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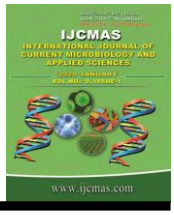

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# Detection of Virulence Genes in *Aeromonas sobria* Isolated from Diseased Fish Rohu, *Labeo rohita* by PCR

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**A B S T R A C T**

The Genus *Aeromonas* belongs to facultative, anaerobic gram negative bacteria. Several mesophilic species of *Aeromonas* cause septicaemia in a variety of aquatic organisms and gastrointestinal diseases in humans. The virulence of *Aeromonas* is considered multifactorial and are known to possess aerolysins, hemolysins, proteases, phospholipases, lipases, adhesions and enterotoxins. Therefore, the importance of present work lies in detecting the virulence genes in *Aeromonas sobria* isolated from diseased fish, rohu. The bacterium was identified using VITEK 2, automated bacterial identification system. Out of four genes screened, three genes namely, *actA* (cytotoxic enterotoxin), *ahh1A* (extracellular hemolysin) and *enoA* (enolase) were detected in *A. sobria* by PCR. However, *astA* gene which encodes for heat-stable cytotonic enterotoxin was not detected.

## Introduction

The Genus *Aeromonas* comprises of a large group of species, which are Gram-negative, oxidase positive and facultative anaerobes. *Aeromonas* spp. are ubiquitous and their occurrence has been reported from fish, milk, meat, poultry and human beings. Several mesophilic species of *Aeromonas* cause septicaemia in a variety of aquatic organisms and gastrointestinal diseases in humans (Janda and Abbott, 1998). The virulence of *Aeromonas* is considered multifactorial and pathogenesis is complex (Sen and Rodgers, 2004). They possess many virulence factors such as aerolysins, hemolysins, proteases, phospholipase, lipase, adhesions and enterotoxins (Parker and Shaw, 2010). These genes responsible for virulence have also been found in *Aeromonas* isolated from municipally treated water (Sen and Rodgers, 2004). In fisheries context, bacterial problems

are more commonly encountered in intensive fish culture because of deterioration of water quality and stress in the host, leading to manifestation of opportunistic pathogens, including *Aeromonads* (Syrova *et al.,* 2018). Some of the mesophilic Aeromonads like *A. hydrophila*, *A. veroni bv sobria A. bestiarum* and *A. salmonicida* are reported as important fish pathogens (Kozinska, 2007).

The present study was aimed at identification of *A. sobria* using VITEK-2 system and detection of virulence genes of *Aeromonas* using PCR. The bacterium was isolated form diseased fish rohu, which is an important aquaculture fish in India. Therefore, the importance of the present work lies in assessing the virulence genes in *A. sobria* for determining its pathogenic potential.

## Materials and Methods

### Bacterial isolation and genus phenotypic identification

Using a sterile loop, kidney of a diseased rohu was streaked on non-selective agar medium, Trypticase soy agar (TSA) and agar plate was incubated at 28 ± $2^0$C for 24 h. Pure bacterial culture was used to perform biochemical tests like Gram staining, motility, oxidase, catalase, glucose utilization, salt tolerance and sensitivity to vibriostatic agent O/129 for presumptive identification of bacteria.

### VITEK-2 identification

Single bacterial colony on TSA was used for bacterial identification using an automated bacterial identification system (VITEK 2 compact, BioMerieux, France).

### DNA extraction

Total chromosomal DNA from *A. sobria* was prepared by thermal lysis of bacterial cells according to Martino *et al.,* (2011) with slight modifications. Briefly, overnight grown fresh single colony was resuspended in 100 µl triple distilled water, vortexed and heated at $94^0$C for 10 min, followed by spinning. The supernatant of the lysed cells was used as DNA template in PCR.

### Detection of virulence factor genes

Four pairs of reported primers (Table 1) were used for the detection of virulence genes; *actA* (cytotoxic enterotoxin), *astA,* (heat-stable cytotonic enterotoxin), *ahh1A* (extracellular hemolysin) and *enoA (enolase)* using PCR. The PCR reactions consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles with denaturation at 94°C for 30 s, annealing temperatures; 54°C for 30 s for *aerA*, 60°C for 30 s for *astA*, *ahh1A* and *enoA*, and extension at 72°C for 1 min. A final extension step was carried out at 72°C for 10 min. The PCR amplicons obtained were analysed on 1.5% agarose gel and observed under UV light.

## Results and Discussion

### Bacterial isolation and identification

Several buff-coloured colonies were observed on TSA medium after 24 hours of incubation and all were of similar morphological appearance. Four-colonies were randomly picked for biochemical characterization. All four colonies were Gram-negative, oxidase-positive, fermented glucose, showed absence of growth in 6.5% salt and were resistant to vibriostatic agent O/129. These characteristics presumptively indicate that the bacterium belonged to the family Aeromonadaceae. Several authors have reported similar kind of findings in presumptive identification of genus *Aeromonas* (Galbis *et al.,* 2002; Abbott *et al.,* 2003). Furthermore, VITEK 2 result also confirmed the identification of bacterium

as *A. sobria* (Table 2). VITEK 2 an automated bacterial identification system provides rapid, reliable and highly reproducible results (Ling at al., 2001; Ling *et al.,* 2003).

## Detection of virulence genes in *A. sobria*

There are reports about clinical strains of *A. sobria* associated with humans infection (Wang *et al.,* 2018) and possesses virulence associated factors (Daily *et al.,* 1981). This bacterium has also been isolated from diseased fish, silver carp (*Hypophthalmichthys molitrix*) demonstrating 15% prevalence rate (Dar *et al.,* 2016). Motile mesophilic species of *Aeromonas*, particularly *A. hydrphila* and *A. sobria* cause septicaemia in cold-blooded animals including fish, reptiles, and amphibians (Janda and Abbott, 2010). The pathogenic potential associated factors have been correlated with the toxin coding genes in *Aeromonas* spp. which can be a predictor for pathogenicity (Heuzenroeder *et al.,* 1999). Hussian *et al.,* (2013) examined hemolytic strains of *A. hydrophila*, *A. sobria* and other *Aeromonas* spp. from fish and fishery products by the PCR amplification of *ahh1* and *asa1* genes. Therefore, we were interested in determining the presence of potential virulence genes in *Aeromonas sobria*, isolated from diseased rohu fish. Out of four virulence factor genes screened, three genes, namely *actA*, *ahh1A* and *enoA* encoding for cytotoxic enterotoxin, extracellular hemolysin and enloase were detected in the bacterium, respectively. Sen and Rodgers (2004) determined the presence of six virulence factor genes in *Aeromonas* spp., viz. elastase (*ahyB*), lipase (*pla/lip/lipH3/alp-1*) flagella A and B (*flaA* and *flaB*), the enterotoxins, *act*, *alt* and *ast*, using PCR. Out of 205 isolates tested, only one isolate contained all the virulence genes, others *Aeromonas spp* were having variety of combinations of these genes even in different strains of the same species. From our study, presence of these genes *(actA, ahh1A* and *enoA)* in *A. sobria* also indicates potential for virulence in rohu. Hence, further work is being carried for *in-vivo* experimental trails and its correlation with pathological changes.

**Table.1** Primers used for amplification of different virulence factor genes

| Name of the gene | Primer sequence (5'-3') | Size of product | Reference |
|---|---|---|---|
| ***actA*, (Cytotoxic enterotoxin)** | F- AGAAGGTGACCACCAAGAACA<br>R- AACTGACATCGGCCTTGAACTC | 232 | (Kingombe, 1999) |
| ***astA*, (Heat-stable cytotonic enterotoxin)** | F- TCTCCATGCTTCCCTTCCACT<br>R- GTGTAGGGATTGAAGAAGCCG | 331 | (Sen and Rodgers, 2004) |
| ***ahh1A* (Extracellular hemolysin)** | F- GCCGAGCGCCCAGAAGGTGAGTT<br>R- GAGCGGCTGGATGCGGTTGT | 130 | (Wang *et al.,* 2003) |
| ***enoA* (Enolase)** | F- CGCCGACAACAACGTCGACATC<br>R- CTTGATGGCAGCCAGAGTTTCG | 598 | (Martino *et al.,* 2011) |

**Table.2** Details of biochemical characteristics of *Aeromonas sobria* strain using VITEK 2 compact (BioMerieux, France)

| Biochemical test | Result |
|---|---|
| Ala-Phe-Pro-arylamidase (APPA) | + |
| L-Pyrrolydonyl- arylamidase (PyrA) | - |
| L- Arabitol (IARL) | - |
| Beta-galactosidase (BGAL) | + |
| Beta –N- acetyl-glucosaminidase (BNAG) | + |
| Glutamyl arylamidase pNA (AGLTp) | - |
| Gamma-glutamyl transferase (GGT) | - |
| Fermentation/glucose (OFF) | + |
| Beta-glucosidase (BGLU) | - |
| D-Maltose (dMAL) | + |
| D-Mannitol (dMAN) | + |
| D-Mannose (d MNE) | + |
| Beta-xylosidase (BXY) | - |
| Beta-alanine arylamidase pNA (BAlap) | - |
| L-Proline arylamidase (ProA) | + |
| Lipase (LIP) | - |
| Palatinose (PLE) | - |
| Tyrosine arylamidase (TyrA) | + |
| Urease (URE) | - |
| D-Sorbitol (dSOR) | - |
| Saccharose /Sucrose (SAC) | + |
| D- Tagatose (dTAG) | - |
| D- Trehalose (dTRE ) | + |
| Citrate (Sodium) (CIT) | - |
| Malonate (MNT) | - |
| 5-Keto -D- Gluconate (5KG) | - |
| L- Lactate alkalinisation (ILATk) | + |
| Alpha-glucosidase (AGLU ) | - |
| Succinate alkalinisation (SUCT) | + |
| Beta-N-acetyl-galactosaminidase (NAGA) | + |
| Alpha- galactosidase (AGAL) | - |
| Phosphatase (PHOS) | - |
| Glycine arylamidase (GlyA) | - |
| ORNITHINE DECARBOXYLASE (ODC) | - |
| Lysine decarboxylase (LDC) | - |
| L- Histidine assimilation (IHISa) | - |
| Coumarate (CMT) | + |
| Beta- glucoronidase (BGUR) | - |
| O/129 resistance [ comp.vibrio.] (O129R) | + |
| Glu-Gly-Arg-arylamidase (GGAA) | + |
| L-Malate assimilation (IMLTa) | + |
| Ellman (ELLM) | + |
| L- Lactate assimilation (ILATa) | - |
| Adonitol (ADO) | - |
| $H_2S$ production ($H_2S$) | - |
| D- Glucose (dGLU) | + |
| D-cellobiose (dCEL) | - |

**Figure 1**

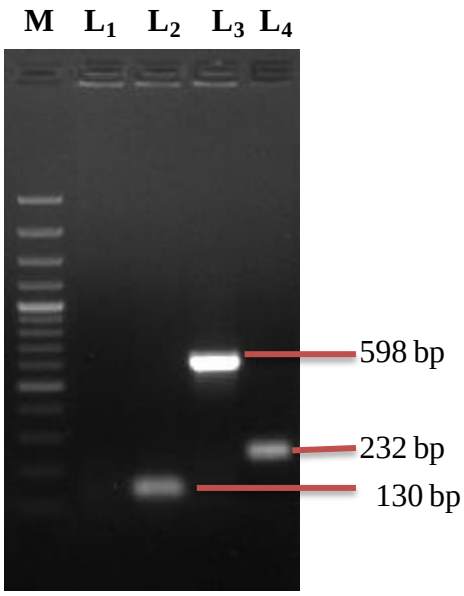


**Fig.1** Agarose gel electrophoresis of PCR products for genes screened for; *astA*, cytotonic enterotoxin; *ahh1A*, extracellular hemolysin; *enoA*, enolase and *actA*, cytotoxic enterotoxin in *Aeromonas sobria*. Marker M: 100bp plus DNA ladder, and Lanes: $L_1$: contain no amplification from *astA* gene; $L_2$-$L_4$: represents amplified products from *ahh1A*, *enoA* and *actA* genes, respectively

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