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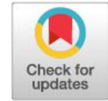

Species Diversity, Existence of Virulence Gene Profile and *in-vivo* Pathogenicity Study of *Aeromonas* spp. Isolated from Diseased *Catla catla*

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CL conducted the study and wrote the manuscript. TN planned the work, analysed the data and supervised the project. TVR, AB and OS edited the manuscript.

Key words
Aeromonas* species, Biochemical characterization, Virulence genes, *In-vivo*, Pathogenicity, *Catla catla

ABSTRACT

This study aimed to isolate *Aeromonas* species from hemorrhagic septicemia diseased *Catla catla* freshwater fish in Andhra Pradesh, India, in order to learn more about them, locate its virulence genes, and evaluate their pathogenicity. A total of nine *Aeromonas* spp., were isolated and identified by API 20E kit. Out of nine *Aeromonas* spp., *A. veronii* bv *veronii* (28%) was most prevalent species than other *Aeromonas* spp. The isolated species were tested for frequency of six virulence genes viz., aerolysin, enterotoxin, elastase, hemolysin, lipase and serine protease. We found heterogeneous distribution pattern of virulence genes among the *Aeromonas* spp. with dominance of elastase (87%). Furthermore, catla were challenged with nine *Aeromonas* spp. with various virulence gene profile by intraperitoneal injection at 10^8cfu/ml and studied for 96 h to determine co-relation between the number of virulence genes and their pathogenicity. *In-vivo* pathogenicity test revealed that the mortality did not depend on number of genes rather it depended on the type of combination of aerolysin, elastage and protease genes. The study concluded that isolated *Aeromonas* spp. harbored various virulence genes indicating their pathogenicity for fishes.

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INTRODUCTION

The Gram-negative, facultative anaerobic bacteria known as aeromonads are seriously risky. They may be found in many natural aquatic habitats (Igbinosa *et al*., 2012). Fresh water fishes especially carps are the important food sources and study models all around the world (Sanyal *et al*., 2018). Most popular amongst carps is *Catla catla* which is a native fish of regions of riverine systems (Shahzad *et al*., 2014). The genus *Aeromonas* has experienced a number of revisions to its classification, adding complexity to an already convoluted subject. *A. hydrophila, A. caviae, A. salmonicida*, and *A. sobria* are the four *Aeromonas* spp. reported in 1980s (Hu *et al*., 2012). Then, recently, the number of *Aeromonas* spp. has increased to 41 (Fernandez-Bravo and Figueras, 2020). These species are responsible for the diseases furunculosis, hemorrhagic septicemia, skin ulcers, fin/tail rot, dropsy, and skin ulcer-causing hemorrhagic septicemia (Reith *et al*., 2008). In humans, some *Aeromonas* species, including *A. caviae, A. hydrophila, A. veronii, A. trota, A. schubertii,* and *A. jandaei,* cause gastroenteritis (Sinha *et al*., 2004), septicemia, peritonitis, meningitis and eye infections (Kelly *et al*., 1993).

Aeromonad's pathogenesis is very complex because of its wide range of virulence factors that interact with one another in a multifactorial manner. Bacterial pathogenesis has been linked to a variety of proteases, including aerolysin, hemolysin, enetrotoxin, temperature-sensitive protease and serine protease (Albert *et al*., 2000; Nawaz *et al*., 2010; Hu *et al*., 2012; Li *et al*., 2020). Aeromonad isolates pathogenicity varies widely in terms of the amount, and presence of virulence genes within and between species. Perhaps there is a geographical explanation for the disparities that exist (Ghenghesh *et al*., 2014). Therefore, the continuous monitor about these species in

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fish culture is necessary to improved disease management and preventative approaches, as it understanding that *Aeromonas* are highly virulent in nature. Therefore, the present study was carried out on *Aeromonas* species identification and characterization of virulence genes with main focus on their pathogenicity.

MATERIALS AND METHODS

Fish sampling

Thirty-two infected *Catla catla* fish were collected from farms in the West Godavari and SPSR Nellore districts of Andhra Pradesh, India. All of the collected samples were brought to the lab at the College of Fishery Science in Muthukur, Nellore district.

Isolation and identification of Aeromonas *spp.*

Infected catla fish showed symptoms of bacterial hemorrhagic septicemia such as hemorrhages on body surface and internal organs, dropsy, pale gills, fin and tail rot, and discoloration of internal organs. Based on severity of infections, inoculums were collected from kidney, liver and gill tissues and were streaked on a rimler shotts (RS) medium. After 18-24 h of streaking on RS plates, the most prominent colonies were sub cultured on trypticase soy agar (TSA). Initially, the standard technique of identifying bacterial isolates relied on biochemical assays through API 20E test kit (Hi-Media, India). Taxonomic keys proposed by Abbott *et al*. (2003), Martinez-Murcia *et al*. (2008) and Beaz-Hidalgo *et al*. (2010) were followed for identification of *Aeromonas* spp.

Virulence genes detection in Aeromonas *spp.*

The DNAs of all the isolated strains were extracted by a DNA extraction kit (Bangalore Genei, Bangalore) according to the company's guidelines with minor adjustments. Briefly, each strain mixed in 110 µl of extraction solution, incubated for 20 min at 35°C then centrifuged for 10 min at 10,000 rpm. 100 µl supernatant collected into another tube having 100 µl 100% ethanol, then centrifuged for 5 min at 10,000 rpm. The DNA pellet was washed for two times at 5000 rpm for 5 min with 100 µl of 95% ethanol. Next allow to dry DNA pellet at room temperature and dissolved in 50 µl of nucleus free water. Aerolysin, cytotonic enterotoxin, elastase, lipase, hemolysin and serine protease virulence genes were amplified by PCR. The virulence genes primers and their thermal cycling conditions are given in Table I. PCR amplification of virulence genes was performed in a reaction volume of 25 µl using a thermal cycler (BioRad, T100, Germany). The reaction mixture consists: 1 µl of template DNA, 2 µl of F and R primer, 10 µl of master mix (Taq DNA polymerase, 2.0 x master mix red, $MgCl_2$ 2.0 mM, Thermo Scientific) and 12 µl of molecular grade water. The amplified product was checked on a 1.2% agarose gel.

In-vivo *pathogenicity study of* Aeromonas *spp.*

To determine the correlation between the number of virulence genes and the pathogenicity mortality rates, In-vivo test was conducted. A total of 330 catla (average weight 25 ± 2 g) were used for this study. Before acclimatization, fishes were treated with 2 ppm potassium permanganate for 10 min (Barkoh *et al*., 2010) and maintained for 15 days. During that period fishes were fed with 35% protein commercial pellet (CP, Chennai) @ 3% of body weight.

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Table I. Primers used in PCR for virulence genes expression of *Aeromonas*.

Virulence gene	Primer sequence (5'-3')	Product size (bp)	Denaturation	Annealing	Extension	Reference
Aerolysin	CCTATGGCCTGAGCGAGAAG CCAGTTCCAGTCCCACCACT	431	94°C, 30 s	55.5°C, 30 s	72°C, 30s	Mansour *et al*. (2019)
Enterotoxin	TGACCCAGTCCTGGCACGGC GGTGATCGATCACCACCAGC	442	94°C, 30 s	63°C, 50 s	72°C, 30s	Nawaz *et al*. (2010)
Haemolysin	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGGG	597	94°C, 30 s	62°C, 30 s	72°C, 2 min	Sreedharan *et al*. (2012)
Lipase	GACTCCCTCAAGGACAGCAG AGAGGCTTTCAGGGCATTG	594	94°C, 45 s	58°C, 30 s	72°C, 30s	U-taynapun *et al*. (2020)
Elastase	ACACGGTCAAGGAGATCAAC CGCTGGTGTTGGCCAGCAGG	540	94°C, 30 s	60.6°C, 30s	72°C, 30s	Mansour *et al*. (2019)
Serine protease	ATTGGATCCCTGCCTATCGCTTCAGTTCA GCTAAGCTTGCATCCGTGCCGTATTCC	911	94°C, 30 s	55°C, 30s	72°C, 30s	Zheng *et al*. (2012)

For bacterial cell suspension preparation, we used the protocol described by Sasmal *et al*. (2014). Initially, a pure culture of *Aeromonas* spp. was obtained from tyrpticase soya agar (TSA) slants, then streaked onto the TSA plate. After 24 h of culture at 32 ± 1°C the young colonies were transferred into 10 ml of trypticase soya broth (TSB). The bacterial cell suspensions were then inoculated into a 250 ml conical flask containing 90 ml of TSB for mass culture and incubated on a shaker at 32°±1°C for 24 h. The cultures were centrifuged at 7500 rpm for 20 min. After three washes with saline, the resulting cell pellet was resuspended in 10 ml of the solution. We used the spread plate method (Collins *et al*., 1989) to determine the number of bacterial cells per ml of neat suspension on TSA after 24 h of incubation at 32 °C.

For challenge test, healthy fish were divided into three categories *viz.,* first category for *Aeromonas* spp. injection, second category for sterile normal saline injection as sham control and third category as control with no injection and maintained as triplicates. Prior to injection, fish were anaesthetized by tricaine methanesulfonate (MS222, Sigma, 150 mg/L) (Das *et al*., 2019). Each catla fish in the first group was injected I/P with 0.2 ml of a bacterial cell solution of *Aeromonas* spp. containing 10^8 cfu/ml. A 0.2 ml I/P injection of sterile normal saline was administered to the second group of fish. All of the fishes in the experimental category were monitored for a full 96 h after the challenge to analyze any changes in behavior, any clinical symptoms, and any deaths that happened. The mortality rate (total number of dead fishes in a time period/ total number of fishes stocked x 100) was calculated based on Sung *et al*. (2000). To confirm Koch's postulates, the moribund/freshly dead fishes with obvious clinical indications were randomly chosen for re-isolation of challenged *Aeromonas* spp.

Statistical analysis

R studio software (3.6.1 version) was used for analysing pathogenicity studies difference by multivariant ANOVA at 5% level of significance.

RESULTS AND DISCUSSION

The motile Aeromonads are the opportunistic Gram negative bacteria responsible to cause various diseases in fish like septicaemia, fin rot, haemorrhages, and dropsy, commonly called motile aeromonas septicaemia (Lewbart, 2001) and in humans, they cause gastrointestinal diseases, peritonitis, meningitis, and eye infections (Reith *et al.*, 2008). The infected samples of the present study also recorded various motile aeromonas septicaemia disease symptoms such as pinpoint haemorrhages on the kidney, on body and fin bases, fluid discharge from a reddened vent, excess fluid in the gut and visceral cavity, pale gills, fin and tail rot, and discoloration of internal organs like the kidney, liver, and spleen.

Table II. Prevalence and distribution of *Aeromonas* spp. between two sampling locations.

S. No	*Aeromonas* species	Prevalence (N=56)	Location wise prevalence	
			W. Go-davari (N=37)	SPSR Nellore (N=19)
1	*A. veroniibv.veronii*	28% (n=16)	n = 9	n =7
2	*A. veroniibvsobria*	17% (n=10)	n = 6	n = 4
3	*A. trota*	10% (n=6)	n = 3	n = 3
4	*A. tecta*	10% (n=6)	n = 4	n = 2
5	*A. schubertii*	7% (n=4)	n = 2	n = 2
6	*A. popoffii*	7% (n=4)	n = 3	n = 1
7	*A. media*	7% (n=4)	n = 4	0
8	*A. aquariorum*	7% (n=4)	n = 4	0
9	*A. allosacharophila*	3% (n=2)	n = 2	0

Characterization of Aeromonas *spp.*

The biochemical characterization of isolated *Aeromonas* spp. are given in Supplementary Table I. Based on the severity of disease condition, inoculums were taken from kidney, liver and gills. A total of 56 of nine *Aeromonas* species were identified from of 32 diseased catla. Table II shows the prevalence and distribution of *Aeromonas* spp. among the two districts. Many researchers isolated *Aeromonas* species from fishes, including *A. hydrophila, A. veronii, A. sobria, A. caviae, A. salmonicida, A. allosaccharophila, A. media* and *A. jandaei* (Nawaz *et al*., 2010; Hu *et al*., 2012; Sanayal *et al*., 2018). Among them, *A. veronii* is a significant cause of infections in fish (Silver *et al*., 2011; Nawaz *et al*., 2010; Li *et al*., 2020). Our study reports also suggested that *A. veronii* bv. *veronii* and *A. veronii* bv. *sobria* are the major pathogenic bacteria causing disease in freshwater fishes. In the present study, we found that *A. veronii* bv. *veronii* (28%, 16/56) was more prevalent spp. when compared to others species. This is completely in accordance with several studies (Hu *et al*., 2012; Li *et al*., 2020; U-taynapun *et al*., 2020; Sayuti *et al*., 2021). Moreover, we have seen variation in species distribution among the two sampling locations. From W. Godavari district, 37 isolates of nine *Aeromonas* spp. recovered, while 19 isolates of six *Aeromonas* spp. found in SPSR Nellore district. Which might be influenced by type of water intake, because in W. Godavari district

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Table III. Mortality rate (Mean±SD) of catla fish challenged with *Aeromonas* species.

Aeromonas species	No. of virulence genes	Virulence gene profile	Bacterial injection (cfu/ml) (10^{-1} dilution)	Mortality (mean number of fish died)					Mortality (%)
				0-12 h	12- 24 h	24 - 48 h	48 - 72 h	72 - 96 h	
A. schubertii	3	*Aer, Ela, AhyB*	2.0 x10^{8}	-	$2.00^{a}\pm0.57$	$3.00^{a}\pm0.57$	$2.33^{b}\pm0.57$	$2.67^{b}\pm1.00$	$100.0^{a}\pm0.00$
A. tecta	4	*Aer, Ela, AhyB, Lip*	2.1 x10^{8}	-	$2.33^{a}\pm0.57$	$2.67^{a}\pm0.57$	$2.33^{b}\pm0.57$	$2.67^{b}\pm0.57$	$100.0^{a}\pm0.00$
A. aquariorum	4	*Aer, Ela, AhyB, Lip*	2.3 x10^{8}	-	$3.00^{a}\pm0.57$	$3.33^{b}\pm0.57$	$2.67^{b}\pm0.57$	$1.00^{a}\pm1.15$	$100.0^{a}\pm0.00$
A. allosaccharophila	5	*Aer, Ela, AhyB, Lip, Alt*	1.4 x10^{8}	-	$2.67^{a}\pm0.57$	$3.67^{b}\pm0.57$	$2.67^{b}\pm0.57$	$1.00^{a}\pm0.00$	$100.0^{a}\pm0.00$
A. media	5	*Aer, Ela, AhyB, Lip, Hly*	1.2 x10^{8}	-	$2.33^{a}\pm0.57$	$3.33^{b}\pm0.57$	$2.67^{b}\pm0.00$	$1.67^{a}\pm0.57$	$100.0^{a}\pm0.00$
A. trota	6	*Aer, Ela, AhyB, Lip, Alt, Hly*	1.4 x10^{8}	-	$5.00^{b}\pm0.57$	$2.67^{a}\pm0.57$	$1.33^{a}\pm0.57$	$1.00^{a}\pm1.00$	$100.0^{a}\pm0.00$
A. popoffii	6	*Aer, Ela, AhyB, Lip, Alt, Hly*	2.1 x10^{8}	-	$5.33^{b}\pm0.57$	$3.67^{b}\pm0.57$	$1.00^{a}\pm1.00$	-	$100.0^{a}\pm0.00$
A. veronii bv. Sobria	6	*Aer, Ela, AhyB, Lip, Alt, Hly*	1.2 x10^{8}	-	$5.33^{b}\pm0.57$	$3.33^{b}\pm0.57$	$1.33^{a}\pm0.57$	-	$100.0^{a}\pm0.00$
A. veronii bv. veronii	6	*Aer, Ela, AhyB, Lip, Alt, Hly*	2.4 x10^{8}	-	$5.67^{b}\pm1.00$	$3.33^{b}\pm0.57$	$1.00^{a}\pm1.00$	-	$100.0^{a}\pm0.00$
Sham control group	-	-	-	-	-	-	-	-	00.00
Control group	-	-	-	-	-	-	-	-	00.00

Aer, Aerolysin; Ela, Elastase; AhyB, Serine protease; Lip, Lipase; Alt, Enterotoxin; Hly, Hemolysin.
*Figures having different super scripts are significantly different

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culture is mainly using creek water as intake where as ground water is a major intake source for Nellore district. Our findings are supported by Altwegg *et al.* (1989) who suggested that the prevalence of *Aeromonas* is likely to vary with geographical locations.

Determination of virulence genes in Aeromonas *spp.*

There is a heterogeneous distribution of virulence genes pattern was observed among the nine *Aeromonas* species. It believed that virulence factors contributing to the severity of many diseases through the release of a wide range of toxins Sen and Rodgers (2004). However, all the *Aeromonas* species cannot produce all the toxins (Chopra and Houston, 1999). The *Aeromonas* pathogenesis process was rather complicated, since no one suspected virulence-associated factor could be definitively linked to any given set of symptoms or disease (Albert *et al*., 2000). Major virulence factors in *Aeromonas* were aerolysin, cytotonic enterotoxin and serine protease (Chopra *et al*., 1996; Sha *et al*., 2002; Nawaz *et al*., 2010; Tomas, 2012). The present study findings showed that, 87% of the isolates possessed at three or more virulence gene. Of the six virulence genes, elastase gene was found to be dominant with prevalence of 87% (49/56) followed by serine protease 73% (41/56), aerolysin 62% (35/56), cytotonic enterotoxin 48% (27/56), lipase 46% (26/56) and haemolysin 39% (22/56).

Similar to our findings, Shuang *et al.* (2020) also found elastase in 100% of *Aeromonas*. Elastase is a zink metalloprotease enzyme that involved in pathogenesis (Tomas, 2012). Further, Serine proteases participate in β-hemolysin precursor activation, which includes the stimulation of aerolysin and other cellular enzymes, which may have an impact on the total virulence of *Aeromonas* (Nawaz *et al*., 2010). In the present study, we found serine protease in 73% of the isolates and aerolysin in 62% of the isolates. The aerolysin gene participates in the secretion of adhesins, hemagglutinins, and several hydrolytic enzymes, all of which are important in pathogenesis (Sreedharan *et al*., 2012). More or less similar frequencies of virulence genes are found by (El-Gohary *et al*., 2020; Li *et al*., 2020; Nawaz *et al*., 2010). The present study isolates had enterotoxin in 48%, which is very similar to (Gashgari and Selim, 2015) who fond 42% in *A. veronii* isolated from sea bream. These enterotoxins are implicated in tissue destruction and the release of fluid in infected fish intestines (Sha *et al*., 2002). Further, lipase gene recorded in 46% of present study isolates, similarly (U-taynapun *et al*., 2020) found 46% of lipase in *Aeromonas* spp. Lipase enzymes involving in the modification of the animal cell membrane, increasing the seriousness of the disease (Tomas, 2012). In our isolates haemolysin gene was found in 39% of the strains, which are involving in lysis of the erythrocytes (Wang *et al*., 2008).

In-vivo *pathogenicity of* Aeromonas *spp. to* Catla catla

The mortality pattern of catla challenged with nine

Aeromonas spp. is shown in Table III. *In-vivo* pathogenicity studies showed that the bacteria were able to induce severe infections in catla fishes. We found 100% mortality in challenged fishes with *Aeromonas* spp. possessed three (elastase, serine protease and aerolysin) to six virulence genes (elastage, serine protease, aerolysin, enterotoxin, lipase and haemolysin). Indicating that, even presence of only three virulence genes such as elastase, serine protease and aerolysin could cause 100% mortality. The *In-vivo* pathogenicity results were supported by the prevalence of virulence genes in isolated *Aeromonas* spp. of the present study. The results completely agreed with those of Nawaz *et al*. (2010); Hu *et al*. (2012) and Li *et al*. (2011, 2020), who suggested that aerolysin, enterotoxin elastase, and protease in *Aeromonas* pose a high threat to the animals. Moreover, we have observed prominent pathological signs after 24 h of post injection, such as hemorrhages on various body parts, tail rot and internal fluid accumulation, which might be due to presence of aerolysin, enetrotoxins and proteases. In addition, mortality was not found in sham control and control groups. Our findings suggested that a bacterial strain's pathogenicity was actually associated with the type of virulence genes possessed rather than its number.

CONCLUSION

Catla fish farms often have problems with *Aeromonas* spp., especially by *A. veronii* bv *veronii* and *A. veronii* bv *sobria.* In addition, the present study isolates found with elastase, enterotoxin, protease and aerolysin genes, indicating the pathogenic potential of our isolates. The *in-vivo* pathogenicity test also confirmed the virulence of *Aeromonas* spp. to catla. It was suggesting that pathogenicity was related to the type of virulence genes possessed by *Aeromonas* spp. but not on number of genes. Hence, our findings suggesting that, *Aeromonas* spp. pose more serious threat to the freshwater fishes.

DECLARATIONS

Funding

The study was funded by College of Fishery Science, Muthukur.

Ethical approval

The catla fishes were used in this study handled with very care as per Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) and study was approved by Sri Venkateswara Veterinary University committee, 2019.

Statement of conflict of interest

The authors have declared no conflict of interest.

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