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PCR INVESTIGATIONS FOR DIAGNOSIS OF PATHOGENIC BACTERIA FROM INFECTED FISH

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Abstract

Many microorganisms causes infection and decrease in the aquaculture productions and bacteria considered as the most common microorganisms, which cause wide range of infections in carps, especially *Aeromonas hydrophila*. This bacteria Gram negative rod shape and has no spores. *Aeromonas hydrophila* causes infection fish and human as well, thus present study used microbiological and molecular methods in complementary for the accurate diagnosis of this bacterium in Dukan lake which located in Kurdistan region of Iraq during a farming season (2018-2019). Results of the present study confirmed that only 51 suspected bacteria were *Aeromonas hydrophila*, also concluded that molecular techniques like PCR diagnosis was applicable as it depends on genotype rather phenotypes characters and also microbiological techniques like Gram stain and culturing was useful in decreasing the number of suspected bacteria.

Key words : Bacteria, infection, diagnosis, polymerase chain reaction.

Introduction

Infections of pathogenic bacteria from fish actually depend on the quality of water, methods of farming, feeding methods, age of fish and other environmental factors (SamCookiyaei *et al*., 2012). Many researches confirmed that more than 37 bacteria species causes fish diseases which cause economical loss in the aquaculture productions (Allen *et al*., 2010).

Motile *Aeromonas* consider as one of the most pathogenic bacteria in aquaculture and the term "Motile *Aeromonas* Septicaemia" (MAS) is used to describe motile aeromonas infections of warm water fish (Allen *et al*., 2010). Many hosts for this bacteria have been reported including *Cyprinus carpio*, *Sparus aurata*, *Dicentrarchus labrax*, *Trachurus mediterraneus*, *Oncorhynchus mykiss*, *Carassius auratus* (Orozova *et al*., 2018; Reyes-Becerril *et al*., 2011; Öztürk and Altýnok, 2014). The genus *Aeromonas* belongs to the gram-negative group of bacteria, characterizing by its rod shape and facultative anaerobic and pathogenic properties. Also, *A. hydrophila* is among highly pathogenic bacterium isolated from the freshwater crayfish, *Pacifastacus leniusculus* (Jiravanichpaisal *et al*., 2009).

Many factors make this pathogenic bacterium to be able to cause infection and diseases including it is virulence factors such as protease, Aerolysin and enterotoxin toxin, which cause disease in humans and fish (Zhu *et al*., 2007). The whole nucleotide sequence of the Aerolysin 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).

The aim of the present study was using microbiological techniques and Polymerase Chain Reaction as complementary method for the diagnosis of pathogenic bacteria (*Aeromonas hydrophila*).

Materials and Methods

Sample collection

All bacteria samples in the present study was collected and obtained from skin of farming fish especially (Common carp), totally 300 carps have been investigated

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from (Dukan lake) for the presence of pathogenic bacteria on their skins during a farming season (2018-2019). Farming fishes were over 20cm in length which weighted between 1500-2000 gm, as shown in fig. 1. 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.

Fig. 1 : Common carp farming fishes which collected from Dukan lake.

Preparation of Media

Blood Agar Medium was prepared by suspending 40.0 grams in 1000 ml distilled water, heated to boiling to dissolve the medium completely sterilized by autoclaving at 15 lbs, 121°C for 15 minutes, finally cooled to 50°C and aseptically added 5% blood. Mixed well and poured into sterile petri plates (Janda *et al*., 1984).

Luria-Bertani medium was prepared by dissolving 10 gm tryptone, 5 g yeast extract, and 10 g NaCl in 950 mL deionized water, after that adjusted the pH of the medium to 7.0 using 1N NaOH and bring volume up to 1 liter, autoclaving on liquid cycle for 20 min at 15 psi., allow solution to cool to 55°C and add antibiotic if needed (50µg / mL of Amp or Kan), stored at room temperature at +4°C (Khushiramani *et al*., 2007).

Preparation PCR and Agarose gel electrophoresis

Designed primers were compared in the GenBank database to verify the correctness of primers for Aerolysin gene amplifications in *A. hydrophila*.

Aero-F:5'CGCGGATCCGGCTTGTCATTGATCATATCC 3'

Aero-R:5'CCGCTCGAGTTATTGATTGGCAGCTGGC 3'

PCR Mixture : reaction mixture is a total of 50 µl consisted 25 µl master mix, 1 µl of each primer, 20 µl of ddH_2O 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).

Agarose Gel Electrophoresis : Gel was prepared by mixing 70 ml of Ix TAE (Tris Acitate EDTA) with 0.70 g agarose to make 1% gel for electrophoresis and high power run off for about 4 minutes. Subsequently, 2.5 µl of the Orange G dye (1/10 sample volume) was added to each sample. To calibrate the gel, 25 µl of 1 kb ladder was prepared as follows; 21.5 µl of molecular water, 2.5 µl of Orange G dye and I µl of ladder (100bp, Promega).

Diagnosis methods

First diagnosis has been done with the microbiological tests such as Gram stain and Culturing, sterile swap taken from skin of suspected fishes (Fish with skin ulcer) and inoculated in Nutrient broth for the amplification of bacteria, and turbidity examination have been done after incubation for about 24hr at 37°C. PCR diagnosis started with the isolation of bacteria genome by using Jena Bioscience kit (Germany) according to the manufacturer's instructions, one set of designed primers for Aerolysin gene have been used for amplification of target gene (1482 bp). Amplicons were separated by gel electrophoresis in order to confirm the size, location and quality of the PCR specific product for specific primers. The electrophoresis tank was prepared using a standard method (De Gregoris *et al*., 2011). The protocol for diagnosis has been shown in fig. 2.

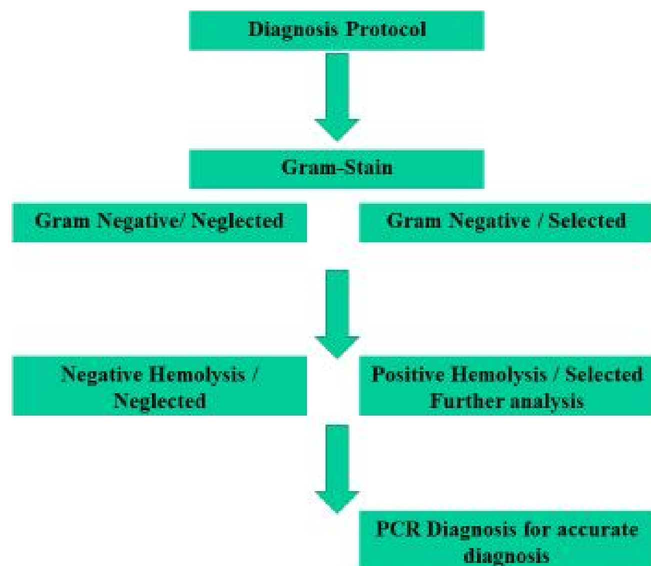

Fig. 2 : Illustrative diagram shows diagnosis protocol of pathogenic bacteria (*Aeromonas hydrophila*).

Results

Gram stain determined the pattern, shape and subclasses bacteria into Gram negative and Gram positive, and blood agar confirmed the presence of *Aeromonas* ssp. by the future and hemolysis activity of colonies while PCR diagnosis confirmed the presence of *Aeromonas hydrophila*. Thus, all three ways together were complementary for diagnosis of pathogenic bacteria.

Results showed that 125 samples out of 300 samples

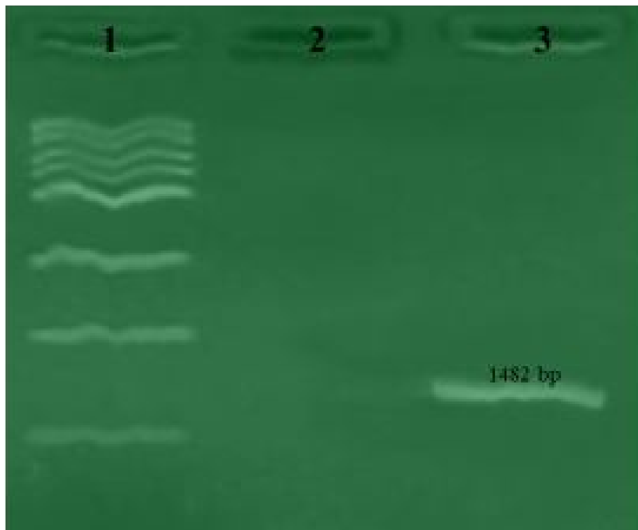

Fig. 3 : PCR products which were examined by Agarose gel electrophoresis (1%) under UV light. Lane 1: Ladder, Lane 2: negative PCR control, Lane 3: positive PCR product.

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/ 300	Positive samples %
Gram-Negative	125	52.1
Blood agar/Hemolysis	82	25.2

were Gram-negative, however only 82 samples showed positive hemolysis on blood agar table 1.

As described, PCR run took place after the designee of special primers for Aerolysin gene, PCR diagnosis confirmed that only 51 samples out of 82 suspected out of 82 *Aeromonas* ssp. were *Aeromonas hydrophila*, 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 fig. 3.

Discussion

Fish is one of the most world-wide source of nutrient which contains many types of essential amino acids (Skibniewska *et al*., 2013). Fish can be infected by viral, bacterial and fungal infection, however bacterial infection consider as the most common factor in decreasing fish products and 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).

Polymerase chain reaction was first used more than 30 ago, thus is consider as a new techniques for the diagnosis of microorganisms, additionally, 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 that way contamination is possible depend on other researches (Pongsachareonnont *et al*., 2017 and Panangala *et al*., 2007) also identification based on differences in one or two phenotypic characteristics does not reveal the real environmental diversity and allows mistaken identification when results obtained in different laboratories are compared (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 (Pincus, 2010).

Pathogenic bacteria usually have more than one virulence factors by which causes infection and molecular techniques, which depend on the diagnosis of genes responsible for virulence factors is more acceptable, in *Aeromonas hydrophila* many studies determined Aerolysin gene for the diagnosis of this pathogenic bacteria (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).

In conclusion, Polymerase chain reaction was applicable in the accurate diagnosis of pathogenic *A. hydrophila* in common carp as it depends on genotype rather than phenotype characters, and also microbiological tests were useful in the decreasing suspected samples.

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