

Research Article

Molecular diagnosis of pathogenic *Aeromonas hydrophila* in *Cyprinus carpio* ulcers

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Abstract

A total of 150 carp fish were collected from Grdarasha Farm (8 kilometers from Erbil-Iraq) and examined for the presence of pathogenic *Aeromonas hydrophila* on their skin ulcers, this bacterium secretes a toxin protein called aerolysin which is a virulence factor and has a role in diarrhoeal diseases, deep wound infections and hemorrhagic septicemia. The present study aimed to determine the presence of *A. hydrophila* in common carp (*Cyprinus carpio*). For this purpose samples were collected from fish ulcer by swap and incubated at 37°C in blood agar to determine the shape and morphology of colonies, and then the colonies were examined by gram stain test for microscopic examination, finally molecular diagnosis was conducted for aerolysin gene with primer design and PCR. According to the diagnosis methods results were different; PCR diagnosis confirmed that only 68 skin lesions were caused by *A. hydrophila*, no visceral lesion was caused by *A. hydrophila* and this bacterium hardly ever seen in the skin surrounding mouth as only 2 lesions surrounding mouth had *A. hydrophila* in PCR diagnosis.

Keywords: Aerolysin, *Aeromonas hydrophila*, *Cyprinus carpio*, Polymerase chain reaction

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Introduction

Fish is an important source of nutrient for human, because it contains many types of essential amino acids that are non-existent in other meats. Also it contains 16-29% of proteins which are considered highly valuable for human body. Additionally it provides 300-1600 calories per pound depending on the type of fish (Skibniewska *et al.*, 2013). The common carp (*Cyprinus carpio* Linnaeus, 1758) is native to Europe and Asia, and is introduced to every part of the world except the poles. This is the third most frequently introduced species worldwide and is used as food in Kurdistan region of Iraq and other parts of the world (Abbott *et al.*, 2003). The bacterial diagnosis of pond fishes of a fish farm is actually depended on the quality of inlet and outlet water of the pond, the type of supplementary food that is used for growing and breeding the fishes as well as age of the fishes (SamCookiyaei *et al.*, 2012).

According to a recent review, only 37 bacteria have been reported to be pathogenic for fish, which is surprisingly a small number considering the large number of fish species and their diverse environments (Allen *et al.*, 2010). Many affected fish in ponds are considerably related to motile *Aeromonas*, in fact the term “motile *Aeromonas* septicemia” (MAS) is used to describe motile *Aeromonas* infections of warm water fish, including common carp (Abdelhamed *et al.*, 2019; Adamek *et al.*, 2019). The motile

species are often ubiquitous members of the aquatic ecosystem where *Aeromonas hydrophila* is a normal inhabitant and carps are constantly exposed to infection. Disease outbreaks are common when carps are under stress caused by crowding, low oxygen, or high temperature (Plumb, 2018).

The goal of the current research was to determine the presence of *Aeromonas hydrophila* in *C. carpio* of Grdarasha ponds in the fish farm of Agriculture College using microbiological diagnosis as well as molecular methods with PCR. There is no previous research on these common carp bacteria in Kurdistan region, but there is a study on ectoparasite infection of them (Al-Marjan and Abdullah, 2009).

Materials and methods

Collection and Examination of samples

A total of 150 specimens of *C. carpio* were collected from Agriculture College fish farm (Grdarasha) from October 2016 to August 2017. Samples were labeled and kept alive in cool boxes containing pond water, and transported to the laboratory of Microbiology, Biology Department, College of Science, University of Salahaddin. The total length and weight of samples were measured to be 22-34 cm (± 2 cm) and 125-245 gm (± 5 gm) (Stone *et al.*, 2016).

Isolation and identification of bacteria

For isolation of bacteria, samples were taken from oral and gut of fishes by using sterile swabs. Then the swabs

were pressed on blood agar medium and Luria-Bertani broth and inoculated. The agar plates and broth were incubated at 37°C for 24-48 hours and the bacterial colonies were examined for further characterization and identification. Morphological characteristics such as color, size, shape and margin of the colonies were recorded. The bacterial colonies were then subjected to gram staining reaction and motility test. Bacteria from Luria-Bertani broth were used for DNA extraction that followed by PCR diagnosis.

Aerolysin gene

The aerolysin gene is used as a virulence marker to identify potentially pathogenic *Aeromonas* (Heuzenroeder *et al.*, 1999; González-Serrano *et al.*, 2002). Aerolysin is considered to bind to eukaryotic cell surface and form holes (channels), leading to destruction of membrane permeability and cell death (Christy *et al.*, 2019). The pathogenicity and immunogenicity of proaerolysin (inactive form) or aerolysin (active form) is currently not well known due mainly to the limitations in purifying large amounts of the bioactive protein from *A. hydrophila* (Li *et al.*, 2019).

Media

Blood Agar Medium: Blood agar is the most commonly used medium and support the growth of most of the common fastidious organisms. Colonies on blood agar can readily be tested for

oxidase test (Joseph *et al.*, 1979). This medium was prepared by suspending 40.0 grams in 1000 ml distilled water, heating to boiling to dissolve the medium completely and sterilizing at 15 lbs./ 121°C for 15 minutes by autoclave. The medium was then cooled to 50°C and added 5% blood aseptically. Finally the medium was well mixed and poured into sterile petri plates (Janda *et al.*, 1984).

Luria-Bertani

This medium was prepared by dissolving 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water. After that pH of the medium was adjusted to 7.0 using 1N NaOH, and volume of the medium was brought up to 1 liter. The medium was sterilized by autoclaving for 20 min at 15 psi on liquid cycle. The solution was allowed to cool to 55°C, and antibiotic was added if needed (50µg/ mL of Amp or Kan). Finally the medium was allowed to store at room temperature at +4°C (Khushiramani *et al.*, 2007).

Gram stain

Gram stain was used for microscopic examination of bacterial shape, according to this stain bacterium can be divided into two groups: Gram negative and Gram positive (Janda *et al.*, 1984).

DNA Extraction

Bacterial DNA was extracted after incubation at 37°C in LB broth by using Jena Bioscience (Germany) and

according to the manufacturer's instructions with some modification.

Primers

The primers were designed using DNA sequences, which was obtained from a

primer blast in the NCBI (National Centre for Biotechnology Information). The primers were designed for Aerolysin gene (This unique primer for the present study was designed by SnapGene program):

Aero-F: 5'CGCGGATCCGGCTTGTCATTGATCATATCC 3' 53%GC contents

Aero-R: 5'CCGCTCGAGTTATTGATTGGCAGCTGGC 3' 57% GC contents

Polymerase chain reaction and conditions

Aerolysin gene of *Aeromonas hydrophila* was amplified by PCR. The reaction mixture was a total of 50 μ L consisting of 25 μ L master mix, 1 μ L of each primer (0.4ng/ μ L for each

primer), 22 μ L of ddH₂O and 10ng of bacterial genomic (Chacón *et al.*, 2003). PCR reaction condition for the diagnosis of aerolysin genes in *Aeromonas hydrophila* is shown in Table 1.

Table 1: PCR condition which contain all cycles regarding Temperature and time.

Steps	Temperature	Time
Initial Denaturation	95°C	6 min.
30 Cycles		
Denaturation	95°C	30 sec.
Annealing	66 (65)°C	35 sec.
Elongation	72°C	1 min.
Final Elongation	72°C	4 min.

Amplicons were separated by gel electrophoresis in order to confirm the size, location and quality of the PCR specific product for each primer. A standard method was used for electrophoresis (1%) preparation (De Gregoris *et al.*, 2011).

Results

A total of 150 fish were measured between 22-34 cm (\pm 2 cm) of total length and weight between 125-245 gm

(\pm 5 gm) that were collected from Agriculture College Fish Farm (Grdarasha Farm). The three types of conducted examinations were macroscopic for lesion and ulcer formation, culturing and gram staining examination for confirmation of pattern and shapes of colonies (bacteria); and Vatik2 for the presence of *Aeromonas hydrophila* which was finally confirmed by PCR for aerolysin gene.

Macroscopic examinations

In the macroscopic examinations of the common carp, brown or red spotted skin lesions of a varying degree were observed along their bodies (Fig. 1).

First examination was done by viewing and observing for skin tissue change like ulcer and lesion formation on the skin, in mouth cavity and intestine, the result showed that out of 150 only 86 fish were infected with ulcer on their skin, only 6 fish had lesion around their mouth, and just 3 visceral organ lesions were present. The results are illustrated in Table 2.



Figure 1: Ulcer and lesions on the skin of infected common carp.

Table 2: Frequency of ulcer formation in 86 *C. carpio* from Grdarash, Kurdistan Region of Iraq.

Site of infection	Number of fish with Ulcer
Skin	86
Mouth surrounding	6
Visceral organ	3

Culturing and gram-stain examination

This examination was conducted after decreasing the number of fish from 150 to 86. The target fish were chosen depending on the results of previous

examination which was conducted by naked eye for lesion observation. Culturing and gram stain examination were conducted by taking swap from each lesion of skin, mouth cavity and stomach. The swap from each lesion was inoculated on to blood agar plate and incubated in 37°C overnight and then gram stained slides were prepared from each colony. The results are illustrated in Table 3.

Table 3: The presence of gram-negative bacteria from each lesion using blood agar.

Site of infection	Lesion with gram negative bacteria
Skin	80
Surrounding Mouth skin	5
visceral organ	none

Gram stain

Aeromonas spp. are gram-negative, straight rods appearing singly or in pairs with or without short chains (Janda and Abbott, 2010).

Media and incubation

Aeromonas spp. grew well on common laboratory media including blood agar, nutrient, and Luria Bertani. On blood agar, *Aeromonas* formed circular colonies that were 1-3 mm in diameter which starts off grayish in color due to beta-hemolysis and after three days became dark green. DNase culture result was positive. Maximal growth was seen when the temperature was

between 37°C and 44°C (Khushiramani *et al.*, 2007; Parker and Shaw, 2011).

Colony morphology

Colony morphology on blood agar was large, round, raised, opaque; most colonies were beta-hemolytic except for *A. caviae*.

PCR diagnosis

Gram negative bacterial lesions from previous tests were examined by Vatik2 technique for the presence of *Aeromonas hydrophila* then the results

were finally confirmed by PCR diagnosis, for that purpose special primers have been designed for aerolysin gene as described in the methodology section. Agarose gel electrophoresis (1%) was conducted for PCR product and amplicons with 1479 bp confirmed the presence of *Aeromonas hydrophila* as shown in figures 2 and 3. The PCR test result is shown in Table 4.



Figure 2: PCR diagnosis for *Aeromonas hydrophila* bacteria which examined through agarose gel electrophoresis (1%) under UV light. Lane 1: DNA Ladder (500-10200bp), Lane 2: negative PCR control, Lane 3: positive PCR control, Lane 4: positive bacterial sample, Lane 5: negative bacterial sample.

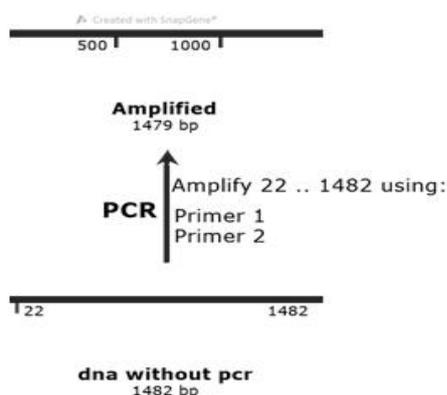


Figure 3: SnapGene program showing the size of amplicons after PCR run.

Table 4: PCR diagnosis showed *Aeromonas hydrophila* resence in the lesion of common carp.

Site of infection	Lesion with <i>Aeromonas hydrophila</i>
Skin	68
Surrounding Mouth skin	2
visceral organ	none

Discussions

As food fish is considered the most world-wide source which contains many types of essential amino acids that are non-existent in other meats (Skibniewska *et al.*, 2013), thus fish diseases due to loss of fish has an adverse effect on human health. According to a recent research a group of bacteria are pathogenic for fish (Allen *et al.*, 2010). The most considerable bacterial group which causes disease in many fish species, including common carp, are motile *Aeromonas* (Allen *et al.*, 2010). The present study aimed to suggest the prevalence of gram negative bacteria especially *A. hydrophila* in infected fish with skin ulcer. Results indicated the presence of *A. hydrophila* in skin of 68 common carp lesions which could be considered as causative agent as it is similarly suggested in other researches (Öztürk *et al.*, 2007). Also PCR study of the present study confirmed that only two mouth surrounding lesions showed positive results for the presence of *A. hydrophila*. It is confirmed that *A. hydrophila* in mouth lesion is a secondary cause, researchers indicated that *Aeromonas* lesion is more related to the tail or fin, gill and eye not to the

skin surrounding mouth (Ozer, 1999). PCR result showed that none of visceral organ lesions were caused by *A. hydrophila* or gram-negative bacteria. This may be related to the time of infection that bacteria need to occupy visceral organ (Geny and Popoff, 2006).

PCR diagnosis is more accurate than culturing and gram stain examinations, because PCR technique depend on the presence of the target gene while culturing technique depend on the morphology, shape and enzymes of the bacteria that contamination is possible as it is mentioned in other researches (Pongsachareonnont *et al.*, 2017). Also detecting aerolysin toxin, which is a virulence factor used by bacteria in order to penetrate the cell and produce pore secondarily ulcer, is recommended compared with diagnosis by culture or gram stain (Geny and Popoff, 2006).

In conclusion, this research reported the following points: only 86 fish (totally 57.3% of Common Carp) out of 150 samples had lesions in their skin according to the microbiological diagnosis; PCR study confirmed presence of *A. hydrophila* in skin ulcers of 68 fish and only 2 lesions surrounding fish mouth were caused by *A. hydrophila* according molecular diagnosis; lesion in the visceral organs are rare as PCR diagnosis confirmed that no of visceral organ lesion was caused by *A. hydrophila*.

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