© by PSP Volume 26 – No. 1a/2017, pages 1108-1115 Fresenius Environmental Bulletin

# ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA FROM DISEASED FRESHWATER FISH *CATLA CATLA* (HAM.)

**Divya Sahni[1], Daoud Ali[2,*], Ali Al-Amer[3], Neeraj Sood[1], D.K Chaudhary[1], Ashish Tiwari[4],SA Hussain[2]**

[1]ICAR-National Bureau of Fish Genetic Resources, Lucknow
[2]Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia
[3]Joint Center for Genomics Research, King Abdulaziz City for Science and Technology and Chinese Academy of Sciences, Prince Turki Road, Riyadh, Saudi Arabia
[4]ICAR Indian Institute of Sugarcane Research, Lucknow

## ABSTRACT

Diagnosis is one of the most important steps for prevention of disease. In the present study, we have isolated bacteria from the muscles of theabdominal infected area of diseased fish*Catlacatla,* whichis the fastest growing Indian major carp and an important freshwater fish in Asian countries. On the basis of primary and secondary biochemical test, it was confirmed that *Edwardsilatarda, Aeromonashydrophila* and *Vibrio cholera* are frequently present. DNA was isolated and amplified with help of PCR and then RFLP was performed, which showed all the three bacterial isolates were pathogenic in nature on the basis of 350bp band pattern. Cell lines designed as *C. catla* blood monocyte was developed from adherent peripheral fish are one of the healthiest and low calorie nutrient rich food sources. Partial sequencing, amplification and alignment of fragments of two mitochondrial genes 16S rRNA and CO1 confirmed that the developed cell line originated from *C. catla*. This cell line could be successfully employed for cytotoxicity assays as revealed by uptake of neutral red. This cell line can be auseful tool to study the role of macrophages in teleost immune response and development of markers for macrophages.

**KEYWORDS:**
*Catlacatla*;*Edwardsila;Aeromonas; Vibrio*sps; RFLP; ELISA

## INTRODUCTION

Fish are the important nutrient rich food source and have significant medicinal value. In developing countries, freshwater reserves are decreasing due to growing populations, increased human consumption, urbanisation and the lack of cost-effective sewage water treatment systems [1].However, fish are susceptible to various pathogens causing diseases. Bacteria are associated with disease outbreaks in aquaculture. These accounts for more than 50% of disease outbreaks among various fish species. Many pathogenic bacteria have been isolated and identified from different fishes such as *Crucian carp, Carassiuscarassius*and*Megalobramaamblycephala*i ncreasing haemorrhagic septicaemia several times [2]. Pathogenic bacteria were isolated from cultured fish farms and characterised phenotypically and by 16S rDNA sequences. These isolates were assayed for hemolytic and proteolytic activity [3]. In fishes, bacteria of *Aeromonadaceae* family cause the well-known diseases haemorrhagicsepticaemia, fin as well as tail rot and results in high mortality in commercial aquaculture [4].

Motile bacteria isolated from commercially raised channel catfish were screened for production of surface-layer protein. However, the surface layer protein of aeromonads was produced only in 58% isolates [5]. Sioutas et al. [6] reported the mortality of carp (*Cyprinuscarpio*) in farms in northern Greece. A bacterium secretes several enzymes which cause the disease such as proteases, DNase, RNase, lecithinase, amylase, lipase, gelatinase, chitinase [7] and cytotoxic/ cytolytic enterotoxins [8] and three haemolysins [9]. Rivero et al.[10] reported that enzymes and toxins are major virulence factors involved in the pathogenesis. Other virulence factors may influence the pathogenicity of organisms and production of surface proteins, lipopolysaccharide, surface layer or outer membrane proteins. Bacterial isolates from water samples may be avirulence or less pathogenic to fish than isolated from diseased fish [11].Polymerase chain reaction is a rapid sensitive and specific molecular tool for detection of pathogens. The biochemically similar bacteria were isolated from fish but they were slight difference was proved by 16S rDNA sequencing [8].

The immunoassays have been accepted for detection and characterise the pathogens. It requires antigen or antibody. The whole bacterial cell can be used for the immunoassay but common antigenic

epitopes give cross-reactivity. Therefore, the purified and specific protein is required for the diagnostics of pathogens.

## MATERIALS AND METHODS

**Isolation and biochemical identification of bacterial isolates.** The bacterial isolates were isolated from diseased fish(*Catla catla*).

**Genus identification (primary test).** A single colony of each isolate was streaked on nutrient agar. These colonies were subjected to the primary tests for genus identification as per the methods described below

**Gram's staining.** Bacterial cells are categorised into groups according to the structure and composition of their cell wall. These are Gram-positive (retain theviolet colour of Gram's stain) and Gram negative (undergo decolourization by washing with acetone after staining with Gram's stain).The slides were observed under the microscope using oil immersion objective (100X). Cells stained red, were Gram negative (- ve).The shape, size and arrangement of cells was noted.

**Motility Test.** Hanging drop method was used for motility of bacterial culture. A drop of overnight grown culture was placed in the coverslip. All corners of coverslip were smeared with paraffin jelly. A dust free cavity slide was taken and inverted over the coverslip, and then it was detcet under the microscope using high power objective (40X).

**Oxidase Test.** The test was done to observed the cytochrome oxidase enzyme in bacteria. The reagents contain tetramethyl-p-phenylenediamine (a substrate for the cytochrome oxidase reaction). the colourless reagent was showed the reduced state and purple colour showed oxidised state. All isolates were used for oxidase activity using oxidase disks (HiMedia, Mumbai) saturated with sterile normal saline. Oxidase disk was placed on glass slide with culture. The appearance of dark purple colour on the disk within the 30 seconds denotes a positive test.

**Catalase test.** This test was used to detect the catalase enzyme. It catalyses the break of the $H_2O_2$ and generates free oxygen. Fresh overnight grown bacteria was re-suspended with normal saline and placed on glass slide. Two drops of 30% $H_2O_2$ were added. Evolution of gas from the culture indicated apositive reaction for catalase.

**Oxidation-fermentation test.** The test was done to determine the bacterium has these enzyme necessary for the aerobic oxidation of the glucose and/or the fermentation of the glucose. OF test was done by growing the bacterium in two tubes of Hugh and Leifson`s [12] medium; in one tube the medium was overlaid with a layer of sterile paraffin oil. The carbon sugar of the medium was glucose. Oxidizers showed acid production in the open tube while as fermenters produced acid in the paraffin-covered tube and starting from the bottom in the open tube.

**Carbohydrate breakdown (gas acid and from glucose):** Nutrient broth containing 5% glucose was prepared and approximately 4ml of it was taken in the test tube into which carefully a small tube (Durham's tube) was inserted. Then a bacterial colony was inoculated into the tube and was kept for incubation. After incubation if a gas bubble is seen within small tube i.e. is positive for the gas test, by theaddition of 100µl of Andrade's reagent, if colour changes to rose pink it means positive test for acid. This was tested in sugar media. Acid production was shown by pink or red colour change of the medium and the gas produced in the small tube.

**Species identification (Secondary test).** Secondary tests are necessary to recognize bacteria up to species level.

**Methyl Red test (MR) and Voges-Proskauer (VP) test.** MR test was employed to observe the production of acid during the fermentation of glucose and pH below 4.5 in an old culture. Five drops of 0.04% solution of methyl red were added to the culture in glucose phosphate medium incubated at 30ºC for five days, mixed well. The red colour was positive while yellow signified a negative test.

VP) test is used to observed the an organism produces acetylmethyl carbinol from glucose fermentation. In the occurrence of alkali and atmospheric oxygen, the small amount of acetyl-methyl carbinol exist in the medium was oxidised to diacetyl ($CH_3.CO.CO.CH_3$), which reacted with peptone broth to give a red colour. The test was performing by adding 0.6ml of a 5% solution of alpha-naphthol in ethanol and 0.4 ml of 40% KOH to thesame tube which was used for MR test. The result was positive if the colour was in the range of pink to magenta and was considered negative if no colour change was detected.

**Carbon source utilisation: citrate utilisation test.** This test was performed to show the ability of amicroorganism to grow in media without organic nitrogen and carbon but having the ability to utilise Sodium citrate as a carbon source and ammonium dihydrogenphosphate ($NH_4H_2PO_4$) as a nitrogen source.

Simmon's medium and Christensen citrate has citrate have the lone source of carbon. The production of turbidity in the medium was indicated ability to use this substance. The slants of medium were prepared in the tube and inoculated the bacterial colony by streaking the loop over the surface of the slope. The slants were incubated at 30ºC and examined after 72 h.

**Decarboxylase reaction.** From a plate culture, tubes of the four media (arginine, lysine, ornithine, and control) were heavily inoculated with a straight wire, through paraffin layer. Alternatively, they were inoculated by adding a drop of a suspension of the organism above the paraffin layer and shaking to distribute the inoculums; the paraffin rose to the top on standing. It was then incubated and examined daily for up to 5 days. The media first became yellow due to acid production from the glucose; if decarboxylation occurred, the medium became purple. The control remained yellow. With non-fermentative organisms, no acid (or insufficient acid) was produced from glucose and there was, therefore, no change of the indicator to yellow.

**Triple sugar iron (TSI) test.** This test was performed to observed the ability of an organism to used specific carbohydrate (glucose, sucrose and lactose).The test was performed by taking a single colony from the plate and streaked on the slant of sterilised TSI slants. Tubes were incubated at 30ºC for 18-24 hr. if the butt changed from red to yellow and slant changed from red to yellow then glucose and lactose were positive.

Some organisms decompose sulphur containing amino acids and produced $H_2S$ among the products. Inoculated a tube of TSI Agar by streaking the slope; observed daily for upto 7 days for blackening only due to $H_2S$ production.

**Indole test.** The test was done to observe the ability of an organism to break tryptophan into indole. This was tested in nutrient broth culture incubated at 30ºC for 48-96hrs. This test demonstrated the formation of indole from tryptophan. Kovac`s reagent (0.5 ml) was added and shaken gently. The red colour indicated a positive test.

**Urease test.** This test was carrying out to observe the production of the enzyme Urease by bacteria. Urease converts urea to ammonia, which makes the condition alkaline. This change of pH was indicated by phenol red (an indicator) whose colour changes from yellow to pink. A slant of urea media was made in a tube and inoculated and examined after 24 hours. Urease positive culture produced a violet colour and urea negative culture remained yellow colour.

**Gelatinase Test.** Differential medium (nutrient gelatin) used to tests the ability of an organism to generate an exoenzyme, called gelatinase, whichhydrolyzesgelatin. When gelatin is below 32°C it is a semisolid form and above 32°C, it is a viscous liquid. Gelatinase break down gelatine into smaller polypeptides, peptides, and amino acids and utilised by the organism. If an organism can break gelatine, the areas where the organism will remain clear zone after adding mercuric chloride.

**Caseinase Test. For the degradation of** casein some bacteria produced an exoenzyme e.i. casease. Casein is a large protein that is responsible for the white colour of milk. This test was performed on milk agar media which was containing casein, peptone and beef extract. If an organism can produce casein, then there will be a clearing zone around the bacterial growth.

**Extraction of DNA from isolated bacteria and PCR amplification for 16S rDNA.** DNA was extracted following Lo et al. [13]. Genomic DNA (5 µl) was loaded on 1% agarose gel electrophoresis.

**TABLE 1**
**Primers used for amplification of 16S rDNA gene**

| Primer | Primer sequence |
|---|---|
| 27 F | AGA GTT TGA TCC TGG CTC AG |
| 1492 R | CTT GTG CGG GCC CCC GTC AAT TC |

The 16S rDNA of bacteria is a highly conserved non-structural gene, which is useful for identification of bacteria. 100 ng DNA (1 µl DNA template solution) was added to PCR tube containing 50 µl of reaction mixture. The forward primer was 27F' and reverse primer was 1492R (Table 1). In 16S rDNA-specific amplicon from this reaction was 1465 bp.10 µl of PCR reaction products were run on 1% agarose gels.

**Restriction fragment length polymorphism (RFLP)**. It refers to a difference between samples of homologous DNA molecules. In RFLP analysis, the DNA sample was digested by restriction enzymes and restriction fragments was separated by agarose gel electrophoresis (according to their lengths).

**Antigen preparation and their quantification and protein profiling of isolated bacteria.** Isolated bacteria was grown in 10 ml NB medium, bacteria were harvested by centrifuge at 5000*g* for 10 min, the pellet was washed twice with PBS and finally resuspended in 500 µl PBS and sonicated. After sonication centrifuge with high

speed (13000*g* for 10 min) and collected supernatant. These proteins were estimated with Lowry method [14]and the concentration was 950 microgram per ml.

Whole cell protein analysis by SDS-PAGE provides a rapid, simple and powerful tool to discriminate and differentiate various strains of bacteria. Whole-cell bacterial proteins were analysed under reducing conditions in a 12% gel. Ten µl of sonicated supernatant and reducing dye (1:1) put on boiling water for 5 min, then loaded on thegel and run at 25 V. After thecomplete run, thegel was stained with astaining solution and observed.

**Antibodies and immunofluorescence.** The cells were grown on coverslips (sterile) for 24 h. Their after cells fixed and permeabilized. For immunostaining, the coverslips incubated overnight at 4°C with mouse anti-cytokeratin and mouse anti-vimentin antibodies. After PBS washing, cells were incubated with rabbit anti-mouse FITC conjugate for 1 h. The coverslips were washed again in PBS, mounted in buffered glycerol and observed at fluorescent microscope.

**Effect of bacterial toxin on cell line.** The bacterial extracellular products from isolated bacteria were tested with cytokeratin-positive cells. The cell lines were grown in 24 well plates using L-15 media with FBS supplemented and incubated at 30ºC. After 24 hr, a monolayer was formed well. For the toxicity test, the cell line was inoculated with 0.1 ml serial dilutions of the bacterial supernatant. Plates were incubated at 30 °C and the effects of the bacterial toxin on the cells were observed.

**RNA Isolation.** TRI reagent is the most effective method for RNA isolation. It isolates a whole spectrum of RNA molecules. Hundred mg fresh tissues were taken and homogenised in TRI-reagent and centrifuged at 12000*g* for 10 min at $4^0$C. The supernatant was taken and 0.2 ml chloroform was added. The tubes were shaken vigorously for 15 sec and allowed to stand for 10 min at room temp. The tubes were centrifuged and theupper layer was taken in another tube. Thereafter 0.5 ml isopropanol was added and the tubes were incubated at RT for 10 min. After centrifugation at

**TABLE 2**
**Results of biochemical tests**

**Primary test:**

| S.N. | Biochemical tests | Results | | | | | |
|---|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** | **6** |
| **1** | Grams staining | -Ve | -Ve | -Ve | -Ve | -Ve | -Ve |
| **2** | Motility | M+ | M+ | M+ | M+ | M+ | M+ |
| **3** | Oxidase test | -Ve | +Ve | +Ve | -Ve | +Ve | +Ve |
| **4** | Catalase test | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve |
| **5** | O/F test | F | O | F | F | F | O |
| **6** | Morphology | R | R | R | R | R | R |

**Secondary test:**

| S.N. | | Biochemical tests | Result of Sample | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **1** | **2** | **3** | **4** | **5** | **6** |
| **1** | | MR test | +Ve | +Ve | +Ve | +Ve | +Ve | -Ve |
| **2** | | VP test | -Ve | +Ve | +Ve | -Ve | +Ve | +Ve |
| **3** | | Indole test | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve |
| **4** | | Urease test | +Ve | -Ve | +Ve | +Ve | +Ve | +Ve |
| **5** | | Carbohydrate breakdown | | | | | | |
| | 1 | Gas test | G- | G+ | G+ | G- | G- | G+ |
| | 2 | Acid test | A+ | A+ | A+ | A+ | A+ | A- |
| **6** | | Citrate utilisation test | | | | | | |
| | 1 | Simmon's citrate | -Ve | +Ve | +Ve | -Ve | +Ve | +Ve |
| | 2 | Christenson test | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve |
| **7** | | Decarboxylase reaction | | | | | | |
| | 1 | Arginine | -Ve | +Ve | +Ve | -Ve | +Ve | -Ve |
| | 2 | Lysine | +Ve | +Ve | +Ve | +Ve | +Ve | +Ve |
| | 3 | Ornithine | +Ve | -Ve | -Ve | +Ve | -Ve | +Ve |
| **8** | | TSI | | | | | | |
| | 1 | Alkaline | K/A | K/A | K/A | K/A | K/K | A/A |
| | 2 | $H_2S$ production | +Ve | -Ve | -V | +Ve | -Ve | -Ve |
| | | Gas | G+ | G+ | G- | G- | G- | G- |
| **9** | | Caseinase | +Ve | -Ve | +Ve | +Ve | +Ve | -Ve |
| **10** | | Gelatinase | -Ve | -Ve | +Ve | -Ve | +Ve | +Ve |

previous speed, the supernatant was removed and the pellet was washed with 70% alcohol and air dried. The pellet was dissolved in RNA storage solution and observed in 1% agarose gel.

**Histological observation in experimentally infected fish.** In the study, mean 50-80 gm fish were taken and acclimatised in thewet lab for 15 days. Bacteria were grown in 9 ml broth for 24 hrs washed the bacteria and 100 µl of 1 x $10^8$ bacteria/ml was injected in fish by intraperitoneal route. After one week, lesions appeared. Then fish was sacrificed and thesample was fixed in 10%NBF. After one week, fixed tissue was dehydrated in ascending grades of alcohol, cleared in chloroform and impregnated with wax. Finally, paraffin block was prepared. The blocks were sectioned at a thickness of 4-5 µ, stained with Haematoxylin and eosin and mounted with DPX. The stained sections were observed under amicroscope.

## RESULTS AND DISCUSSION

**Biochemical tests.** We have done biochemical test for identification genus (primary test) and species (secondary test). On the basis of biochemical tests, these isolates are isolate no 1 and 4, *Edwardsiellatarda*(Table 2),isolate no 2, *Pseudomonas aeruginosa*(Table 2),isolates no 3 and 5, *Aeromonashydraphila*(Table 2*)* and isolate no. 6, *Vibrio cholera* (Table 2).

**Results of biochemical test.** On the basis of biochemical tests, these isolates are-

Isolate no 1 & 4, *Edwardsiella tarda*
Isolate no 2, *Pseudomonas aeruginosa*
Isolates no 3&5, *Aeromonas hydraphila*
Isolate no 6, *Vibrio cholera*

**DNA extraction from isolated bacteria and PCR amplification for 16S rDNA.** Genomic DNA was isolated from bacteria and Fig. 1 shows bacterial genomic DNA.

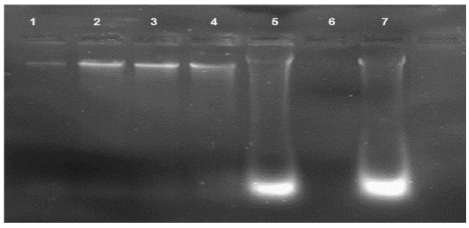


**FIGURE 1**
**Lane 1-7. Agarose gel electrophoresis of bacterial genomic DNA on 0.8% gel.**

Hassan et al., [15] has been reported that 16S rDNA has been widely used for the identification of micro-organisms at genus, family or species levels. In this study, a PCR-mediated identification assay was designed using species-specificsegments of 16S rDNA as targets. 16S r DNA is anon-structural gene of thebacterial genome. This is ahighly conserved region of bacteria genome, this product was amplified by the using 27F and 1492R primer. The size of this product is approximately 1500 bp (Fig 2).

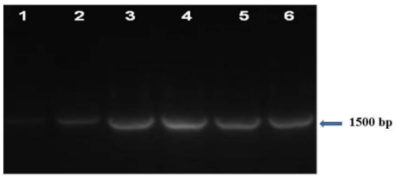


**FIGURE 2**
**Agarose gel electrophoresis of bacterial 16S rDNA gene, amplified using universal primers Lane 2 to 6- PCR product(~1500 bp), lane 1 negative control**

Targets containing conserved regions and areasof variability for specific identification of bacteria arethe genes encoding 16s and 23s rRNA [16]. In addition, astretch of DNA between the 16S and 23S rDNA, the16S-23S rDNA intragenic spacer region, proved to be a comparable speciesspecific segment.

**RFLP.** In this study, PCR-amplified 16s ribosomal DNAs of strains were analysed on the basis of their restriction fragment length. Tetra cutter restriction enzyme was used for digestion; it is useful for screening of bacterial strains.Fig. 3shows amplified 16s rDNA gene restriction analysis in 3% agarose gel. Lane 3, 100 bpladders, Lane 1,2,4, 5 restriction analysis patterns of isolates.

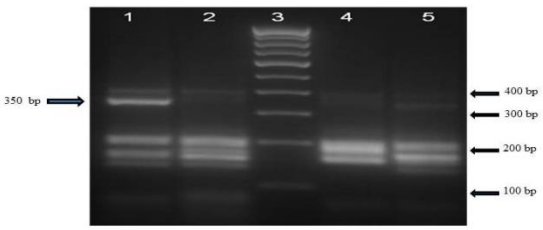


**FIGURE 3**
**Amplified16S rDNA generestriction analysis in 3% agarose gel Lane 3, 100 bpladder, Lane 1,2,4,5, -restrictionanalysispattern of isolates.**

Yoon et al. [17] identify *Saccharo monospora* strains by using RFLPs analysis of PCR-amplified 16s ribosomal DNA.

**Antigen preparation and their quantification and protein profiling (SDS-PAGE) of isolated bacteria.** Bacterial cultures (24 hr old) were used for isolation of protein, the concentration of this protein was estimated by

Lowry method [14] and it was 950 µg/ml. The isolated bacterial protein can be used in immunoassays or for immunisation. These proteins were used for differentiated of strains bacteria on the basis of their band pattern. Fig. 4shows total protein profile of bacteria and serum (lane 3, 4, 5, 6), serum protein normal fish (lane 1), serum protein infected *Catla* fish (12%, lane 2).

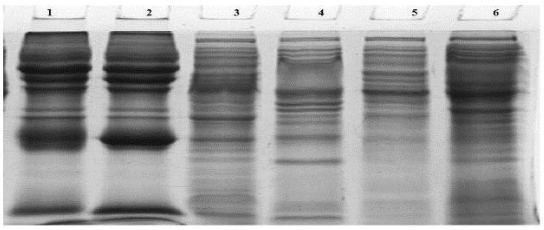


**FIGURE 4**
**Lane 3,4,5,6 Total protein profile (SDS-PAGE) of bacteria and serum, Lane 1, Serum protein normal fish, Lane 2, Serum protein infected Catla fish(12%)**

**Antibodies and immunofluorescence.** This is an antibody-based test, in this, we have observed the effect of the toxic material of bacteria on the related fish cell line, here we confirmed that these cells are epithelial-like by using this antibody. Fig. 5 shows immunofluorescent staining of cultured cells for a demonstration of cytokeratin.

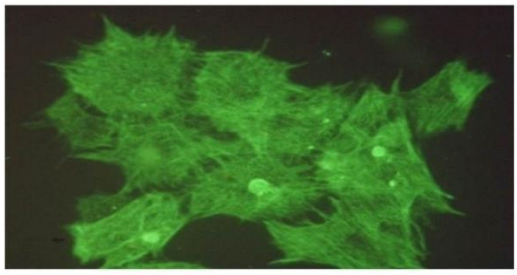

**FIGURE 5**
**Immunofluorescent staining of cultured cells for demonstration of cytokeratin.**

**Effect of bacterial toxin on cell line.** 24 hrs old cultures supernatant were used in a different concentration, after 48 hr it was observed that cells were going dead.Fig. 6 B shows cytotoxic effects of bacterial extracellular products on cultured cells of catla.

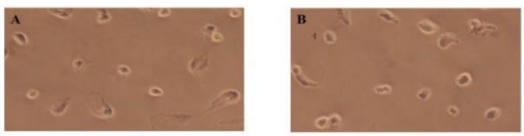


**FIGURE 6**
**Cytotoxic effects of bacterial extracellular products of cultured cells of catla**
**A. Control cells B. Exposed cells**

**Expression of RNA.** Different tissues of fish such as liver, kidney, spleen were taken for RNA isolation. Fig. 7shows agarose gel electrophoresis of RNA extracted from fish tissues; total RNA from spleen, kidney and liver (Lane 1, 2, 3), respectively.

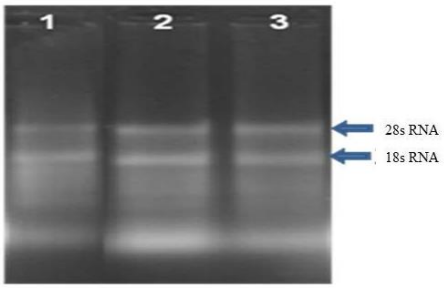


**FIGURE 7**
**Agarose gel electrophoresis of RNA extraxted from fish tissues; Lane 1,2,3- Total RNA from spleen, kidney and liver, respectively.**

**Histology of kidney from infected *Catla catla.*** Apparently, healthy fish was challenged with isolated bacteria and after two-week tissue was fixed for observation of effect on kidney tissues. Fig. 8 shows a section of a kidney from *C. catla* and it was stained with Haematoxylin and Eosin.

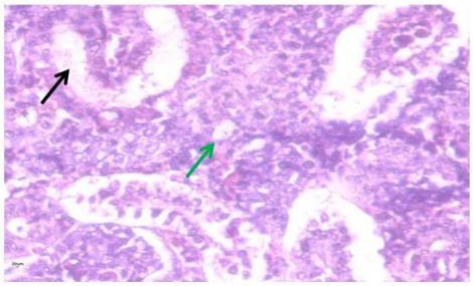

**FIGURE 8**
**A section of kidney from infected *Catla catla* fish stained with Haematoxylin and Eosin. Black colour arrow indicated enlargement of bowman capsule and green arrow vacuole formation.**

Kaleeswaran et al [18] also reported that *Aeromonas hydrophila* infected *C. Catla* showed the symptoms such as haemorrhages erosion of the pectoral fins, ulcers on the skin surface and septicaemia, kidney and spleen with clear histopathological alterations in morbid fish. Moreover, histopathological changes have been widely used as biomarkers for evaluating the health of fish. It was also observed that histopathological alterations are good biomarkers for field measurement [19].

Witeska and Jezierska [20] and Kondera and Witeska [21] have been reported heavy metals such as cadmium damaged the hematopoietic renal tissue of fish e.g. common carp. Diagnosis is one of most important step for prevention of disease. There are so many diseases possible in fish such as bacterial,

fungal, viral and parasitic etc. Bacterial disease in fishes is possible throughout the year.In this study, we have isolated bacteria from diseased fish and characterised through the biochemical test, molecular and protein level difference we have observed. We have also observed the effect of bacteria and their toxin on the cell.

## ACKNOWLEDGEMENT

The Project was supported by King Saud University, Deanship of Scientific Research, College of Science - Research Centre.

No potential conflict of interest was reported by the authors.

## REFERENCES

[1] Carr, R.M., Blumenthal, U.J., Mara, D.D. (2004) Guidelines for the safe use of wastewater in agriculture: revisiting WHO guidelines. Water Sci. Technol., 50,31-38.

[2] Nielsen, M.E., Hoi, L., Schmidt, A.S., Qian, D., Shimada, T., Shen, J.Y., Larsen, J.L. (2001) Is *Aeromonas hydrophila* the dominant motile *Aeromonas* species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China? Dis. Aquat. Organ. 46 (1), 23-29.

[3] Kozinska, A. (2007) Dominant pathogenic species of mesophilic aeromonads isolated from diseased and healthy fish cultured in Poland. J. Fish Dis., 30 (5), 293-301.

[4] Janda, J.M., Abbott, S.L., Cheung, W.K., Hanson, D.F. (1994) Biochemical identification of citro bacteria in the clinical laboratory. J. Clin. Microbial. 32 (8), 1850-1854.

[5] Ford, L.A., Thune, R.L. (1991) S-layer positive motile aeromonads isolated from channel catfish. J. Wildl. Dis., 27 (4), 557-561.

[6] Sioutas, S., Hoffmann, R.W., Pfeil-Putzien, C., Fischer-Scherl, T. (1991) Carp erythro dermatitis (CE) due to an *Aeromonas hydrophila* infection. Casuistic and experimental results. Zentralbl Veterinarmed. B., 38 (3), 186-194.

[7] Pemberton, J.M., Kidd, S.P., Schmidt, R. (1997) Secreted enzymes of *Aeromonas.* FEMS Microbiol. Lett. 152, 1-10.

[8] Chopra, A.K., Houston, C.W., Peterson, J.W., Jin, G.F. (1993) Cloning, expression and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila.* Can. J. Microbiol., 39 (5), 513-523.

[9] Hirono, I., Aoki, T. (1991) Nucleotide sequence and expression of an extracellular hemolysin gene of *Aeromonas hydrophila*. Microb. Pathog. 11,189-197.

[10] Rivero, O., Anguita, J., Mateos, D., Peniagua, C., Naharro, G. (1991) Cloning and characterization of an extracellular temperature-labile serine protease gene from *Aeromonas hydrophila.* FEMS. Microbiol. Lett. 81, 1-8.

[11] De Figueiredo, J., Plumb, J.A. (1977) Virulence of different isolates of *Aeromonas hydrophila* in channel catfish. Aquaculture. 11, 349-354.

[12] Hugh, R., Leifson, E. (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative rods. J. Bacteriol. 66, 24-26.

[13] Lo, Y.M., Tein, M.S., Lau, T.K. (1998) Quantitative analysis of foetal DNA in maternal plasma and serum: Implications for non-invasive prenatal diagnosis. Am J Hum Genet. 62,768-75.

[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the Folin phenol reagent.J Biol Chem. 193(1), 265-75.

[15] Hassan, A.A., Vossen, A., Lammler, C., Siebert, U., Fernandez-Garayzabal, J.F. (2008) PCR amplification of species specific sequences of 16S rDNA and 16S-23S rDNA intragenic spacer region for identification of *Streptococcus phocae*. Microbiological Research 163, 132-135.

[16] Kenneth, H.W., Rhonda, B.B., Ronald, C.G. (1990) Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. Journal of Clinical Microbiology, 28(9), 1942-1946.

[17] Yoon, J.H., Sung, T.L., Kim, S.B., Kim, W.Y., Goodfellow, M., Park, Y.H. (1997) Restriction fragment length polymorphism analysis of PCR-amplified 16s ribosomal DNA for rapid identification of *Saccharomonospora* Strains. International Journal of Systematic Bacteriology, 47 (1), 111-114.

[18] Kaleeswaran B., Ilavenil S., Ravikumar S. (2012) Changes in biochemical, histological and specific immune parameters in *Catla catla* (Ham.) by Cynodon dactylon (L.). Journal of King Saud University Science 24, 139-152.

[19] Alagappan, K.M., Deivasigamani, B., Kumaran, S., Sakthivel, M., (2009) Histopathological alterations in estuarine catfish (*Arius maculatus*; Thunberg, 1792) due to *Aeronomas hydrophila*. Infect. World J. Fish Mar. Sci. 1, 185-189.

[20] Witeska, M. and Jezierska, B. (2003). The effects of environmental factors on metal toxicity to fish. Fresen. Environ. Bull. 12 (8), 824-829.

[21] Kondera E and Witeska M. (2012). Cadmium-induced alterations in head kidney

hematopoietic tissue of common carp. Fresen. Environ. Bull. 21(3a), 769-773.

**Received:** **30.08.2016**
**Accepted:** **15.11.2016**

**CORRESPONDING AUTHOR**

**Dr. Daoud Ali**
Associate Professor
Department of Zoology,College of Science KingSaud University, BOX 2455 Riyadh 11451, Saudi Arabia

e-mail: daudali.ksu12@yahoo.com