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In vitro and In vivo virulence study of Aeromonas hydrophila isolated from fresh water fish

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KEYWORDS

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A B S T R A C T

The isolation, biochemical characterization, *In vitro* and *In vivo* virulence study of 12 isolates of *Aeromonas hydrophila* along with reference strain MTCC 646 isolated from diseased fish as rohu, catla, mrigal, cat fish, gold fish, *Channa* spp., were done in the present experiment. *A. hydrophila* strains along with reference strain MTCC 646 produced small, round, smooth, convex and translucent, yellow colonies on Rimler Shott's medium and were Gram negative short rod, motile by polar flagella and fermentative. All strains of *A. hydrophila* were β-haemotytic against fish and animal blood and binds to Congo red producing slime and biofilm on glass tubes stained with safranine. The *in vivo* pathogenicity studies of *A. hydrophila* (Ah1-Ah12) strains were done against fries of *Labeo rohita* by immersion challenge and the LD_{50} value varied from 1.72 X 10^7 to 1.14 X 10^8 cfu/ml. All the twelve strains of *A. hydrophila* possess haemolysin gene, 1079 bp and aerolysin gene, 1236 bp as revealed in simplex polymerase chain reaction (PCR)

Introduction

Diseases have now become a primary constraint to the culture of many aquatic species, impending both economic and social developments and a significant constraint on aquaculture production and trade (Smith, 2006). It is recognized that bacteria are one of the important causative agents of fish diseases (Yesmin et al., 2004), the most frequently encountered bacterial agents associated with fish diseases in the tropical environments are vibrios in marine and brackish water systems and motile aeromonads in freshwater environments (Otta et al., 2003).

Congo red agar binding assay, haemolytic activity using rabbit RBCs of *A. hydrophila* strains isolated from Tunisian aquatic biotopes and diarrhea patients and water samples in USA was studied by Saidi et al.,

(2011) and Khajanchi et al., (2010). The *in vivo* virulence test of *Aeromonas hydrophila* was carried out using formalin killed, heat killed and lipopolysaccharides (LPS) in different fish with varied bacterial load with several routes of challenges (Das et al., 2009; Du et al., 2011; Dehghani et al., 2012). Aerolysin and hemolysin are significant toxins secreted by *Aeromonas hydrophila*, which contributes pathogenicity of fish to humans. Virulence factors such as aerolysin, haemolysin, cytosine, enterotoxin, proteolytic activity, lipolytic activity, gelatinase, slime production and antimicrobial peptides have been identified in *A. hydrophila* (Castro-Escarpulli et al., 2003; Illanchezian et al., 2010). The aim of this experiment was to study the virulence properties of *Aeromonas hydrophila* isolated from different freshwater fish from Odisha and Andhra Pradesh.

Materials and Methods

Isolation and characterization of *Aeromonas hydrophila*

Isolation of *Aeromonas hydrophila* was done from diseased fish viz. catla (*Catla catla*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), gold fish (*Carassius auratus*), Anabas (*Anabas testidenus*) and clarias (*Clarias betrachus*), cat fish and Channa species (*Channa punctatus* & *Channa marulius*) showing gross pathological lesions as ulcerated skin, tail and fin rot, from different experimental and culture ponds of Central Institute of Freshwater Aquaculture (CIFA), commercial farms of Odisha and Andhra Pradesh. The biochemical characterization of *Aeromonas hydrophila* was done according to the methods of West and Colwell, (1984).

In vitro virulence test of *Aeromonas hydrophila* isolates

In vitro virulence test was done by presence of virulence factors of isolates and ascertained by the following tests viz, haemolytic test and Congo red binding assay.

Hemolytic test

Haemolytic test was done according to the methods of Seethalakshmi et al., (2010) with slight modifications. For haemolytic test, blood from catla, rohu, sheep, goat and rabbit were taken and it was mixed well with equal volume of sterile Alsever's solution. After properly mixing, the sample was centrifuged at 2500 rpm for 15 minutes. Supernatant was removed and collected RBC added to 100 ml sterile TSA (7% v/v) separately. The inoculums were spot inoculated onto haemolytic plates and incubated at 37^0C for 24-48 h and subsequently the type of haemolysis and zone diameter was measured. The range of the zones, 0-10 mm was taken as weakly positive (+) whereas within 11-15 mm as strongly positive.

Congo red binding assay

Congo red binding assay was done according to the methods of Illanchezian et al., (2010); and Hassan et al., (2011) with slight modifications. Congo red agar was prepared by adding 0.03 % (w/v) Congo red in sterile pre-boiled TSA and sterilized at 121^0C for 15 minutes. The inoculums were spot inoculated on Congo red plates and incubated at 37^0C for 24-72 h. Virulent strains showed deep red, raised colonies.

In vivo virulence test of *Aeromonas hydrophila* isolates

The *in vivo* virulence test of 12 *Aeromonas hydrophila* isolates was done by immersion challenge method and LD50 value was determined. Groups of rohu (*Labeo rohita*) fry (average weight 1-1.5 g, 20 nos. fish per FRP tank each containing 30 liters water with continuous aeration at room temperature) were challenged with 10-fold dilution of the isolates (10^5, 10^6, 10^7 and 10^8 cfu/ml). The different isolates of *A. hydrophila* were bath challenged against 20 fries of *L. rohita* to each dilution in triplicate set with the control and the challenge experiment against fries of *L. rohita* has the ethical committee clearance report from CIFA, Bhubaneswar. The observation was made at 24 h interval up to 96 h. The test inoculums were prepared by streaking 18-24 h old bacterial culture on TSA plate and cells were harvested using sterile tryptone soya broth after 24 h at 37^0C. Different doses of inoculums were made by dilutions and the viable cell counts of the suspensions were checked by plating on TSA and by measuring at a wavelength of 546nm. The dilutions were carried out with sterile milli Q water. Each experiment included a control group where fishes were kept without the inoculums. During the experiment the fishes were monitored twice daily and dead or moribund fishes were sampled for the presence of challenged bacterium. The fry were fed with pelleted feed containing rice bran, ground nut, oil cake and fishmeal. LD_{50} values were calculated as per the method of Reed and Muench, (1938).

Detection of aerolysin and haemolysin genes of *A. hydrophila* isolates

The aerolysin and haemolysin genes of *A. hydrophila* isolates were detected according to the method of Abdullah et al., (2003) with slight modifications. The nucleotide sequences of aerolysin and haemolysin genes were PF1 5'-TTTGGAACCCAT TTCTCGTGTGGC-3' and PR1 5'-TCGA AGTAGTCCGGGAAGGTCTTGG-3', *Hly*AF 5'-CCACGCAAATTCATCACG-3' and *Hly*AR 5'-ATCCTTGTT CACCTC GAC-3' respectively. Prior to amplification, DNA samples of *A. hydrophila* isolates were diluted to a concentration of 25 ng/µl. DNA samples were mixed with 2.5 µl 10X Buffer (Genei Pvt.Ltd Banglore, India), 1 µl of a deoxynucloside triphosphate mixture (Genei Pvt. Ltd Banglore, India), 5 pmol primer (Operon Technologies, INC., Alameda, USA), 1U Taq DNA polymerase (Genei Pvt. Ltd Banglore, India). For aerolysin and haemolysin gene detection, the final volume of 25 µl was adjusted with sterilized double distilled water. The tubes were then placed in the thermal cycler (Veriti, AB Applied Biosystems, USA) with program setting as follows: one cycle of initial denaturation step at 95^oC for 5 min, followed by 30 cycles of 20 S for 94°C (denaturing temp), 55°C (annealing temp) for 30 S and 72°C (extension temp) for 2.0 min. The cycling was concluded by an additional final extension at 72°C for 8 min and the reaction products were stored at 4°C until further analysis. The amplified products were runed in 1.2% agarose gel electerophoresis stained in EtBr solutions and visualized and photographed by gel Documentation system (Model UVI Tec., Techne, UK).

Results and Discussion

Isolation and Biochemical characterization of *A. hydrophila* strains

Total of 197 samples were collected from skin lesion, liver, kidney, intestine, gill and spleen of different diseased fish as *Channa* spp., mrigal, catfish, goldfish, rohu, catla etc. and after Gram staining, a series of

biochemical tests and sugar fermentation tests; only 12 isolates confirms as *Aeromonas hydrophila*. In our study, we found that *A. hydrophila* isolates (Ah1-Ah12) produced small, round, smooth, convex, translucent, yellow colonies on Rimler Shott's (RS) medium and microscopically *A. hydrophila* was a Gram negative short rod, motile by polar flagella with swarming movement. *A. hydrophila* isolates including reference strain (MTCC 646) were fermentative, motile by polar flagella, Vibriostatic compound (O/129, 150 µg), and novobiocin- resistant, showed positive reaction towards oxidase, catalase, produced gas and acids from glucose, utilize citrate for growth and produced acetoin, produced indole, reduced nitrate, oxidate gluconate, produced amylase enzyme and decompose starch, showed positive reaction towards lipase test, gelatinase test, caseinase test and DNase test.

In vitro virulence test

In our study of *in vitro* virulence test of Congo red binding assay and haemolytic test against blood of different animals and fishes of 12 isolates of *A. hydrophila* including MTCC 646 were shown in Table 1. All the isolates of *A. hydrophila* were virulent as they shown positive reaction to haemolytic test and Congo red binding assay and produced slime and biofilm on glass tubes stained with crystal violet and safranin; but the degree of binging to Congo red and the zone of β-haemolysis varies. Haemolytic test (β haemolysis) against bloods of sheep, rohu, catla, goat and rabbit varied from 6-15 mm respectively. The β-haemolysis zone was same in Ah1, Ah8 (6 mm), Ah2, Ah6 (9 mm), Ah10, Ah11 and Ah13 (13 mm) respectively. The zone of β-haemolysis in Ah3, Ah4, Ah5, Ah9, Ah10, Ah11 and Ah13 were maximum against bloods of sheep, goat, rabbit, rohu and catla. The *A. hydrophila* strains as Ah3, Ah4, Ah5, Ah9, Ah10, Ah11 and Ah13 were strongly bind with Congo red and hence assumed to be more virulent as compared to other strains. Similar results of *in vitro* virulence tests as Congo red binding assay and β-haemolytic test on sheep blood agar plates against *A. hydrophila* and *F. columnare* was reported by several workers (Dash et al., 2009; Saidi et al., 2011; Al Alzainy, 2011; Sahu et al., 2012).

In vivo virulence test

In the present experiment, we observed similar results as *in vivo* virulence study of *A. hydrophila* against fish showed that mortality occurs from 36 h and subsequent deaths in the 72 h. The fry of *L. rohita* challenged with *A. hydrophila* heat killed and formalin killed cells, showed reduced appetite, prostrate in the swimming the day before of their mortality, internally haemorrhagic patches were found in liver, intestine, kidney, gills and swollen-eye (exophthalmia) condition. The cumulative mortality and LD_{50} value of fry of *L. rohita* against *A. hydrophila* isolates varied from 10% to 100% and 1.72 X 10^7 to 1.14 X 10^8 cfu/ml respectively (Figure 1-3). Similar *in vivo* virulence studies with different LD_{50} values of virulent isolates of *A. hydrophila* was reported by various workers as, 10^4–10^5 cfu/ml (Mittal et al., 1980), 2.1x10^4 cfu/ml in tilapia at 5 days challenge with viable cells (Khalil and Mansour, 1997), 1.7x10^4 cfu/ml was lethal to rohu and the lethality of ECP was decreased by heating and completely inactivated by boiling at 100^0C for 10 min (Sahu et al., 2011). The LD_{50} of *P. putida* from freshwater fishes ranged from 10^5-10^7 cfu/ml (Das et al., 2009), of *F. columnare* (MS2) virulent strain was found to be 6x10^4 cfu/ml after experimental infection to *L. rohita* (Dash et al., 2009).

Detection of aerolysin and haemolysin genes of *A. hydrophila* isolates

The major virulence factors of *Aeromonas* species include protease, lipases, haemolysis, aerolysis, flagellation and biofilm production. The detection of virulence factors of *A. hydrophila* is a key component in determining potential pathogenicity because these factors act multifunctionally and multifactorially. In our study, it was noticed that all the twelve isolates of *A. hydrophila* possess haemolysin gene, 1079 bp (Figure 4) and aerolysin gene, 1236 bp (Figure 5) as revealed in simplex polymerase chain reaction (PCR). The intensity of amplified aerolysin and haemolysin gene varied among the strains of *A. hydrophila*, which may be due to the degree of virulence or the lethal toxins within the cultures, as reported earlier by Abdullah et al., (2003). Hexaplex-PCR was developed for rapid detection of six virulence factors of *Aeromonas* spp. isolated from water samples from a trout farm of Pyungchang, Kangwondo and Republic of Korea as aerolysin (*aer*), GCAT (*gcat*), serine protease (*ser*), nuclease (*nuc*), lipase (*lip*) and lateral flagella (*laf*), (Nam and Joh, 2007), aerolysin gene (primers sensitivity limit 5 pg) was detected in 85% out of twenty-five isolates of *A. hydrophila* recovered from fish and pond water (Singh et al., 2008), *aer*A (416 bp) and *hly*H (597 bp) was detected in *A. hydrophila* from screened fish samples by PCR (Yoganantha et al., 2009)..

Table.1 *In vitro* pathogenicity test of different *Aeromonas hydrophila* strains (Ah1-Ah12) and reference strain of *A. hydrophila* (MTCC 646) in Congo red binding assay and Haemolytic test

Isolates	Haemolytic test[a]					Congo red binding assay
	Sheep blood	Rohu blood	Catla blood	Goat blood	Rabbit blood	
Ah 1	+ (8)	+ (7)	+ (9)	+ (8)	+ (6)	+
Ah 2	+ (10)	+ (9)	+ (9)	+ (10)	+ (9)	+
Ah 3	++ (12)	++ (13)	++ (12)	++ (13)	++ (14)	++
Ah 4	++ (13)	++ (12)	++ (11)	++ (14)	++ (13)	++
Ah 5	++ (13)	++ (11)	++ (14)	++ (12)	++ (15)	++
Ah 6	+ (8)	+ (7)	+ (6)	+ (7)	+ (9)	+
Ah 7	+ (9)	+ (10)	+ (9)	+ (8)	+ (10)	+
Ah 8	+ (7)	+ 8)	+ (7)	+ (9)	+ (6)	+
Ah 9	++ (14)	++ (13)	++ (11)	++ (15)	++ (12)	++
Ah 10	++ (15)	++ (12)	++ (11)	++ (14)	++ (13)	++
Ah 11	++ (12)	++ (11)	++ (15)	++ (13)	++ (13)	++
Ah 12	+ (8)	+ (9)	+ (10)	+ (9)	+ (8)	+
Ah13 MTCC 646	++ (11)	++ (12)	++ (15)	++ (14)	++ (13)	++

N.B. [a] Values in the parentheses indicates zone of haemolysis in millimeter, in Congo red test ++, strongly positive, +, weakly positive.

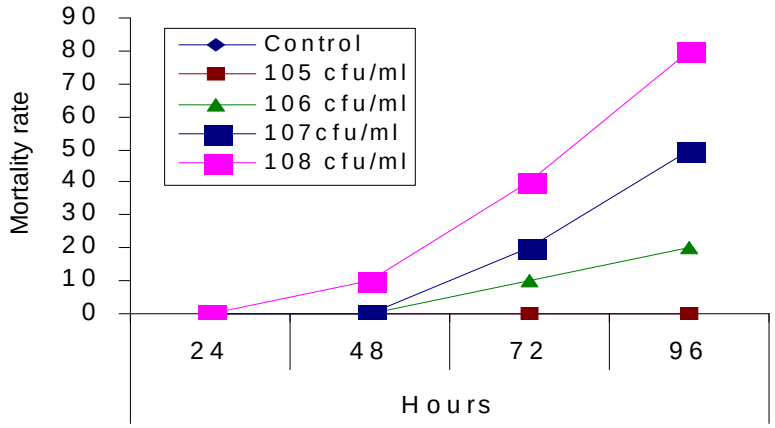

Figure.1 *In vivo* pathogenecity test of *A. hydrophila* (Ah1) on fry of *L. rohita*

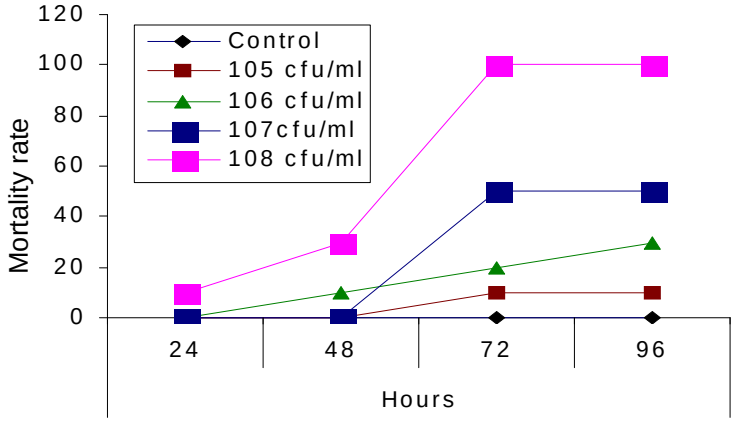

Figure.2 *In vivo* pathogenecity test of *A. hydrophila* (Ah6) on fry of *L. rohita*

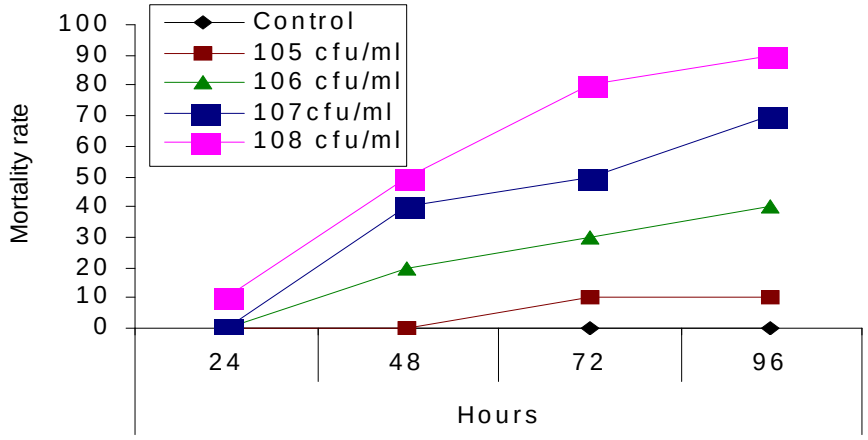

Figure.3 *In vivo* pathogenecity test of *A. hydrophila* (Ah12) on fry of *L. rohita*

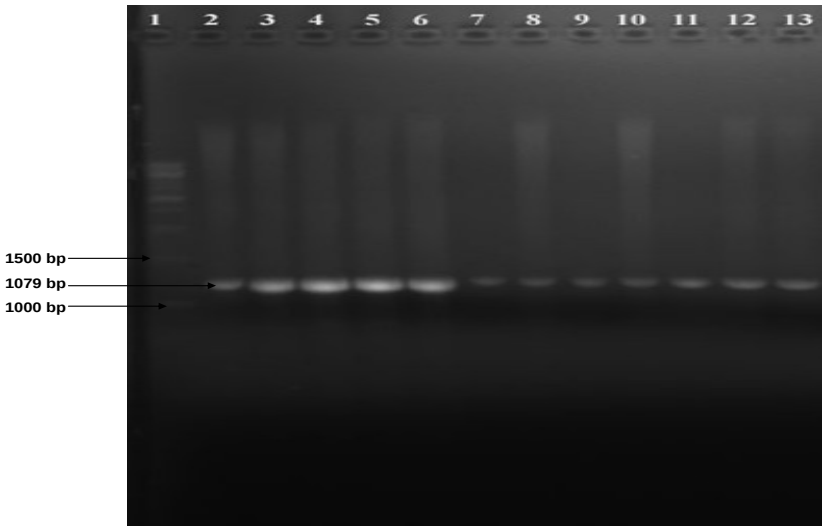

Figure.4 Haemolysin gene detection of 12 strains of *A. hydrophila*. (L-R: Lane 1, 1kb DNA Ladder, Lane 2-13, *hly*A genes of *A. hydrophila* (Ah1-Ah12) strains

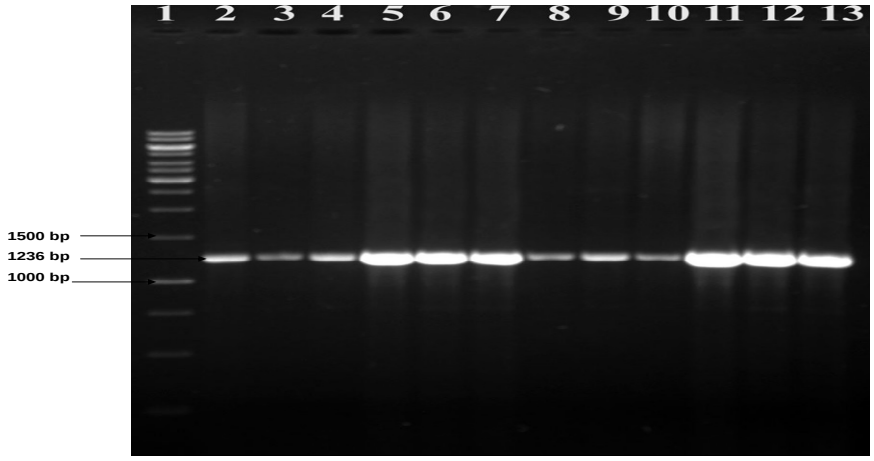

Figure.5 Aerolysin gene detection of 12 strains of *A. hydrophila*. (L-R: Lane 1, 1kb DNA Ladder, Lane 2-13, *Aer*A genes of *A. hydrophila* (Ah1-Ah12) strains.

Conclusions

The findings of the present study indicate the involvement of A. hydrophila from the disease affected fish and also confirmed the presence of haemolysin and aerolysin in the virulence mechanism. Future studies on other virulent genes of A. hydrophila can be done and this could be used for diagnosis of aeromoniasis in different outbreak and epidemiological condition.

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Conflict of Interest

Conflict of interest declared none.

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