which reported to be present in human pathogenic isolates using a specific series of primers for amplification (12,13).

Materials and Methods

Fish Samples

(N=100) fish samples included, infected fish in which there is typical disease signs as external hemorrhage, inflammation and ulcers with average weight about 1500-2000 gm collected during the period December 2019- November 2020 randomly from different areas of Mosul governorate, Iraq. Each sample was delivered sterilely in a labeled bag to the laboratory in Department of Microbiology, College of veterinary medicine, Mosul University. Swaps obtained from hemorrhagic or ulcerative area on the skin, gills, air sac and viscera for each common carp fish sample randomly collected from different ponds and areas in Mosul city.

Bacterial isolation, phenotypically

The swabs inoculated on broth of brain heart infusion (BHI) for enrichment, after incubation for one day at 28°C. The morphological aspects of bacterial colonies were observed after sub culturing on plates of Ampicillin sheep blood agar (2), pure colonies were selected for bacterial identification.

Suspected bacterial colonies examined and several biochemical tests were achieved for identification (3, 14) then stored in 30% glycerol at -20°C (15). The virulence of isolated bacterial strains evaluated by PCR (through PCR amplification of aer and alt genes).

Bacterial DNA Extraction and PCR amplification of aer and alt genes.

The isolates were amplified using BHI agar and extraction of DNA was performed with a DNA extraction kit (gSYNC™ Geneaid extraction kit), thermal extraction method was applied (16).

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Bacterial cultures after overnight incubation lysed by 20 mg/ml enzyme of Proteinase K, and transferred to a spin tube, after washing step and the DNA was prepared as indicated in the instructions for the kit used.

The presence of aer and alt genes were confirmed by using PCR specific primers which shown in Table -1-.

Table (1): Sequence of forward and reverse primers of virulence genes (aer, alt).

Primer type	Primer sequence (5' to 3'), F/R	Primer size (pb)
Aer	F-5-CCTATGGCCTGAGCGAGAAG-3 R-5-CCAGTTCCAGTCCCACCACT-3	431
Alt	F: 5-TGACCCAGTCCTGG-3 R: 5-GGTGATCGATCACC-3	442

(8,17).

Selected 3-4 pure colonies were homogenized by 200 µl of SDD water in a 1.5 ml tube. The mixture was exposure to vortex for 2 second then heated at 96ºC for 10 min; after that it was centrifuged. 200 µl supernatant then can be used as a template DNA for PCR, then stored at (-20) until it used (18). The aer and alt PCR amplification applied in 25 µl volum, contained 12.5 µl of premix, 1 µl of each of forward and reverse primer for each gene separately, 3 µl of sample DNA (50-150 ng/µl), the rest completed by 7.5 µl water free of nuclease. Amplified in thermal cycler as explained by Latif-Eugenín *et al.* (19). The program of PCR was performed as in Table (2).

Table 2: PCR Thermocycler program for *A. hydrophila*

Cycle	Temp. °C	Time
	95	3 min.
35	94	1 min.
	56	1 min.
	72	1 min.
1	62	5 min.

Gel Electrophoresis

The products of PCR were definite by 1.5% agarose gel using Ethidium bromide dye (Biometra, Germany). Amplification success was evaluated when bands seen at the size of 431 bp 442 bp for each aer and alt genes, and as standard a 100 bp DNA ladder used.

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Results

Phenotypically detected *Aeromonas hydrophila*

Of the 100 infected fish samples with obvious signs. The pathogenic bacteria isolated from four site of each fish sample including gills, air sac, skin and intestine as totally 134 gram negative bacterial isolates, 45 (34%) of them were identified as *Aeromonas hydrophila* strains, the distribution of these isolates according to sites of isolation showed in Table (3).

Table (3): Percentage of *A. hydrophila* isolated according to examined organs

Suspected organs	Number of isolates	Percentage of isolates %
Gills	19	42.2
Air sac	22	48.9
Skin	3	6.6
Intestine	1	2.2
Total	**45**	**100**

Detection of aer and alt genes (virulence genes) in isolated bacterial strains

The analysis of Conventional PCR of gene profiles based on allocation of the two virulence-genes (aer and alt), among the isolated *Aeromonas hydrophila* strains revealed different virulence patterns as it shown in Table (4). The findings of conventional PCR were showed that among the 45 (34%) *Aeromonas hydrophila* isolates 36 (80%) reversed specific (431 and 442 bp) bands by amplification of aer and alt genes while 7 (15%) were positive only for Aerolysin and the rest of isolates 2 (4.4%) show positive results for cytotoxic enterotoxin gene.

Table (4): The rates of virulence genes combination in *A. hydrophila* isolates

Gene combinations	Number of isolate	Occurrence (%)
Aerolysin + Cytotoxic Enterotoxin	36	80
Aerolysin	7	15.6
Cytotoxic Enterotoxin	2	4.4

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Gel electrophoresis

As it shown in Figures (1) and (2) The results of conventional PCR analysis.

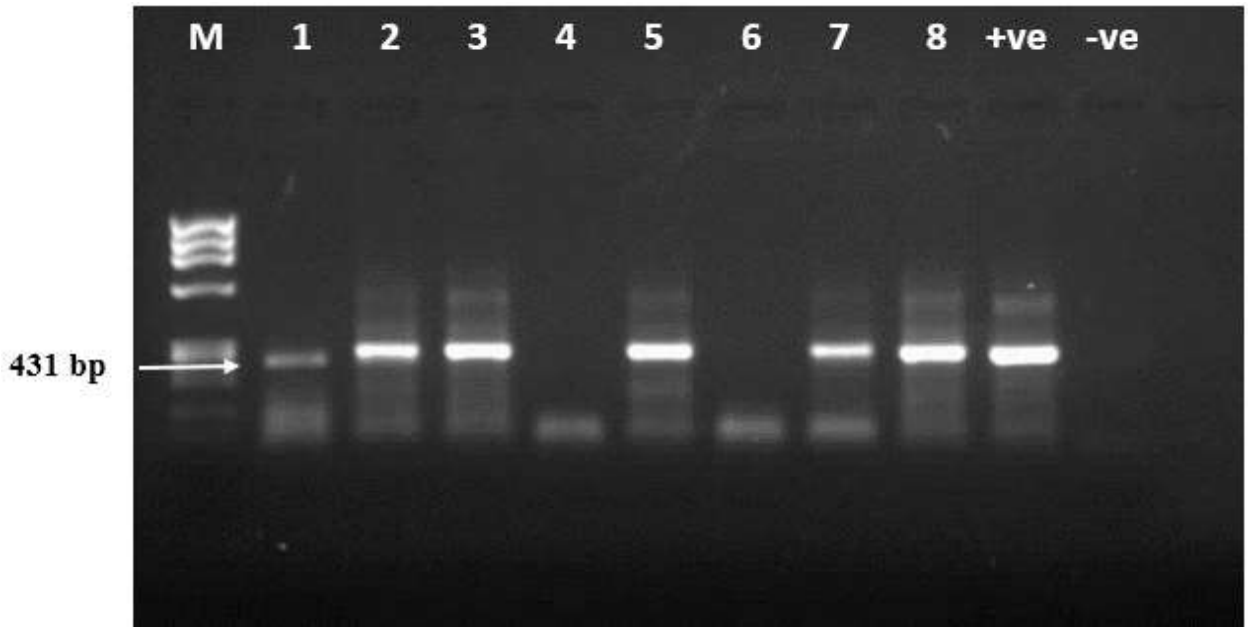

Figure (1): analyzes of PCR products in Ethidium bromide stained agarose gel to perform amplification of 431 bp of aer gene amplicons in *A. hydrophila*. Lane M: DNA marker, +ve: positive control, -ve: (negative) control, Lane 1-3, 5, 7, 8: (positive) strains, Lane 4,6: negative strains.

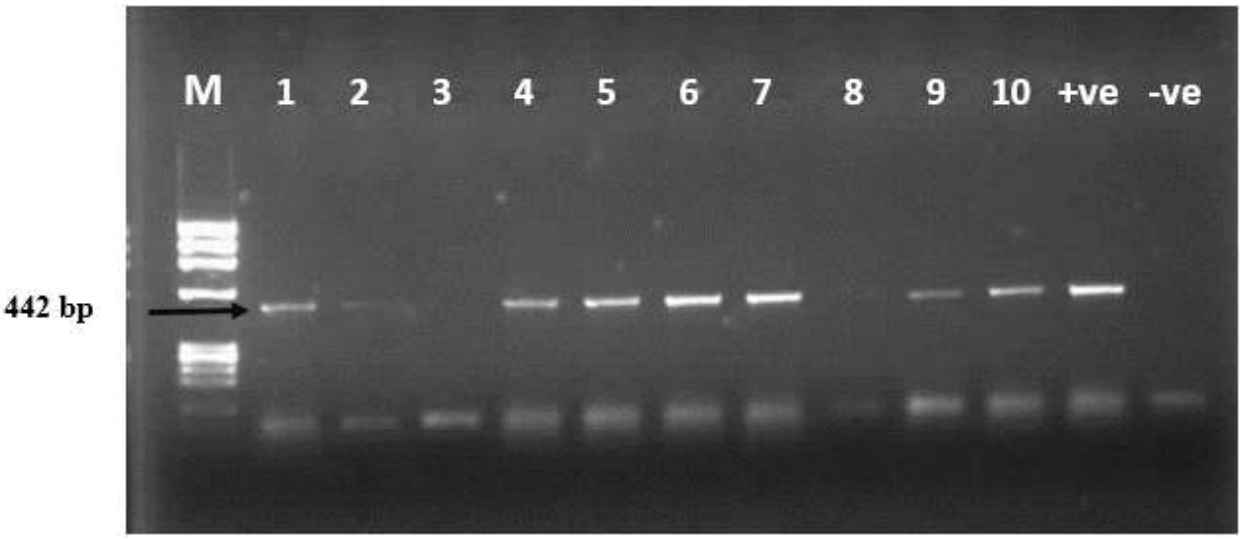

Figure (2): Analyzes of PCR products perform amplification of 442 bp of alt gene amplicons in *A. hydrophila*. Lane M: DNA marker, +ve: (positive) control, -ve: (negative) control, Lane 1,4-7,9,10 positive strains, Lane 2,3,8: negative strains.

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Discussion

Aquaculture is very important source of protein but it is threatened by highly virulent bacterial species such as *Aeromonas hydrophila* (8). The results of bacterial isolation in this study revealed that *Aeromonas hydrophila* is the prevalent species, it was isolated, distinguished from other different species in which symptomize clinical cases and others without obvious clinical syndroms. *Aeromonas hydrophila* assumed to be the most virulence within the Aeromonas complex (20).

The rate of recurrence of various Aeromonas species. Bacterial isolates depend on detection of 16S rRNA gene were 48/76, (63.2 %) from infected fish (3), this disagree with data in current literature including isolation rate of about (34%) from 134 bacterial isolates based on bacteriological examination. While it was near with (7), who showed that from 115 isolated bacteria only 60 isolates negative to Gram stain, while 40 bacterial isolates characterized as motile and 29 strains revealed positive results regarding to hemolysis on blood agar Ibrahim *et al*. (7) and (Al-Haider *et al.* (21) with recording rate of about (58.76%) for *A. hydrophila* obtained from gills, intestine and skin of fish (common carp) in Hilla river (21). The infection with these bacteria increased by altering temperature of water from 25°C to 32°C, salinity, organic substances and pH 5-8 (3).

The virulent gene content of Aeromonas spp. highlights the substantial role of these bacteria in many cases of infection (22). The distribution of complete virulence genes confirms that *A. hydrophila* is a genetically heterogeneous wide spreed species harboring environmental patterns with different pathogenic prospect to human as well as animals (6).

Molecular screening of the two virulence genes (aer) and (alt) confirmed detection of 36(80%) isolate from 45 isolated *Aeromonas hydrophila* having combined genes while the rest 9 isolate have one of them separately, which confirms the data in the study of Samira *et al*. (20), that only 12.28% (14/114) of isolates lack virulence genes coding factors, but not compatible with result of molecular diagnosis dealing with Ibrahim *et al*. study that only 18 bacterial isolates have Aerolysin gene (7).

According to distribution of the genes coding specific virulence factors in positive *A. hydrophila* strains, these isolates confirm (60.6%), while the bacteria lacking virulence genes represent (39.4%). What supports our research results in which 80% from identified *A. hydrophila* carry at least two of virulence factors. This is compact with previous researches relating with the extent of haemolytic factors and their occurrence which can be used as indicator for both pathogenicity and virulence of *A. hydrophila* (23, 3).

The predominant aerolysin (aer) gene indicate high RBCs and cellular lysis for the cells (17). The enterotoxins including: Alt, Act, and Ast have been considered as a primary virulence factors in diarrhea (23). PCR experiments in some researches do not succeed magnifying the Aerolysin, haemolysin genes, and cytolytic enterotoxin. Although these

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factors are important for virulence of Aeromonas bacteria (24). Genetic resemblance among some isolated strains from different geographical areas may reveal that isolates possess the same virulence factors what makes it an important source of infection about physiological adaptation of the environments that they exist (25).

Conclusion

This study demonstrated isolation of *Aeromonas hydrophila* and confirmed the virulence factors in the isolated strains using polymerase chain reaction. The revealing of virulence genes aer and alt in *Aeromonas hydrophila* in fish make the bacteria source of harmful pathogenic agents for humans and animals.

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