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Research Article

MOLECULAR DETECTION OF *Aeromonas hydrophila* ISOLATED FROM INFECTED CARP *Cyprinus carpio* BREEDING IN AQUAFARMING IN BASRA, IRAQ

Rasha M. Othman[1], Fayhaa S. Al-Thahe[2], Rana A. Faaz[1] and Hanan Y. Jassim[3]

[1]*Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basrah, Iraq.*

[2]*Department of Pathology, College of Veterinary medicine, University of Basrah, Iraq.*

[3]*Department of Internal and Preventive Medicine, College of Veterinary Medicine, University of Basrah, Iraq.*

Abstract

In this present study, we used the polymerase chain reaction technique for the diagnosis of pathogenic bacteria (*Aeromonas hydrophila*) in infected fish and their environment. This method was considered contemporary and accurate techniques in the diagnosis of pathogen as well as it is fast, more sensitive and less time taking. Of the 130 infected fish samples with clinical investigation of distended abdomen, exophthalmia, skin ulceration, prominent scales and eroded fins, only 76 (58.5 %) have cultural *Aeromonas* spp. On the other hand, out of 130 water samples approximately 91 (70 %) capable of culture as *Aeromonas* spp. Additionally, the percentage of frequency of *Aeromonas* spp. isolates based on 16S rRNA gene were 48/76 (63.2 %) and 53/91 (58.2 %) from infected fish and water samples respectively. Furthermore, the results of multiplex PCR were revealed that among the 48 and 53 *Aeromonas hydrophila* isolates only 31 and 44 given a specific (130 and 309 bp) bands by amplification of *ahhl, AerA* genes.

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1. Introduction

Aeromonas spp. was considered as the global bacteria (Janda and Abbott, 2010). *Aeromonas* is a common inhabitant in rivers, lakes, sewage, drinking water and sea water (Pinto *et al*., 2011; Kingombe *et al*., 2014; Ottaviani *et al*., 2016). *Aeromonas hydrophila* is the most common serious pathogen. It is a Gram negative, non - spore forming, rod shaped, facultative anaerobic, mesophilic bacteria (Nakano *et al*., 1990; Ashbolt *et al*., 1995).

* *Corresponding author*: **Rasha M. Othman**
University of Basrah, Basrah, Iraq.

Aeromonas spp. causes many pathologic conditions such as dermal ulceration, fin rot, hemorrhagic septicemia (motile *Aeromonas septicemia*), red sore disease, exophthalmia, erythrodermatitis and scale protrusion especially for common carp *Cyprinus carpio* (Cipriano *et al*., 2001; Austin and Austin, 2007).

The main virulence factors of *Aeromonas* spp. that play an important role in the pathogenicity of the disease are the presence of extra cellular proteins including exotoxins and exoenzymes, endotoxin, lipopolysaccharide layer, the presence of S-layers and fimbriae and the ability of production of Capsular layers

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(Blair *et al*., 1999). Symptoms of infected fishes vary, ranging from sudden death in hemorrhagic septicemia, or it may show the following signs: swimming abnormalities, exophthalmia, redness of the skin, accretion of fluid in the scales pockets (Faktotovich, 1969). Other symptoms are include distention in the abdominal area and projecting scales, internally gills, liver, kidney, spleen, pancreas, skeletal muscle are also affected by bacteria. But, the target organs in the disease are the liver and kidneys. The liver may become pale or green, kidneys may become swollen and friable due to the attack of bacteria that make them loss their integrity (Huizinga *et al*., 1979). Several methods were used to identify and classify *Aeromonas* spp. either complement or alternative to biochemical tests, including gene - based techniques such as Polymerase chain reaction (Ash *et al*., 1993). Determination of virulence factors by molecular methods such as PCR is attitude to identify the potential pathogenicity of *Aeromonas* sp. Many virulence genes of *Aeromonas* sp. has been described, including genes that encode for lateral flagella (Gavin *et al*., 2002). Similarly, genes encode for the secretion of extracellular proteins such as nuclease, lipase, protease and aerolysin (Nam and Joh, 2007; Pemberton *et al*., 1997). Many other factors, like pili, amylase, chitinase, elastase, gelatinase and lecithinase are also considered as virulence factors of *Aeromonas* (Kirov *et al*., 2000). So, our study was directed for isolation and identification of the *Aeromonas hydrophila* from infected fishes isolated from several aquafarming in different areas in the province of Basra, Iraq and confirm the identification of these bacteria by PCR assay using a specific sets of primers for amplification the 16S rRNA and hemolysin genes (*ahhl* and *AerA*).

2. Materials and Methods

Sample collection & clinical investigation

A total of 260 samples included (130 infected fishes and 130 water samples were collected from several aquafarming in different areas in the province of Basra, Iraq during the period of April to August 2016 from several aquafarming in different areas in the province of Basra, Iraq. The fish and water samples were placed in sterile plastic bags and taken immediately to the Laboratory of Microbiology for investigation and the clinical signs of infected fishes were done immediately after collection.

Bacterial Isolation

The swabs were carefully chosen from different parts of fish body including head, gills, fins and skin lesions and another swab from each water samples and both were immediately cultured on Tryptic Soya Agar and incubated at 28 °C for 24 hrs. Several bacteriological and biochemical tests such as Gram stain, culturing on Brain Heart Infusion Agar and MacConkey agar. Mannitol Salt Agar, Triple Sugar Iron, Oxidase test, Catalase test, Indole producing and Urease test) were carried out for the further identification of *Aeromonas* spp. According to the previous methods of Ho;t *et al*. (1994); Health Protection Agency (2003); Abbott *et al*. (2003) and Martin Carnahan and Jpseph (2005), following purification and sub-culturing, the isolated bacterial colonies further identifying using PCR.

Bacterial DNA Extraction

Five colonies from the agar plate of suspected as *Aeromonas* spp. cultures were transferred into an Eppendorf tubes containing 100 µl distilled water. The tubes were vortexed and incubated at 100 ºC for 15 min. Then, 900 µl of distilled water was added and mixed well until the solution was homogeneous. The solution was centrifuged at 12,000 rpm for 10 min. The supernatant which contain the genomic DNA was transferred into an new Eppendorf tubes for PCR technique.

PCR analysis

The PCR was achieved to confirmation the identification of *Aeromonas hydrophila* isolates on the previously extracted DNA samples using Green master mix (Promega, USA) and a specific set of oligonucleotide primer for identify 16S rRNA gene (Choresca *et al*., 2010) and the presence of *ahhl* and *AerA* haemolysin genes were detected in the template DNA by Multiplex PCR as designated early by (Sanaa Rahman Oleiwi, 2013). The sequence of the primers which used in the current study was illustrated in Table - 1.

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Table – 1: Sequence of forward and reverse primers of (16S rRNA, *ahhl, AerA)* genes

Primer type	Primer sequence	Primer size
16S rRNA	F-5-GGGAGTGCCTTCGGGAATCAGA-3 R-5-TCACCGCAACATTCTGATTTG-3	356 bp
Ahhl	F-5-GCCGAGCGCCCAGAAGGTGAGTT-3 R-5 -GAGCGGCTGGATGCGGTTGT-3	130 bp
AerA	F-5-CAAGAACAAGTTCAAGTGGCCA-3 R-5- ACGAAGGTGTGGTTCCAGT-3	309 bp

Conventional PCR for 16S rRNA gene was accomplished in a Thermocycler type (*Esco*, Singapore) in a total reaction volume of 25 µl containing 12.5 µl of master mix (*Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers), 0.5 µl of primer and 9.5 µl of template DNA. Amplification condition was obtained with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, and annealing at 59 °C for 30 sec, 72 °C for 30 sec and final extension 72 °C for 5 min (Puthucheary *et al*., 2012). The multiplex PCR of *ahhl* and *AerA* genes were carried out in a total reaction volume of 25 µl containing 12.5 µl of master mix (*Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers), 0.5 µl of each set of primer and 9.5 µl of template DNA. The amplification condition of PCR was performed with the following protocol; an initial denaturation step at 95 °C for 5 min followed by 35 cycles at 94 °C for 30 sec and annealing at 62 °C for 30 sec, 72 °C for 30 sec and final extension 72 °C for 5 min (Puthucheary *et al*., 2012).

Gel electrophoresis

Ten microliters of PCR products were analyzed on 2 % Agarose gel and run at 75 V for 4 hrs. Gels were examined and photographed under UV illumination (E - graph – ATTO - Japan). Fragment size of approximately 356, 130 and 309 bp was verified as positive for 16S rRNA, *ahhl* and *AerA Aeromonas* spp. genes respectively. A 100 bp DNA ladder (Bioneer, Korea) was used as a molecular size standard.

3. Results

Of the 130 infected fish samples with clinical investigation of distended abdomen, exophthalmia, skin ulceration, prominent scales and eroded fins as in Figure - 1, only 76 (58.5 %) have culturable *Aeromonas* spp. On the other hand out of 130 water samples approximately 91 (70 %) capable of culture as *Aeromonas* spp. Additionally, the results of Gram staining, culturing on TSA and biochemical tests were shown in Figure - 2, 3, 4 and 5). On the other hand, the percentage of frequency of *Aeromonas* spp. isolates based on 16S rRNA gene were 48 (63.2 %) and 53 (58.2 %) from infected fish and water samples respectively. Furthermore, the results of multiplex PCR were revealed that among the 48 and 53 *Aeromonas* spp. isolates only 31 and 44 gave specific (130 and 309 bp) bands by amplification of *ahhl* and *AerA* genes respectively. The results of conventional and multiplex PCR analysis were revealed in Figures - 6 and 7.

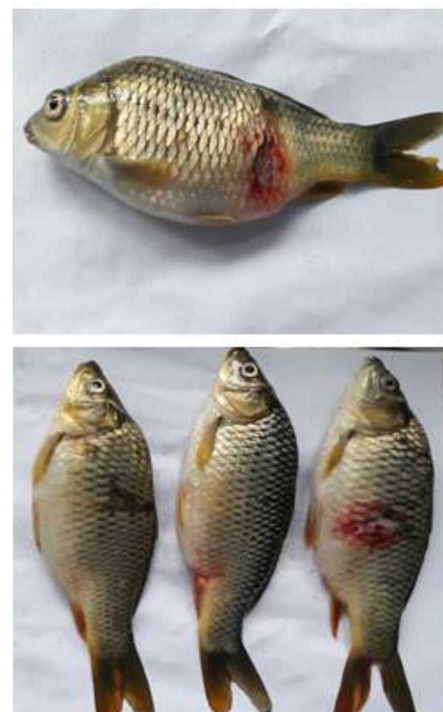

Figure - 1: Distended abdomen, exophthalmia, skin ulceration, prominent scales and eroded fins of infected carp *Cyprinus carpio*

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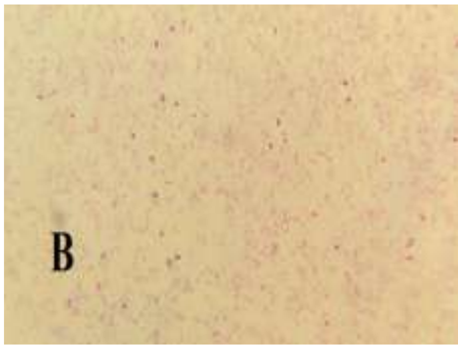

Figure - 2: A) Culturing of suspected *Aeromonas* spp. isolates on Tryptic Soya Agar; B) Gram negative of suspected *Aeromonas* spp. isolates

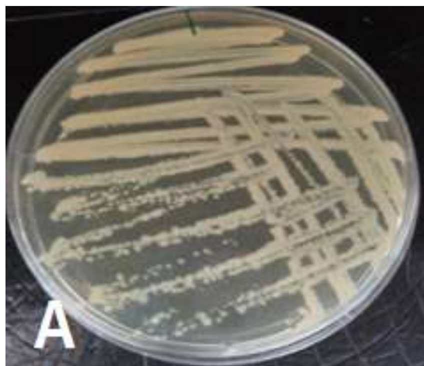

Figure - 3: A) Culturing of suspected *Aeromonas* spp. isolates on Brain heart infusion agar; B) Culturing of suspected *Aeromonas* spp. isolates on MacConkey agar

Figure - 4: A) Catalase positive of suspected *Aeromonas* spp. isolates; B) Oxidase positive of suspected *Aeromonas* spp. isolates

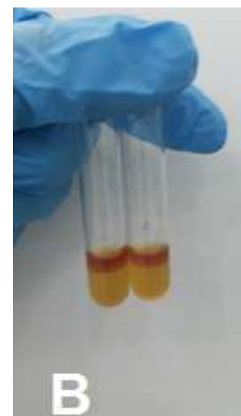

Figure - 5: A) Culturing of suspected *Aeromonas* spp. isolates on TSI; B) Indole positive of suspected *Aeromonas* spp. isolates; C) Urease negative of suspected *Aeromonas* spp. isolates

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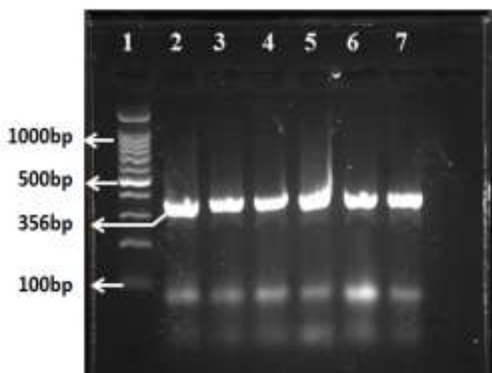

Figure - 6: PCR Products of 16S rRNA *Aeromonas hydrophila* gene. Lane - 1: 100 bp DNA Ladder. Lanes - 2 to 7: PCR Products of 16S rRNA *Aeromonas Hydrophila* gene

Figure - 7: PCR Products of (*ahhl, AerA*) *Aeromonas hydrophila* genes. Lane 1: 100 bp DNA Ladder. Lanes 2 to 5: PCR Products of 130bp *ahhl* and 309 bp *AerA* genes respectively

4. Discussion

Fish is considered as an essential source of food in Iraq. In recent years, the development and spread of aquaculture and fishes farms have provide a good fishes resource and subsequently are a complicate by the appearance of different fish diseases, most commonly bacterial infections disease like hemorrhagic septicemias. One of the mainly causes of fish hemorrhagic septicemias was *Aeromonas* spp.

Aeromonas hydrophila are common inhabitants of aquatic environments giving rise to infections in both fish and humans. Identification of *Aeromonas* to the species level was difficult and complicated due to their phenotypic and genotypic diversity (Puthucheary *et al*., 2012). In this study, we are focused to use the PCR as quick, current and definitive technique to diagnostic the *Aeromonas* spp. As the characterization and identification of the members of the genus *Aeromonas* by conventional biochemical methods was often inaccurate. So, we use three species specific primers to amplification the 16S rRNA and haemolysin genes (*ahhl, AerA*) in the identification of *Aeromonas*. On the basis of the conventional PCR results, the presence of an amplification product at 356 bp characteristic for the genus *Aeromonas hydrophila*. Additionally, the multiplex PCR results were approved the presence of haemolytic gene fragments 130 bp and 309 bp for *ahh1* and *aerA*, respectively. This is an agreement with former studies that the incidence of haemolytic factors in *Aeromonas* was established and their existence can be used as indicators for both virulence and pathogenicity of *Aeromonas hydrophila* (Zhang *et al*., 2000; Furmanek – Blaszk, 2014). Moreover, in 2013 were established the percentages of *Aeromonas* from river water was 72.52 %, and from wells water was 35 % based on biochemical tests and PCR (16S rRNA, hemolysin and aerolysingenes). On the other hand, Yousr *et al*. (2007) (found that *AerA* were mainly associated with *Aeromonas veronii* biovar *sobria* (66.6 %), while (52.6 %) and (44.7 %) of *A. hydrophila* and *A. caviae harbored AerA*. It was interesting to note that primers designed from the *AerA* gene sequence of the *A. hydrophila* were found to give the expected size of the amplicon with *A. caviae* and *A. veronii* biovar *sobria* isolates. Moreover, the specificity of the PCR assay for the hemolytic strains of *Aeromonas* sp. from fish and fishery products has been confirmed by other researchers (Pollard *et al*., 1990; Hussain *et al*., 2014).

Finally, we concluded that the *Aeromonas* strain and their environment have a virulence factors that lead to suggests that the aquatic environment may act as a reservoir and source of virulent and pathogenic *Aeromonas* spp. Furthermore in observation of pathogenic natural of *Aeromonas* spp., the breeding of fishes will be necessary to put under accurate observing in order to determine the quality of water which is critical to reduce the human health risk due to *Aeromonas* spp.

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