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Virulent *Aeromonas veronii* Strain BLB-01 Associated with Mass Mortality of *Clarias batrachus* (Linnaeus, 1758)

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The Asian catfish, *Clarias batrachus* of the family claridae, commonly known as 'magur' has high commercial importance in India. *Aeromonas veronii* has been reported as an important bacterial pathogen for aquatic animals and humans. Several reports have demonstrated the pathogenic effect of other *Aeromonas* species on aquatic animal; however in farmed *C. batrachus* no definitive data are available about *A. veronii* infection. In the present study, a virulent *A. veronii* strain BLB-01was isolated from the infected *C. batrachus* collected from freshwater fish farm Balabhadrapuram, East Godavari district, Andhra Pradesh, India and its identification was confirmed by 16S rRNA amplification and sequencing (Gen Bank accession number.MF370515). In addition two genes encoding aerolysin (*aerA*), haemolysin (*hlyA*) were found present in the isolate which further confirmed its potential virulence. Histologically, the infected muscle tissue showed erythrocyte and leukocyte infiltration due to bacterial infection. *In vitro* antimicrobial susceptibility test was also conducted by using effective antibiotic drugs to guide the treatment of the disease.

Introduction

The Asian catfish, *Clarias batrachus*, (Linnaeus, 1758) commonly known as 'magur', are one of the most widespread catfish genera, found in inland waters in most part of the world (Thomas *et al.,* 2013). It is one of the most popular and economically important indigenous freshwater food fishes in the countries like India, Bangladesh, Sri Lanka, Myanmar and Malaysia (Argungu *et al.,* 2013; Mookerjee and Mazumder, 1950). *C. batrachus* culture has high commercial importance in India and it has been identified

as one of the potential national priorities in Indian aquaculture (Paul *et al.,* 2015). *Aeromonas* species are ubiquitous inhabitants in nature and increasingly being reported as important pathogen for both human and lower vertebrates, including fish (Janda and Abbott, 1998). Motile aeromonads have been associated with mass mortalities of fish around the globe and consider as the most common and troublesome diseases of warm and cold water fish, resulting in huge economic losses (Janda and Abbott, 2010; Paul *et al.,* 2015). Many reports demonstrated that *Aeromonas salmonicida*, *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas sobria* were frequently occurring species of *Aeromonas* that have been considered important in fish pathology (Janda and Abbott, 2010; Paul *et al.,* 2015).

Recently, the panorama of species has been expanded with the discovery of many new *Aeromonas* species (Martinez *et al.,* 2013). Among them *A. veronii* has been described as an important fish and human pathogen (Singh *et al.,* 2012). *A. veronii* pathogen has been isolated from *Ichtalurus punctatus* (Nawaz *et al.,* 2006), *Coilisa lalia* (Hossain, 2008), *Misgurnus anguillicaudatus* (Qin *et al.,* 2008), *Acipenser baerii* (Ma *et al.,* 2009), *Cyprinus carpio* (Gong *et al.,* 2010), *Ictalurus lunetas* (Huang *et al.,* 2010), *Leiocassis longirostris* Gunther (Cai *et al.,* 2012), *Astronotus ocellatus* (Sreedharan *et al.,* 2011), *Oreochromis niloticus* (Eissa *et al.,* 2015), Channel catfish, *Ictalurus punctatus* (Liu *et al.,* 2016) and *Carassius gibelio* (Sun *et al.,* 2016). However, there is no definitive data on pathogenic association of *A. veronii* infection in cultured *C. batrachus*.

In this paper, in order to investigate the cause of the disease, a virulent *A. veronii* strain could be isolated from the infected *C. batrachus* at freshwater fish farm Balabhadrapuram, East Godavari district, Andhra Pradesh, India and confirm the etiology through phenotypic, molecular characterization and histopathological alterations caused by the bacteria.

Materials and Methods

Isolation and phenotypic characterization of bacteria from *C. batrachus*

The moribund *Clarias batrachus* samples were collected from three culture ponds at freshwater fish farm Balabhadrapuram, East Godavari district of Andhra Pradesh. Where affected fish had developed similar pathological signs and almost 60% of the animal died. The infected fish swam slowly on the surface of the water and had different degrees of ulcer, fin rot. 20 no's of infected fish samples were aseptically transported to laboratory within one hour using sterile plastic bag containing aerated water (water temperature 26 ± 2.0ºC). The physico-chemical parameters *viz.* dissolve oxygen (DO), pH and water temperature were analyzed during sampling in the infected pond following standard procedure. The dissolved oxygen was analyzed by the Winkler method (APHA, 2005). Fish muscle and kidney were used for microbial examination. The infected fish with typical clinical signs were sanitized with 75% alcohol and the bacteria were isolated from muscle and kidney under aseptic condition. The specimens were inoculated onto nutrient agar (Himedia, India); tryptone soya agar supplemented with 0.5% NaCl (Himedia, India) and brain heart infusion agar (Himedia, India) plate and incubated aerobically for 24 h at 28ºC and further observed upto 48 h, 72 h and 96 h at 28ºC. After incubation, the dominant colony on nutrient agar, tryptone soya agar and brain heart infusion agar were re-streaked onto the respective agar media to obtain pure isolate. Colonies of different shape and sizes were chosen for Gram staining. All the purified

isolates were stored on nutrient agar slants and tryptone soya agar slants at 4ºC for later use and maintained frozen in nutrient broth (Himedia, India) and tryptic soy broth (Himedia, India) with 20% (v/v) sterile glycerol at -20ºC. The phenotypic characterization involve morphological and biochemical tests were performed according to the methodologies described by Bergey's Manual of Systematic Bacteriology (Vos *et al.,* 2009) and HIIMVIC biochemical test kit, Himedia India. The tests include Gram staining, motility, Oxidase test, Catalase test, Voges- proskauer test, Nitrate reduction, citrate utilization, Indole production, Arginine dihydrolase, O/129 sensitivity, Trehalose, Mannose, D-Ribose, Mannitol, Ornithine decarboxylase, Esculine hydrolysis, Lactose, 6.5% Sodium chloride, Urease, Sorbitol, Inulin, Arabinose, Raffinose, Methyl-red, Oxidation of ONPG, carbohydrate fermentation etc.

Molecular characterization of the isolated bacteria

The culture isolate were subjected to molecular analyses in order to compare and test the phenotypic determination. The genomic DNA was extracted from the isolated bacteria using uniflex DNA isolation kit (Himedia, India) as per manufacturer's protocol. The 16S rRNA gene was amplified by PCR using universal primers 27F, 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1541R, 5'-AAG GAG GTG ATC CAG CCG CA-3' (Zhang *et al.,* 2011).

The nucleotides of the 16S rRNA sequence were matched with the other microbes in the database of National Centre for Biotechnology Information (NCBI) using the programme Basic Local Alignment Search Tool (BLAST). The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method. The analysis involved 9 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1480 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 software.

Virulence gene assay

The genomic DNA was extracted from the pure cultures using a uniflex DNA isolation kit following the instructions of the manufacturer (Himedia, India). Two virulence genes encoding aerolysin (*aerA*), haemolysin (*hlyA*) were respectively amplified by PCR using specific *aerA*, *hlyA* gene primers (Table 1) as recommended by Kong *et al.,* (2002), Wong *et al.,* (1998). PCR amplification of virulence genes was carried out in a reaction volume of 25 µl by using a Hi-PCR Kit (Himedia, India). The final concentrations in the PCR mixture were 2x Taq PCR master Mix 12.5 µL, 0.5 µL forward and 0.5 µL reverse primers, respectively, 10.5 µL ddH_2O and 1 µL DNA as template. The thermal cycling conditions were optimized as an initial denaturation at 95ºC for 5 min followed by a total of 35 cycles of denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 1 min. The final extension was done at 72ºC for 10 min. A reagent blank except template DNA for which sterile distilled water was used. The PCR products were electrophoresed on 1% agarose gel stained with ethidium bromide (1 mg/mL) and visualized through ultraviolet trans-illumination. A 50-bp DNA ladder was used as the size standard.

Pathogenicity of isolated *A. veronii* strain BLB-01 on *C. batrachus*

To study the virulence of the bacterial pathogen, a challenge study was conducted in healthy *C. batrachus* using the bacteria

Aeromonas veronii strain BLB-01 isolated from the infected *C. batrachus*. *C. batrachus* weighing approximately 70 ± 2.22 g were maintained in FRP tubs (100 L capacity) for 15 days in order to adapt to laboratory conditions at 26-28°C.

All fishes were anaesthetized with clove oil (Himedia, India) and then injected (intramuscular injection) with 0.1 mL bacterial suspensions (24 h bacterial culture, 10^4-10^8 CFU/fish).

The LD_{50} tests were conducted with batches of 10 fish/dose following Reed and Muench (1938). Sterile PBS was injected into other group of fish as parallel controls. The challenged fish were observed daily for pathological signs and mortalities were recorded for 15 days post injection with bacterial inoculum. The dead fish were processed for bacterial analyses in all the cases.

Histopathology

The muscle tissue of infected *C. batrachus* was fixed in 10% neutral buffer formalin solution and then embedded in paraffin wax following standard techniques. Tissue sections in 5 µm width were stained with haematoxylin and eosin according to Roberts, 2012.

In vitro antimicrobial susceptibility test

In vitro antimicrobial susceptibility testing was performed followed by disk diffusion method of Kirby-Bauer (Bauer *et al.,* 1996). All filter-paper discs were obtained from Himedia, India. After 24 h of incubation at 28°C the zones of inhibition were measured. The isolates were classified as resistant (R), moderately sensitive (M) and sensitive (S) on the basis of the size of the zone of inhibition described by the National Committee for Clinical Laboratory Standards (2000).

Results and Discussion

Isolation and phenotypic characterization of bacteria from *C. batrachus*

At the beginning stage of the disease, the fish have a low mortality. Infected fish were lethargic and swam along the surface of the pond. The principle clinical signs of the affected *C. batrachus* were fin rot and ulcerated red area on body surface (Fig. 1. a, b) and deep skin ulcers on different side of the body surface become noticeably visible (Fig.1 c, d). At later stage of infection fish stop feeding and died, mortality was recorded up to 60%.

The range of physico-chemical parameters of all the three pond water *viz.* temperature (26 - 30°C), pH (6.5 -7.0) and dissolved oxygen (4.3-5.5 mg/L) were reported. After 24 h of incubation under aerobic conditions, colonies appeared on the nutrient agar, tryptone soya agar and brain heart infusion agar plate.

The colonies were round or ellipse in shape with 1–3 mm diameter size in all the media and creamy white color in nutrient agar; colorless, transparent, smooth in tryptone soya agar; white color in brain heart infusion agar. After 48 h, 72 h and 96 h incubation at 28ºC only one type of dominant colony was observed in all the plates.

All the colonies were tested by Gram's staining method and viewed under a microscope and same type of gram-negative bacillus was isolated from the diseased fish as identified with biochemical and phenotypic analysis (Fig. 2).

The phenotypic identification protocol revealed that, the suspected micro biota was Gram negative, motile bacteria. The strain were positive for Oxidase test, Catalase test, Voges- proskauer test, Oxidation of ONPG, Nitrate reduction, citrate utilization, Lysine

decarboxylase, Indole production, Arginine dihydrolase, O/129 sensitivity, Trehalose, Mannose, D-Ribose, Mannitol. The strain were negative for Ornithine decarboxylase, Esculine hydrolysis, Lactose, 6.5% Sodium chloride, Urease, Sorbitol, Inulin, Arabinose, Raffinose, Methyl-red. The strain had the ability to ferment carbohydrate. Based on the morphological and biochemical test the suspected strain was presumed as *A. veronii* (Table 2).

The bacteria *A. veronii* belongs to genus *Aeromonas* and it was first reported by Hichman-Brenner *et al.,* (1987). It usually infects human beings and causes diseases like septic arthritis, bacteremia, spontaneous bacterial empyema and severe pneumonia (Wang *et al.,* 2000; Roberts *et al.,* 2006; Li *et al.,* 2008). It has been seen that, *A. veronii* not only infect human being but also infects fish to spread diseases quickly (Huang *et al.,* 2010).

Molecular characterization and phylogeny of the isolated bacteria

NCBI blast search analysis confirmed that, the isolated bacteria were *A. veronii* strain BLB-01. The PCR products of 16S rRNA were about 1500 bp after an Agarose gel electrophoresis was run (Fig. 3) and it was demonstrated that the 16S rRNA PCR products were 1480bp by sequencing.

Phylogenetic and evolutionary analysis of the 16S rRNA sequence revealed that, the *Aeromonas veronii* strain BLB-01shared at 99-100 % similarity to other *A. veronii* strain (Fig. 4). The sequence were deposited in NCBI database, the strain name and gene bank accession number were *Aeromonas veronii* strain BLB-01; MF370515. Phylogenetic analysis based on the 16S rRNA gene is universally used and considered as an appropriate tool for the reconstruction of evolutionary history and phylogenetic relationship of bacterial genera (Stackebrandt and Goebel, 1994). In the present study, all the sequences of 16S rRNA gene from the representative isolated bacterial strain *A. veronii* strain BLB-01 showed high similarity (99-100 %) with other *Aeromonas spp.*in the GenBank database (NCBI) confirming *A. veronii* strain BLB-01 was the responsible pathogen for mortality in *C. batrachus* at freshwater fish farm Balabhadrapuram, Andhra Pradesh, India.

Virulence gene assay

The specific virulence gene *aerA* and *hlyA* fragments were obtained with the *A. veronii* strain BLB-01 using a pair of *aerA*-specific primers (720 bp region) and *hlyA* specific primers (597 bp region) respectively (Fig. 4) provide a direct way to manifest the virulence of the bacterial strain in farmed *C. batrachus* as these genes are closely related with the pathogenicity of *Aeromonas* species (Sun *et al.,* 2016). Moreover, the virulence factor genes are also considered as good markers for identifying the microorganism pathogenicity (Sun *et al.,* 2016). Wilmsen *et al.,* (1990) reported aerolysin can leads to cell lysis through the oligomerization process and forming channels in the cell membrane. The production of haemolytic toxins (*hlyA* and *aerA*) provides a strong evidence of aeromonads potential pathogenicity (Santos *et al.,* 1999).

Pathogenicity of *Aeromonas veronii* strain BLB-01 on *C. batrachus*

Challenged with the bacterial suspensions of *A.veronii* strain BLB-01into *C. batrachus* was lethal to the fish, in comparison to the control groups injected with sterile PBS. The LD_{50} of the strain live cells for *C. batrachus* was about 3.14×10^6 CFU/fish in intramuscular injection (Table 3). The moribund or dead fish exhibited the same signs as the diseased fish on ulcerative condition in the ponds.

Table.1 PCR primers, targets, amplicon sizes used in the study

Sl. no.	Name of gene	Primer sequence (5'-3')	Product size (bp)	Length (bp)	Reference
1.	*aeroA*	Aero-F TGTCGGSGATGACATGGAYGTG	720	22	Kong *et*
		Aero-R CCAGTTCCAGTCCCACCACTTCA		23	*al.*,2002
2.	*hlyA*	hlyA-F GGCCGGTGGCCCGAAGATACGGG	597	23	Wong *et*
		hlyA-R GGCGGCGCCGGACGAGACGGG		21	*al.*,1998

Table.2 Phenotypic identification of *A. veronii* strain BLB-01 form farmed *C. batrachus*

Sl. No.	Test items	Reaction
1.	Gram's staining	-ve
2.	Motility	+ve
3.	Voges- proskauer	+ve
4.	Oxidation of ONPG	+ve
5.	Oxidase	+ve
6.	Catalase	+ve
7.	Trehalose	+ve
8.	Nitrate	+ve
9.	O/F test	Fermentative
10.	Lysine	+ve
11.	Arginine	+ve
12.	Mannose	+ve
13.	Bile esculine 40%	-ve
14.	Lactose	-ve
15.	6.5% Sodium chloride	-ve
16.	D-Ribose	+ve
17.	Ornithine	-ve
18.	Simmons citrate	+ve
19.	Urease	-ve
20.	Sorbitol	-ve
21.	Inulin	-ve
22.	Indole	+ve
23.	Mannitol	+ve
24.	Arabinose	-ve
25.	Raffinose	-ve
26.	Methyl-red	-ve
27.	O/129 sensitivity	+ve

Table.3 Pathogenicity tests of isolated *A. veronii* strain BLB-01 intramuscularly injected into *C. batrachus* during 15 days observation

Dilution of bacterial culture	Number of death and live fish during 15 d period of observation		Cumulative live fish	Cumulative death in fish	Mortality ratio	% of mortality	LD_{50} value (CFU $fish^{-1}$)
	Live	dead					
10^4	0	10	0	29	29/29	100	
10^5	3	7	3	19	19/22	86.36	
10^6	7	3	7	10	10/20	50	
10^7	9	1	19	3	3/22	13.63	$3.14x10^6$
10^8	10	0	29	0	0/29	0	
Control (PBS)	10	0	-	-	-		

Table.4 Sensitivity of the isolated *A. veronii* strain BLB-01to various antimicrobial agent

Antimicrobial	MIC*($\mu g ml^{-1}$)	Sensitivity[#]
Cephalothin	≤8	R
Chloramphenicol	≤8	S
Ciprofloxacin	>2	R
Clarithromycin	>4	R
Clindamycin	>2	R
Erythromycin	4	M
Fosfomycin	≤32	S
Fusidic Acid	16	M
Gentamicin	≤4	S
Levofloxacin	4	R
Moxifloxacin	>1	R
Netilmicin	≤8	S
Oxacillin	>2	R
Rifampin	≤1	S
Synercid	>2	R
Teicoplanin	>16	R
Tetracycline	8	M
Vancomycin	>16	R

*MIC-Minimum Inhibitory Concentration; [#]R-resistance; S-sensitive; M-moderately sensitive

Fig.1 Bacterial infection caused by *Aeromonas veronii* in farmed *Clarias batrachus*.
(a) Infected fish showing fin rot and ulcerated red area on body surface.
(b) A stock of fish showing fin rot and ulcerated red area on body surface.
(c) & (d) Deep skin ulcers on different side of the body surface become noticeably visible

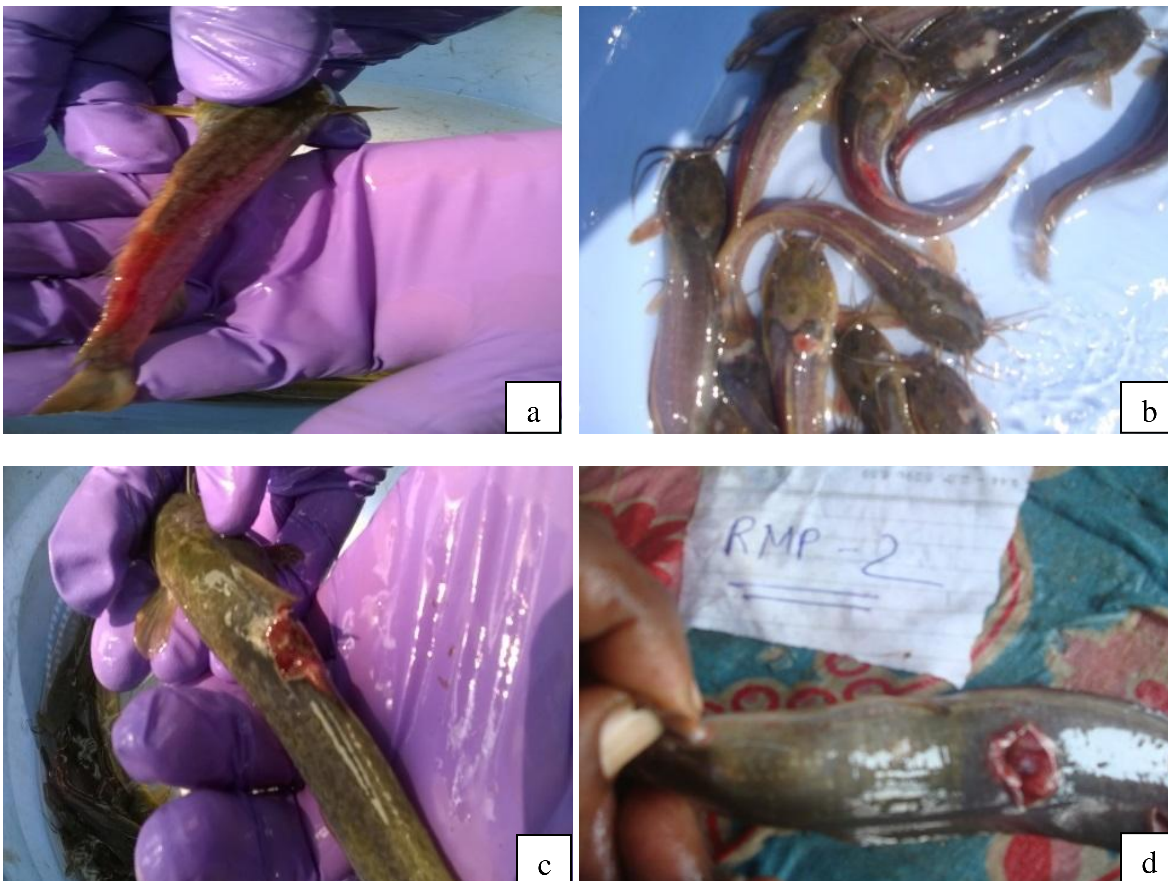


Fig.2 Typical morphology of a gram negative bacilli *Aeromonas veronii*

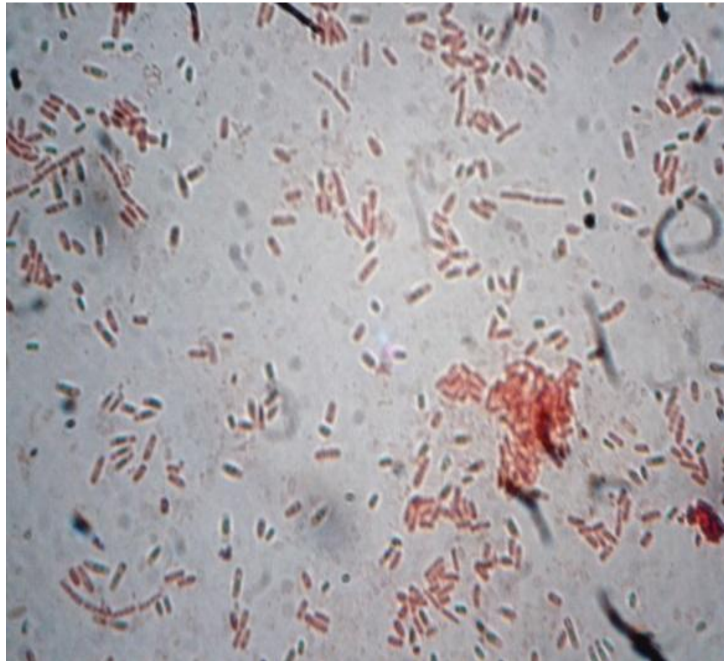


Fig.3 PCR amplified product with 16S rRNA oligonucleotide primer. Lane 1: ladder, lane 2: amplification of 1500 bp product from the template DNA

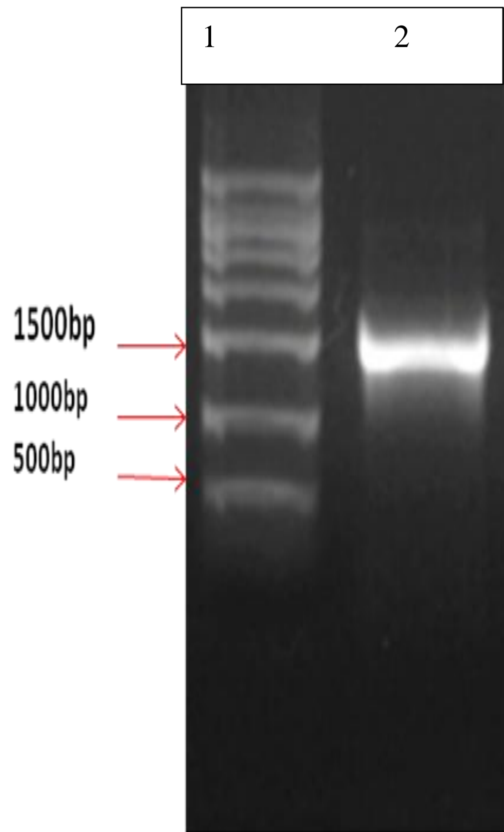


Fig.4 Nucleotides homology and phylogenetic analysis of the microbe based on 16S rRNA gene sequence data compare with other *A. veronii* in the database

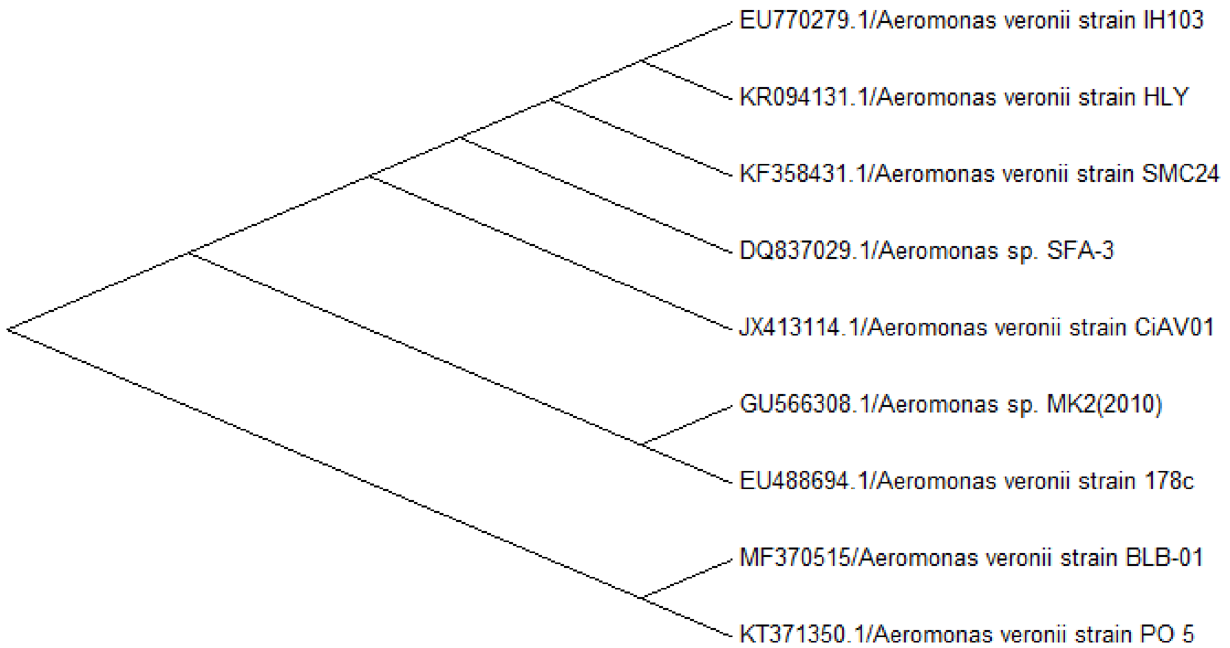


Fig.5 A-B: (A) lane-1: amplification of the 720-bp region of the aerA gene from the template DNA, lane-2: -ve control and lane-3: 50-bp DNA ladder. (B) Lane-1: 50 bp DNA ladder, lane-2: amplification of the 597-bp region of the hlyA gene and lane-3: -ve control

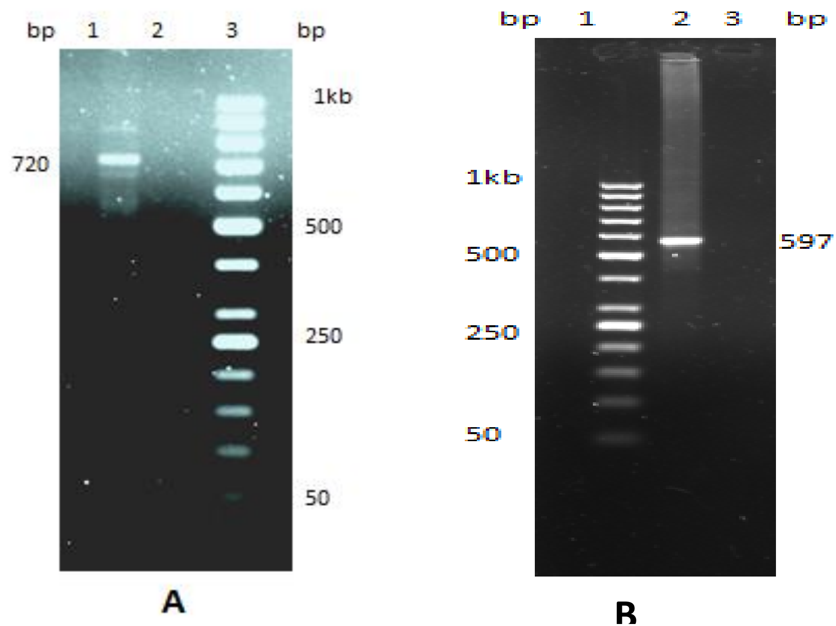
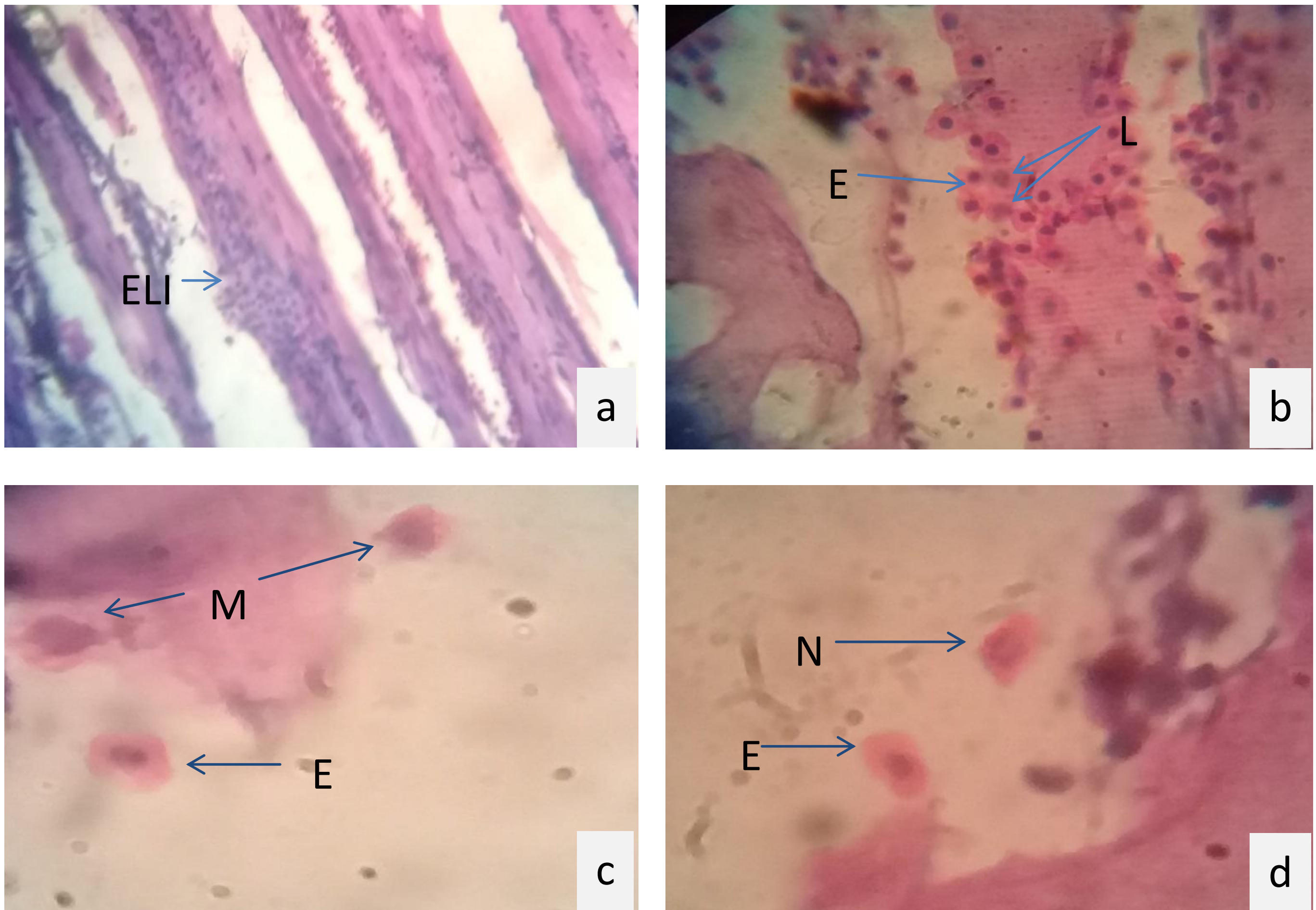


Fig.6 Histological details of the ulceration of the connective tissues: (a) Erythrocyte and leukocyte infiltration (ELI) in the connective tissue of fish muscle due to bacterial infection (X10). (b) Erythrocyte (E) and lymphocytic infiltration (L) (X100). (c)Monocyte and erythrocyte infiltration (X100). (d) Neutrophil and erythrocyte infiltration(X100). H&E staining



The pathogenic capacity of the strain has been verified by fulfillment of Koch's postulates. Han *et al.,* (2008), reported that *A. veronib v. veroni* strain RY001 was virulent to goldfish with an LD_{50} value of 1.6×10^6 CFU/fish which was also in line with the present strain. Cai *et al.,* (2012), reported that *A. veronibv. veroni* strain PY50 was virulent in Chinese long snout catfish when injected intraperitoneally with an LD_{50} value of 3.47×10^4 CFU/ fish which is much lower compared to the present strain. The i.p. administered median lethal dose values ranged from 5×10^3 - 5.2×10^9 CFU/fish in gilthead sea bream challenge against several strains of *A. veroniibiovarsobria* (Gashgari and Selim, 2015) were found to be within the range of the present value.

Higher LD_{50} values (2×10^7 CFU/fish) in *Anabas testudineus* when intramuscularly injected with *A. hydrophila* (Hossain *et al.,* 2011). This difference could be due to different mode of injection, strain or host studied (Cai *et al.,* 2012).

In conclusion, we confirmed that the present bacteria *A. veronii* strain BLB-01 is highly pathogenic to *C. batrachus* and caused mass mortality in *C. batrachus* cultured ponds.

Histopathology

Histologically, the infected muscle tissue of *C. batrachus* showed erythrocyte and leukocyte infiltration (ELI) due to bacterial infection (Fig. 6). Similarly lymphocytic, neutrophil infiltration has been observation in the muscle of ulcerated *C. gariepinus* infected with *Edwardsiella tarda* (Abraham *et al.,* 2015) and Leukocyte infiltration infected with *Aeromonas hydrophila* (Laith and Najiah, 2013). This observed histopathological changes in the muscle tissue indicated that blood cell movement has happened from within the blood into the diseased or infected tissues to neutralize the pathogenic effect caused *A. veronii* strain BLB-01.

In vitro antimicrobial susceptibility test

Antimicrobial susceptibility test showed that the isolated *A.veronii* strain BLB-01was multi-resistant to most frequently used antimicrobial agents in India such as Cephalothin, Ciprofloxacin, Clarithromycin, Clindamycin, Levofloxacin, Moxifloxacin, Oxacillin, Synercid, Teicoplanin, Vancomycin but sensitive to Chloramphenicol, Fosfomycin, Gentamicin, Neltilmicin, Rifampin of the antibiotic tested (Table 4). The present results also in line with the fact that most of *Aeromonas* species are susceptible to chloramphenicol (Awan *et al.,* 2009). The present study also supports the fact that most strains of *Aeromonas* are susceptible to chloramphenicol but resistant to vancomycin, clindamycin in USA (Lupiola-Gomez *et al*., 2003). However present findings differ with the findings of Petersen and Dalsgaard (2003) who reported that the *Aeromonas* strains were resistant against the antibiotic chloramphenicol. It has been a common phenomenon that, the resistant to different antibiotic are variable with respect to area and country, therefore antimicrobial resistance should be performed when the bacterial pathogen are isolated from the samples, in order to avoid the development of serious clinical symptoms and spread of the pathogen in the environment with secretive and excretive products which may ultimate results in therapeutic failures (Cai *et al.,* 2012). *In vitro* antibiotic sensitivity test had helped us to select susceptive antibiotic to prevent and treat the disease. To negate the harmful effect of antibiotic residue sufficient withdrawal period should be given as applied in the present study. The need of the hour is to go for better management practices (BMP) for the *C. batrachus* so that the culture operation can be sustainable.

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