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RESEARCH PAPER

Detection of Aerolysin Gene in *Aeromonas hydrophila* from Suspected Farming Fishes (*Cyprinus carpio*), Erbil Province / Iraq

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A B S T R A C T:
Aeromonas hydrophila consider as the most common pathogenic bacteria which infect many farming fishes and due to many economical losses annually this bacteria found in fresh, brackish waters and ponds. The present study conducted to determine the most reliable method for the diagnosis of *A. hydrophila* from common carp (*Cyprinus carpio*), in Agriculture college fish farmduring a farming (May 2017 to October 2018).Results of the present study showed that 60 bacterial samples out of 115 bacterial samples were gram-negative, however 40 samples were motile and only 29 samples showed positive hemolysis on blood agar. Result of molecular diagnosis showed that only 18 bacterial samples have Aerolysin gene (1482 bp).

KEY WORDS: Aerolysin, *Aeromonas hydrophila*, *Cyprinus carpio*, Polymerase chain reaction.
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1.INTRODUCTION :

Many fishes in ponds are considerable affected by the disease from "motile Aeromonas septicaemia" (MAS) which infect warm water fish including common carp, *A. hydrophila* is one of the most common species which associated indeed relationship with human diseases as well (Allen *et al.*, 2010) and (Barzani and Mustafa, 2015).
Aeromonasis the Gram-negative rods, facultative anaerobic, motile and non spore forming bacteria, usually present acutely in aquatic ecosystems (Barzani and Mustafa, 2015),either surface or underground water such as fresh, estuarine, marine, groundwater, drinking water and ponds (Nakano *et al.*, 1990), (Ashbolt *et al.*, 1995) and (Janda and Abbott, 2010).

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There are many virulence factors in *A. hydrophila*, including protease, aerolysin, and enterotoxin toxin, which cause disease in humans and fish (Zhu *et al.*, 2007). The whole sequence of the aerolysin nucleotide gene is located on a 1.8-kb ApaI-EcoRI fragment and consists of 1,479 bp that contain an ATG initiation codon and a TAA termination codon (Igbinosa *et al.*, 2017), (Singh *et al.*, 2010) and (Singh *et al.*, 2009).
The present study aimed to determine the most reliable method for the diagnosis of *A. hydrophila* from common carp (*Cyprinus carpio*), and comparing between molecular diagnosis by PCR (Polymerase Chain Reaction), standard microbiological diagnosis (Gram-stain, Culturing and other techniques) and Vitek II system

2.Materials and methods

Bacterial sample isolated from115 common carp farming fishes (30-39 cm in length and 1200-2000 gm in weight as shown in figure (1) which were collected from Agriculture College fish farm (Grda-Rasha 8 km away from capital

Erbil city) during a farming season (May 2017-October 2018). The fishes labeled and transported alive in a cool box containing the fish's water pond to the laboratory of Microbiology, Biology Department, College of Science, University of Salahaddin- Erbil for microbiology examination.

Figure 1. Common carp (*Cyprinus carpio*) farming fishes.

3.Collection, isolation and identification

Microbiological tests have been done by taking sterile swab from 115suspected farming fishes, exactly swabs have been taken from ulcers which were visible by naked eye examination throughout the body of collected fishes, suspected fishes for pathogenic *A. hydrophila* from the present study were shown in figure (2).All samples immediately transferred to laboratory and subjected directly to microbiological examinations. All swabs were inoculated on blood agar and incubated aerobically at 37°C for 24h then sub cultured on nutrient agar, suspected bacterial colonies were purified based on the size, shape, color and patterns of hemolysis and non-hemolysis on 5% and were subjected to Gram's staining(Parker and Shaw, 2011). In addition, oxidase and catalase test done according to the method by (Sørum, 2006).

Figure 2.suspected common carp with ulcers on their bodies for pathogenic *Aeromonas hydrophila.*

3.1Gram 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) and(Ashith et al., 2019).

3.2Vitek II system

Biochemical profiles with the Vitek II system (bioMérieux, Lyon) was utilized for identification of *A. hydrophila* after selection of the appropriate card according to the gram stain and growth condition of bacterial samples(Izzati, 2019)

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3.3Detection of Aerolysin gene by Polymerase Chain Reaction

The bacterial DNA extracted after incubation at 37°C in Luria Bertani (LB) broth by using Jena Bioscience kit (Germany) and according to the manufacturer's instructions with some modification has been done and primers were designed for Aerolysin gene (1482 bp). Following, the constructed primers were then matched concurrently with other sequences in the GenBank database in order to check their identity and resemblance with the gene Aerolysin from *A. hydrophila* and other bacterial species. Aero-F: 5'CGCGGATCCGGCTTGTCATTGATCATAT CC 3'

Aero-R:
5'CCGCTCGAGTTATTGATTGGCAGCTGGC 3'

Aerolysin gene of *A. hydrophila* was amplified by PCR. The reaction mixture is a total of 50µl consisted 25 µl master mix, 1 µl of each primer, 20 µl of ddH2O and 3 µl (30 ng)of bacterial genomic. Amplification condition was obtained with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles at 95°C for 30 sec, and annealing at 65°C for 30 sec, 72 °C for 1 min and final extension 72°C for 4 min (Chacon et al., 2003).

To confirm the size, location and quality of the PCR-specific product for specific primers, amplicons were separated by gel electrophoresis. A standard technique was used to prepare the electrophoresis tank (De Gregoris et al., 2011).

Gel was ready to produce 1 percent gel for electrophoresis by combining 70 ml of Ix TAE (TrisAcitate EDTA) with 0.70 g agarose and high electrical power run off for about 4 minutes. Each sample was then added 2.5 µl of the Orange G dye (1/10 sample quantity). 25 µl of 1 kb ladder was prepared as follows to calibrate the gel; 21.5 µl of molecular water, 2.5 µl of Orange G dye and I µl of ladder (100bp, Promega).

4.Results:

All bacterial samples (115 samples) were addresses into microbiological tests such as (Gram stain, Motility test, Blood agar diagnosis, Catalase and Oxidase test) then they were diagnosed by vitek II and finally PCR run for Aerolysin done for the positive samples of vitek II test.

Results showed that 60 samples out of 115 samples were Gram-negative, however 40 samples were motile and only 29 samples showed positive hemolysis on blood agar (Table 1) and showed positive result for both test of catalase and oxidase.

Table 1. Number of positive for *Aeromonas hydrophila* samples by microbiological tests (Gram-stain, Mobility test, and blood culturing).

Microbiological tests	Number of positive samples/ 115	Positive samples %
Gram-stain	60	52.1
Motility test	40	34.7
Blood agar	29	25.2

Vitek II system analysis done after the selection of the appropriate card according to the gram stain and growth condition of bacterial sample (*A. hydrophila*), the result of this analysis confirmed that only 20 samples were *A. hydrophila* out of 29 suspected sample from final microbiological results.

Genotypic detection of aerolysin gene was done by PCR, special primers for aerolysin gene designed as mentioned in methodology section, out of 20 infected fishes with *A. hydrophila* (Vitek) only 18 samples showed the presence of whole coding region of Aerolysin gene and the size(1482 bp), Agarose gel electrophoresis done for PCR product and amplicons with (1479 bp) were considered as positive samples which confirm the presence of *A. hydrophila* as showed in figure (3).

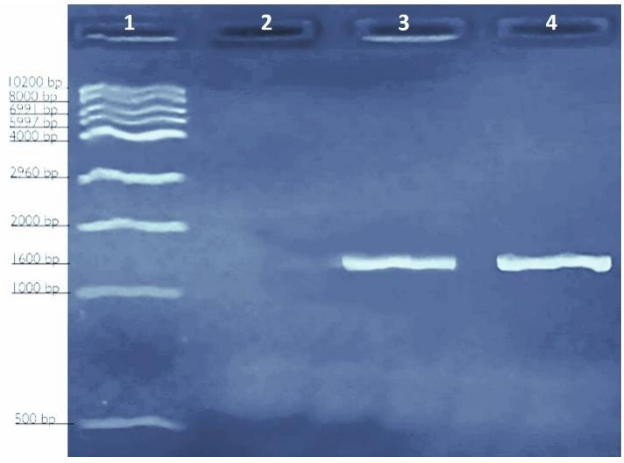

Figure 3. Polymerase chain reaction products on gel electrophoresis (1.5%) for Aerolysin gene

(1482) bp gene for *Aeromonas hydrophila.* Lane 1: 1 kb Ladder, Lane 2: negative PCR control, Lane 3: positive PCR control and Lane 4: positive bacterial sample.

5.Discussions

Fish considered as the most world-wide source of nutrient which contains many types of essential amino acids (Skibniewska*et al.*,2013), *C. carpio* present in Asia, Europa and other part of world like Iraq-Kurdistan region (Aziz and Muhammad, 2017). The most considerable bacterial group which cause disease to many fish including common carp are motile Aeromonas (Allen *et al.*, 2010). Many researchers reported various disease caused by Aeromonas spp. such as gastroenteritis (Chopra et al., 1993), endocarditis (Brouqui and Raoult, 2001) and meningitis (Ouderkirk *et al.*, 2004). Aerolysin toxin considered as the most common virulence factor of *A. hydrophila* (Igbinosa *et al.*, 2017).

PCR diagnosis is more accurate than culturing gram stain examination and Vitek II system because PCR technique depend on the presence of the target gene while culturing technique depend on the morphology, shapes and enzymes of the bacteria (Pongsachareonnont *et al.*, 2017), (Panangala *et al.*, 2007) and (Balaky *et al.*, 2019). Furthermore, identification based on variations in one or two phenotypic features does not show true environmental diversity and enables inaccurate identification when comparing outcomes acquired in distinct laboratories (Cherkaoui *et al.*, 2010). Also identification by Vitek II system is not enough accurate which depend on the purity of sample, thus various qualitative levels of identification were assigned based on the numerical probability calculation *(Izzati, 2019).*

Detecting the Aerolysin toxin is more recommended which is a virulence factor used by bacteria in order to penetrate the cell and produce pore secondarily ulcer than diagnosis by culture or gram stain (Geny and Popoff, 2006).

The present study conducted to detection Aerolysin gene by PCR for the diagnosis of *A. hydrophila* from common carps, PCR assay confirmed that 18 of infected fishes with *A. hydrophila* showed positive Aerolysin gene similar result concluded by (Wang *et al.*, 2003). Previous study concluded only 79% of infected fishes with same bacteria showed cytotoxic gene positive (Ørmen and Østensvik, 2001). The present study is totally agreed with recent study (Leitner *et al.*, 2013), which preferred molecular techniques over the microbiological methods for the diagnosis of *A. hydrophila*in common carp. More-ever previous study consider microbiological methods consider as the basic for the diagnosis of pathogenic bacteria (Agger *et al.*, 1985).

In conclusion, Polymerase chain reaction is a method that takes less time and more accuracy to diagnose pathogenic *A. hydrophila* in common carp as it depends on genotype rather than phenotype characters.

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