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RESEARCH ARTICLE

MOLECULAR IDENTIFICATION OF THE PARASITE *Aeromonashydrophila* FROM *Clariasbatrachus* (BURCHELL, 1822)

***Sarlin, P. J., Megha, M. and Jeniffer Ann Thomas**

Department of Zoology, Fatima Mata National College (Autonomous), Kollam, India

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ABSTRACT

The parasitic infestation in the commercially important fish *Clariusbatrachus*was investigated. The study based on examination of *Clariasbatrachus* under aseptic conditions collected from a commercial farm from Punalur, Kollam, Kerala revealed the bacterial parasite of the catfish to be fairly rich comprising *Aeromonashydrophila*. The gut region samples were serially diluted, incubated and plated.The edited sequence (16 S rRNA sequence) were then used for similarity searches using BLAST (Basic Local Alignment Search Tool) program in the NCBI Genbank DNA database for identifying the sample. The 16S rRNA gene was identical and exhibited 100% sequence similarity with the other known isolates of *A. hydrophila* available in the GenBank. *Aeromonashydrophila* is a heterotrophic, Gram-negative, rod-shaped bacterium mainly found in areas with a warm climate. This bacterium can be found in fresh or brackish water. It can survive in aerobic and anaerobic environments, and can digest materials such as gelatinand hemoglobin. *A.hydrophila* was isolated from humans and animals in the 1950s. It is the most well-known of the species of *Aeromonas*. In this paper, we reported the isolation and molecular detection of *A. hydrophila*from a *Clariusbatrachus.*

Key words: *Aeromonashydrophila, Clariusbatrachus,* BLAST, 16sRNA, Gen Bank

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INTRODUCTION

Fish is important to human populace in trade and economy. It is of importance in the diet of different countries especially in the tropics and subtropics where malnutrition is a major problem. As the human population inevitably increases, the demand for fish as source of protein also grows. In recent times, there has been tremendous increase in the development of fish farming and culture attribute to the increased need for affordable animal protein especially in the tropics, therefore, fishes of the family Claridae are increasingly being used for freshwater aquaculture owing to several favorable cultural characteristics. Parasitic infection and diseases are some of the factors hindering high productivity in fish farming. Most fish especially in the wild population are likely to be infested with parasites, but in the great majority of cases, no significant harm to the host may be ensued or identified; thus, there are only few reports of parasites causing mortality or serious damage to the fish populations, but this may be largely because such effects go unnoticed. Fishermen or consumers often observe parasites in wild fish only when they are so obvious as to lead to rejection of fish. In culture fish population, on the other hand, parasites often cause serious outbreak of disease. Fish are infected by several groups of parasites. Those can be found in the dermis, gills, and internal organs, and their presence in the fish depends largely on their habitats, life cycle of parasites, host physiology and associated functions in ecosystems, among others (Pellitero, 1988; Munoz *et al*., 2006). Nematodes are one of the most common parasites found in fish. The presence of dense populations of fish kept in particular environmental conditions may favour certain parasites so that the parasite population increases to a very high level. According to Roberts *et al*., 2000, parasites are the most diverse and common pathogens the aqua culturist may likely encounter, and parasitic diseases are very common in fish all over the world and are of particular importance in the tropics. Fish parasites result in economic losses not only mortality, but also from treatment expenses, growth reduction during and after outbreak of disease and this militate against expansion of aquaculture. According to Klinger and Francis-Floyd (2000) protozoa are a vast assemblage of eukaryotic organisms and that most of the commonly encountered fish parasite are protozoa, which with practice are the easiest to identify and easiest to control. Protozoan parasites cause serious losses in fishponds and wild worldwide, and their lesions render the fish unmarketable. Fish carrying protozoa parasites are capable of passing on the infective disease to man after its consumption. Parasitism is a constant and frequent phenomenon in fish (Paperna, 1991; Luque *et al*., 2004; Olivero-Verbel*et al.,* 2006). Even though parasitic exposure from fish consumption can be prevented using appropriate

Corresponding author: **Sarlin, P. J.**
Department of Zoology, Fatima Mata National College (Autonomous), Kollam, India.

treatments, the risk associated with the ingestion of parasites cannot be totally removed by physical processes such as refrigeration, freezing or salting (Uradznki *et al*., 2007; Stormo *et al*., 2009). Although these parasites can infect humans after consumption of raw fish or insufficient cooking (Smith and Wootten, 1978; Cheng, 1982; Olson *et al*., 1983; Yagi *et al*., 1996; Esteve *et al*., 2000; Audicana *et al*., 2002, Audicana and Kennedy, 2008; Marzocca *et al*., 2009), there is growing evidence suggesting that the risk depends not only the presence of living organisms. All of the major groups of animal parasites are found in fish, and apparently healthy wild fish often carry heavy parasite burdens. Parasites with direct life cycles can be important pathogens of cultured fish; parasites with indirect life cycles frequently use fish as intermediate hosts. Knowledge of specific fish hosts greatly facilitates identification of parasites with marked host and tissue specificity, whereas others are recognized because of their common occurrence and lack of host specificity. Examination of fresh smears that contain living parasites is often diagnostic. DNA barcoding aims to provide an efficient method for species-level identifications and, as such, will contribute powerfully to taxonomic and biodiversity research. As the number of DNA barcode sequences accumulates, however, these data will also provide a unique 'horizontal' genomics perspective with broad implications. For example, here we compare the goals and methods of DNA barcoding with those of molecular phylogenetics and population genetics, and suggest that DNA barcoding can complement current research in these areas by providing background information that will be helpful in the selection of taxa for further analyses.

DNA barcoding is gaining more attention nowadays, because of its accuracy compared to other methods of taxonomy, though it has many limitations in the following paragraphs, work of earlier researchers has been described. DNA barcoding aims to provide an efficient method for species-level identifications using an array of species specific molecular tags derived from the 5' region of the mitochondrial cytochrome c oxidase I (COI) gene. The efficiency of the method hinges on the degree of sequence divergence among species and species-level identifications are relatively straightforward when the average genetic distance among individuals within a species does not exceed the average genetic distance between sister species. Fishes constitute a highly diverse group of vertebrates that exhibits deep phenotypic changes during development. In this context, the identification of fish species is challenging and DNA barcoding provide new perspective in ecology and systematic of fishes (Hubert N*, et al.*, 2008).

The use of DNA barcodes for the identification of described species is one of the least controversial and most promising applications of barcoding. There is no consensus, however, as to what constitutes an appropriate identification standard and most barcoding efforts simply attempt to pair a query sequence with reference sequences and deem identification successful if it falls within the bounds of some pre-established cutoffs using genetic distance. Since the Renaissance, however, most biological classification schemes have relied on the use of diagnostic characters to identify and place species (Lowenstein JH, *et al*., 2009). Inter and intraspecific sequence comparisons using phylogenetic analysis and a BLAST search algorithm provide rigorous statistical metrics for species identification (Kyle CJ, *et. al*., 2007). Species identification by DNA barcoding is based on sequencing a short standardized genomic region of the target specimen and comparing this information to a sequence library from known species. The proposed standard barcode sequence for animal species is a 650-bp fragment of the mitochondrial gene cytochrome *c* oxidase I (COI). Many benefits of DNA bar-coding for species identification and discovery have been discussed although the concept continues to be hotly debated. In addition to species identification, the construction of barcode database could expose novel DNA barcodes that may indicate provisional new species. Genetic interrelationships of Cyprinid subfamilies have been extensively investigated from morphological, anatomical and molecular perspectives. Two studies mainly based on morphological and anatomical characters have investigated phylogenetic relationships among genera and species and explored the taxonomic status of these fishes but, the molecular identification based mtDNA COI gene are somewhat understudied for the highly specialized *Schizothorax*species in the Garhwal Himalaya.

MATERIALS AND METHODS

A total of 45 freshwater *Clariasbatrachus* (Family Clariidae) were collected alive from fish farms in Punalur and transported alive to laboratory in large plastic bags partially filled with water and supplied with a good aeration. The collection was made between November 2016 and April 2017. The total length of each fish were measured in centimeters (cm) using measuring tape, while the weight of each fish was taken in grams (g) using a weighing balance. Then, the collected fish samples were dissected and the mesenteric cavity examined for parasites. The gastrointestinal tract was then dissected from the rectum to the oesophagus and all parasitic encountered were carefully detached from the stomach or intestinal mucosa. The internal organs of each fish were also examined for parasites or cysts. The parasites from each fish were then fixed in 70% alcohol.

Isolation of bacteria

Fish samples were collected from the local area of Punalur, Kollam during lactation period under aseptic conditions in a sterile screw cap tubes, used for further studies. The gut region samples were serially diluted and incubated at 23ºC for 30 min before plating. Diluted samples were plated on to nutrient agar and incubated at 37º C for 48hrs.

Serial dilution

Labelled tubes as 10^{-1}, 10^{-2}, 10^{-3} up to 10^{-7}. Each bottle contained 9ml of sterile water. Prepared 10^{-1} dilution using appropriate volume of sample (1g/1ml). This first tube now had a 1:10 dilution of the original sample. Using a new sterile 1ml pipette, 1ml was transferred out of the first bottle (1:10 or 10^{-1}) and added this to the test tube labelled 10^{-2} and tubes shaken thoroughly. Using a new sterile 1ml pipette, transferred 1ml from the 10^{-2} tube and added it to the 10^{-3} tube. Repeated the shaking procedure and continued up to 10. Inoculated 100µl sample from 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} into separate petri plates with medium. Using 'L' shaped glass rod spreaded the inoculums on the surface. Incubated the plates at room temperature.

Spread plate method

Prepared proper dilutions of the sample. Removed the upper lid of the plate and placed about 0.1ml of diluted sample in the

center of the plate. The inoculums were spread over the medium by pushing the glass spreader (L-rod) backward and forward while rotating the plate. Replaced the petri dish lid and allowed for drying before inverting and incubating. Incubated the plates for 48 hours at room temperature.

Identification of the predominant bacteria by 16S rDNA

Genomic DNA extraction from bacteria

Extraction and purification of nucleic acids was the first step in most molecular biology experiments. The extraction of nucleic acids from biological material requires cell lysis, inactivation of cellular nucleases and separation of the desired nucleic acid from cellular debris. The lysis procedure was a compromise of techniques and must be rigorous enough to disrupt the complex starting material, yet gentle enough to preserve the target nucleic acid. To isolate in a pure form, cells were first lysed by cell lysis buffer containing detergent (SDS) and chelating agent (EDTA), accomplishing cell disrupture and nuclease inhibition respectively. TRIS added permeabilizes the membrane along with maintaining the pH. Lipids and proteins were precipitated by chloroform. Isoamyl alcohol acts as an antifoaming agent. The large negative charge of the DNA molecule was neutralized by the positive sodium ions (When added sodium acetate) in solution. The neutralization of the negative charges on DNA allowed it to precipitate in alcohol.

Agarsose gel electrophoresis

Electrophoresis is a technique used to separate and sometimes purify macromolecules especially proteins and nucleic acid that differ in size, charge, or conformation. When charged molecules were placed in an electric field, they migrated towards either the positive or negative pole according to their charge.

Standard DNA markers

Commercially available DNA markers were used as standard molecular weight DNA marker to determine the weight of DNA / PCR amplicon.

Setting of Agarose Gel Electrophoresis System

Installed the running tray by placing it firmly on the casting tray. Placed the comb on the rim of the casting tray and adjusted the bottom of the comb so that it is about 1.0mm from the running tray.

- Prepared 0.8 % agarose (0.24g in 30ml 1X TBE) solution by heating and cool it.
- Added Ethidium Bromide (0.5ml EtBr for 10 ml TBE) to the gel.
- Poured the agarose solution into the tray. Checked that the comb was vertical to prevent well shape distortions. Allowed a minimum of 30min for the gel to set.
- Once the gel was set, removed the comb carefully.
- Then removed the running tray with the gel and placed it on the running platform of the electrophoresis unit.

Protocol

- Loaded 10µl of DNA with 4µl DNA loading dye in agarose gel.
- Ran the gel at constant voltage of 100V till the dye has travelled 3 cm from the wells.
- Viewed the gels on UV trans illuminator with the safety shield.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a biochemical technique to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. A typical amplification reaction includes target DNA, a thermo stable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphate (dNTPs), reaction buffer and magnesium. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension. The initial step denatured the target DNA by heating it to $94^{o}C$ or higher for 15 seconds to 2 minutes. In the denaturation process, the two intertwined strands of DNA template for replication by the thermo stable DNA polymerase. In the next step of a cycle, the temperature is reduced to approximately 40-$60^{o}C$. At that temperature, the oligonucleotide primers can form stable associations (anneal) with the denatured target DNA and served as primers for the DNA polymerase. This step lasts approximately 15-60 seconds. Finally, the synthesis of new DNA began as the reaction temperature is raised to optimum for the DNA polymerase.

PCR Consist of 3 basic steps

Denaturation: Two strands melted open to form a single stranded DNA and all enzymatic reactions stop.

Annealing: Primers get annealed with target DNA

Extension: Taq polymerase added dNTPs complimentary to the template at the 3' end of the primers

Cycles of PCR amplification – 16S rDNA

Cycle	Step	Process	Temperature	Time
1	I	Denaturation	$94^{0}C$	3 minutes
II	II	Denaturation	$94^{0}C$	30 seconds
II	III	Annealing	$58^{0}C$	30 seconds
II	IV	Primer extension	$72^{0}C$	30 seconds
		Go to step 2 for 29 times		
III	V	Final elongation	$72^{0}C$	7 minutes
IV			End or $4^{0}C$ forever.	

Universal primers

16S rRNA gene amplification

Forward primer: 5'- GAGTTTGATCCTGGCTCAG – 3'
Reverse primer: 5' – GAATTACCGCGGCGGCTG – 3'

16S rDNA Sequencing

PCR product were send for sequencing at RGCB, Trivandrum.

Blast

The edited sequence (16 S rDNA sequence) were then used for similarity searches using BLAST (Basic local alignment

search tool) programme in the NCBI Genbank (www.ncbi.nlm.nih.gov) DNA database for identifying the sample.

RESULT

Infestation of *Clariasbatrachus*

Out of 45 specimen of *C.batrachus* were collected from Punalur, Kollam and examined of which 20 fishes were infested.

Bacteria, *Aeromonashydrophila* in habiting the gut were identified. The isolated parasite belonged to family Aeromona daceae. *Aeromonashydrophila* is a heterotrophic, Gram-negative, rod-shaped bacterium mainly found in areas with a warm climate. This bacterium can be found in fresh or brackish water. It can survive in aerobic and anaerobic environments, and can digest materials such as gelatinand hemoglobin. *A. hydrophila* was isolated from humans and animals in the 1950s. It is the most well known of the species of *Aeromonas*. It is resistant to most common antibiotics and cold temperatures and is oxidase and indole positive. The bacterium *Aeromonashydrophila*is one of the dangerous disease causing bacteria in freshwater fish farming. The bacteria attack the catfish that is one of the leading commodities in freshwater and can infect fish in all sizes which can lead to death until it reaches 80%, resulting in huge losses in the freshwater fish farming ventures.

Isolation of bacteria from fish gut

Isolation of bacteria from fish was done to get well isolated colonieson nutrient agar.

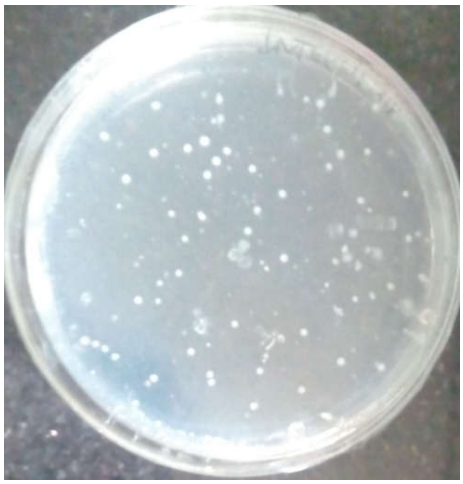


Fig. 1. Bacteria isolated on nutrient agar

Isolation of bacterial genomic DNA

For the PCR amplification of 16S rDNA gene, isolation of genomic DNA was carried out and the qualitative analysis of the isolated DNA (Fig. 2).

Amplification of 16S rDNA gene by PCR

The molecular identification of the organism includes DNA isolation, 16S region amplification. Isolated DNA sample undergone PCR process for 16s region amplification. After sequencing the organism similarity was done using BLAST. The DNA sample was electrophoresed in 1.5% agarose gel with 5% of TBE buffer and 3µl of EtBr. 10µl sample and 5µl of loading dye was loaded in the 1% TBE buffer. And the sample was viewed under

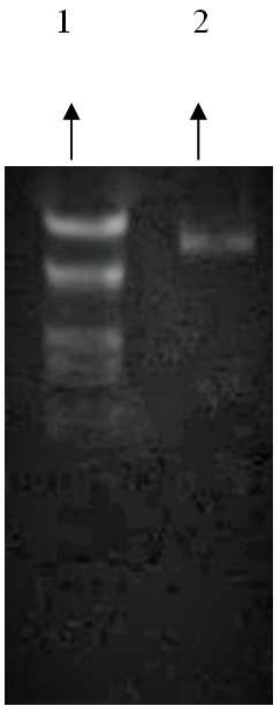


Fig. 2. Agarosegel electrophoresis

Lane1 marker (EcoR1 Hind III double digest product of lambda phage fermantas).
Lane 2 represents genomic DNA (~10,000 kb).

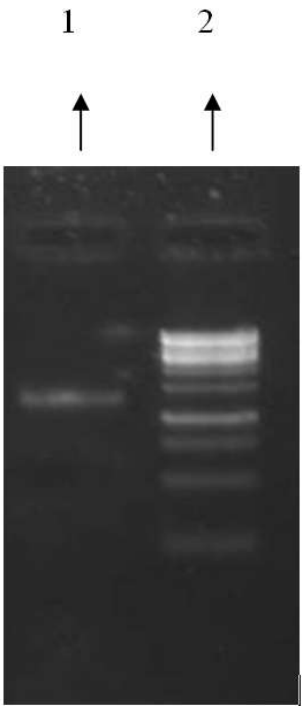
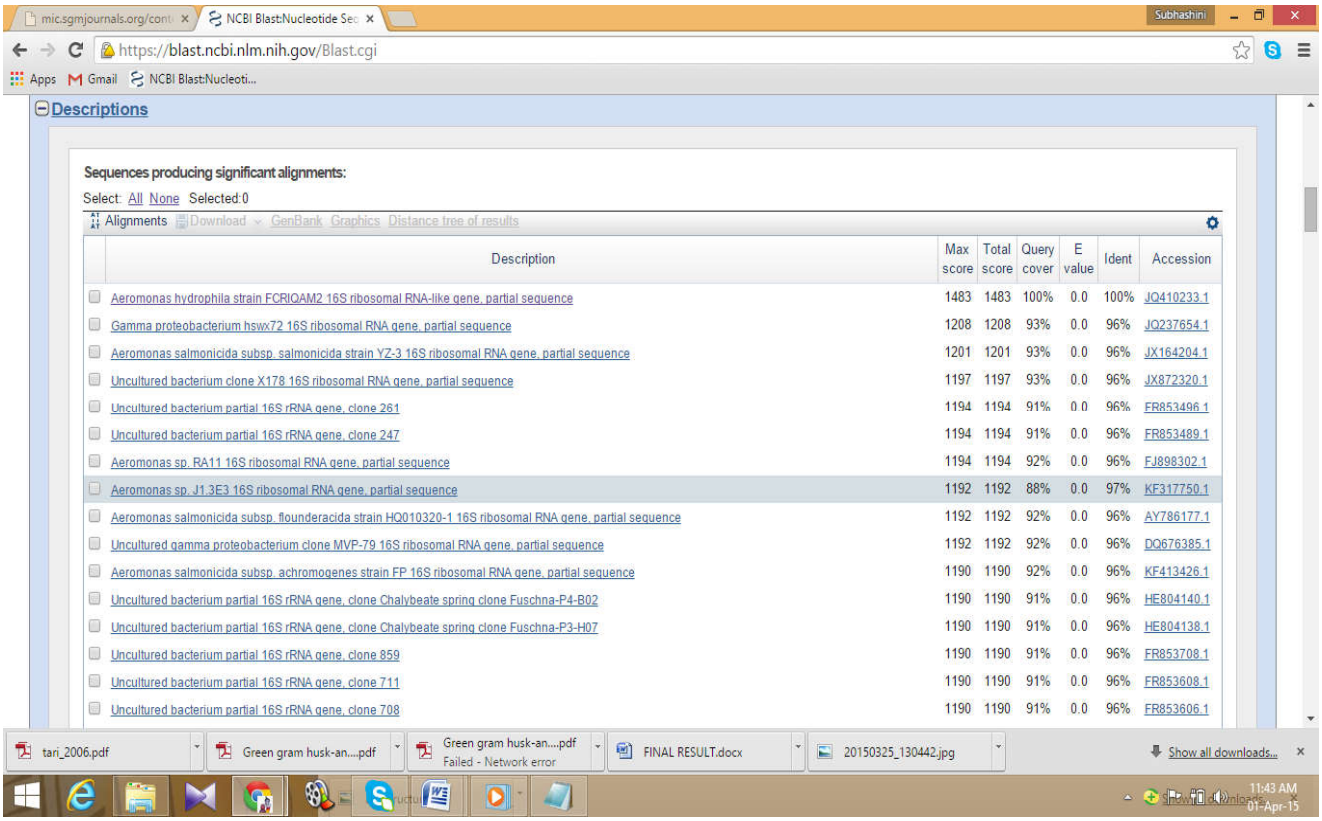


Fig. 3. Agarose gel electrophoresis

Lane 1 shows PCR amplified product (1.5kb).
Lane 2 shows the marker (1kb).

Blast result for 16s region



Inference: shows 100% identity with "*Aeromonashydrophila*" species

Fig. 4. Blast result for 16S region

Sequences of 16s region

GTGGGGGGGGGCTAAAATGCAAGTCGAGCGGCAGCG
GGAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGAC
GGGTGAGTAATGCCTGGGGATCTGCCAGTCGAGGGG
GAAACAGTTGGAAACGACTGCTAATACCGCATACGC
CCTACGGGGGAAAGGAGGGGACCTTCGGGCCTTTCG
CGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTG
GGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGT
CTGAGAGGATGATCAGCCACACTGGAACTGAGACAC
GGTCCACACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGCACAATGGGGGAAACCCTATGCAGCCATGCCGC
GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTC
AGCGAGGAGGAAAGGTTGGCGCCTAATACGTGTCAA
CTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTC
CGTGCCAGCAGCCGCGGTAATACGGA

DISCUSSION

The study based on examination of 45 specimens of *Clariasbatrachus* collected from Punalur, Kollam revealed the bacterial parasite of the catfish to be fairly rich comprising *Aeromonashydrophila*. Parasitic infections often give an indication of the quality of water they, since parasites generally increase in abundance and diversity in more polluted waters. The scenario in Punalur also is pointing to the same. The fish is reared in ambience that is undesirable. The water quality is highly unsatisfactory and that is the reason for the high prevalence of *Aeromonashydrophila* in almost all the individuals collected from the sampling farm. It also is an indication of the lax attitude of the aquaculturist who is least concerned about the quality of the produce of the farm.

The single minded aim at profit could also be shattered if the infestation goes out of control due to any change in other parameters that maintain the host parasite equilibrium. The ignorance of the farmer about the nature of infection that is spreading in his farm and the reluctance to seek professional advice is evident and it will cost him dearly. The bacterium *Aeromonashydrophila*is one of the dangerous disease-causing bacteria in freshwater fish farming. The bacteria attack the Catfish that is one of the leading commodities in freshwater and can infect fish in all sizes which can lead to death until it reaches 80%, resulting in huge losses in the freshwater fish farming ventures. It is a highly opportunist parasite that attacks and takes over, when the hosts' immunity is compromised. Fish parasites result in economic losses not only by mortality, but also from treatment expenses, growth reduction during and after outbreak of disease and this militate against expansion of aquaculture. Parasites cause serious losses in fishponds and wild, and their lesions render the fish unmarketable. Fish carrying bacterial parasites are capable of passing on the infective disease to man after its consumption. *Aeromonashydrophila* is a heterotrophic, Gram-negative, rod-shaped bacterium mainly found in areas with a warm climate. This bacterium can be found in fresh or brackish water. It can survive in aerobic and anaerobic environments, and can digest materials such as gelatinand hemoglobin. *A.hydrophila* was isolated from humans and animals in the 1950s. It is the most well-known of the species of *Aeromonas*. It is resistant to most common antibiotics and cold temperatures and is oxidase and indole positive. Studies on the immune response of *C.batrachus*by experimental or wild infection as well as by microbial toxins enable our understanding of host parasite interaction, disease resistance mechanism and risk factors of culture practices. Effects on a host with a pathogen load may

be useful to propose preventive protocols and vulnerability assessment. The study employs DNA barcoding to provide a standardized measure of sequence. Specifically, examine the extent of sequence diversity in the 648-bp region of the cytochrome *c* oxidase 1 (COI) gene that has been adopted as the standard barcode for members of the animal kingdom. DNA barcoding of the parasites was utilized to aid in the precise identification of the parasites. The study employed DNA barcoding to provide a standardized measure of sequence, specifically examining the extent of sequence diversity in the 648-bp region of the cytochrome *c* oxidase 1 (COI) gene that has been adopted as the standard barcode for members of the animal kingdom. For the PCR amplification of 16S rDNA gene, isolation of genomic DNA was carried out and the qualitative analysis of the isolated DNA. From the blast results for the DNA barcoding, we find that the CO1 sequence shows only 100% similarity to *Aeromona shydrophila*so it was interpreted to belong to Aeromonas sp. But phenotypically and based on peculiarities of growth on fish it was tentatively identified as *Aeromonashydrophila.*

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