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Molecular characterization, virulence gene profiling and multidrug resistance profiles of *Aeromonas hydrophila* isolated from raw fish samples collected in and around Tirupati, Andhra Pradesh, India

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Abstract
Aeromonas hydrophila is a food borne pathogen of emerging importance. In the present investigation, a total of 200 fish samples were procured from different fish markets located in and around Tirupati, Andhra Pradesh. Out of 200 samples collected, 87 (43.5%) isolates were positive for *Aeromonas* spp. based on colony characteristics. On further confirmation with biochemical tests, it was found that 71 presumptive isolates were identified as *Aeromonas* spp., among which only four isolates (5.7%) were confirmed as *Aeromonas hydrophila.* Among these four presumptive isolates, only three were confirmed as *Aeromonas hydrophila* by PCR targeting the genus specific and species specific genes *viz.,* 16S rRNA and *gyrB* gene respectively. On genotypic characterization of virulence genes, it was found that none of the isolate harboured *aer* virulence gene whereas *act* virulence gene is present in all the three *Aeromonas hydrophila* isolates. In antimicrobial resistance studies, all the three *Aeromonas hydrophila* isolates have shown different antimicrobial patterns against ampicillin–sulbactam, cefepime, ceftriaxone, gentamicin, tetracycline ciprofloxacin, co-trimoxazole and chloramphenicol by using standard disc diffusion method. The present study indicates the presence of multidrug resistance *A. hydrophila* isolates in fish samples which is a public health hazard and the need for continuous monitoring and surveillance of this pathogen.

Keywords: *Aeromonas hydrophila*, antimicrobial resistance, PCR, virulence genes

Introduction
Fish and its products have great importance in human nutrition worldwide that provides many health benefits and can be source of various foodborne pathogens. Fish and fish products-related zoonotic infections may be either topically acquired infections due to contact with aquatic animals or foodborne infections due to ingestion of raw or undercooked aquatic products such as fish (Sorsa *et al*., 2019) [1]. According to WHO (2015) [2], unsafe food resulted in 600 million cases of food borne diseases and 4, 20,000 deaths worldwide. Among which 30% deaths were reported in children below 5 years of age.

Aeromonas spp. is considered as emerging foodborne pathogen because of its habitat in aquatic environment such as fresh water, coastal water as well as in sewage, virulence factors *viz.,* enterotoxins, hemolysins, cytolytic proteins and its psychotropic nature (Yucel and Balci, 2010) [3]. Till to date, 14 species of the *Aeromonas* have been isolated and included in Bergey's Manual of Bacteriology among which *Aeromonas hydrophila*, *Aeromonas sobria*, and *Aeromonas caviae* have been incriminated as the main causes of *Aeromonas* associated human diseases (Abd-El Malek, 2017) [4]. *Aeromonas* species are responsible for intestinal as well as extra intestinal infections such as septicemia, cellulitis, wound infections, urinary tract infections, hepatobiliary tract infections, soft tissue infections and occasionally meningitis and peritonitis in humans (Khajanchi *et al.,* 2010) [5].

The incidence of *Aeromonas* infections in developing countries is more generally attributed to routine endemic exposure to contaminated water and foodborne pathogens than was originally considered (Igbinosa *et al.,* 2012) [6]. Therefore, the present study was undertaken to characterize *Aeromonas hydrophila* isolated from fish samples intended for human consumption at both phenotypic and molecular level, virulence gene profiling as well as to document its antimicrobial resistance patterns.

Material and Methods

Sampling details

A total of 200 fish samples of different varieties *viz.*, Catla, Rohu and Murrel were collected from retail fish markets located in and around Tirupati, Andhra Pradesh, India over a period of 3 months from September 2020 to November 2020 and the sampling details were represented in Figure 1 and 2. All the samples were collected aseptically in sterile zip lock covers with the protocol given by International Commission on Microbiological Specifications for Foods (ICMSF, 1978) [7].

Isolation and identification of *Aeromonas hydrophila* by the cultural method

Briefly, 25 gm of each sample was collected, homogenized and transferred into 225 ml of Alkaline Peptone Water (APW) and incubated at 37 ^{0}C for 18 to 24 hrs. A loopful of inoculum was streaked on selective agar *viz.*, Ampicillin Dextrin Agar (ADA) and Starch Ampicillin Agar (SAA) plates followed by incubation at 37 ^{0}C for 18 to 24 hrs. The yellow to honey-coloured colonies on SAA agar and round light yellow colonies on ADA agar were considered as presumptive colonies of *Aeromonas* species which were further confirmed as *Aeromonas hydrophila* by grams staining and biochemical tests *viz*., Oxidase test, Catalase test, Indole test, Vogues – Proskeur's test, Mannitol motility test, Lysine decarboxylase test, Esculin hydrolysis, Sugar utilization tests, Arginine dihydrolase test, Urease test, Triple sugar iron test (Table 1). The isolates were further preserved in glycerol stocks at -20 ºC until future use.

Extraction of template DNA from bacterial cultures

The template DNA was extracted by boiling and snap chilling method as per the procedure given in Didugu *et al.* 2016[8]. A 1.5 ml of overnight grown bacterial culture was taken in micro centrifuge tubes and centrifuged at 10,000 rpm for 5 min at 4 ^{0}C. The supernatant was discarded and the pellet was resuspended with 1ml of sterile distilled water and was kept in a boiling water bath at 99 ^{0}C to allow the cell lysis and release of DNA. Then, the tube was immediately transferred to ice (-20 ^{0}C) for 10 minutes. After snap chilling, the cell lysate was centrifuged and the clear supernatant was transferred to a new tube and used as a DNA template for subsequent PCR amplification.

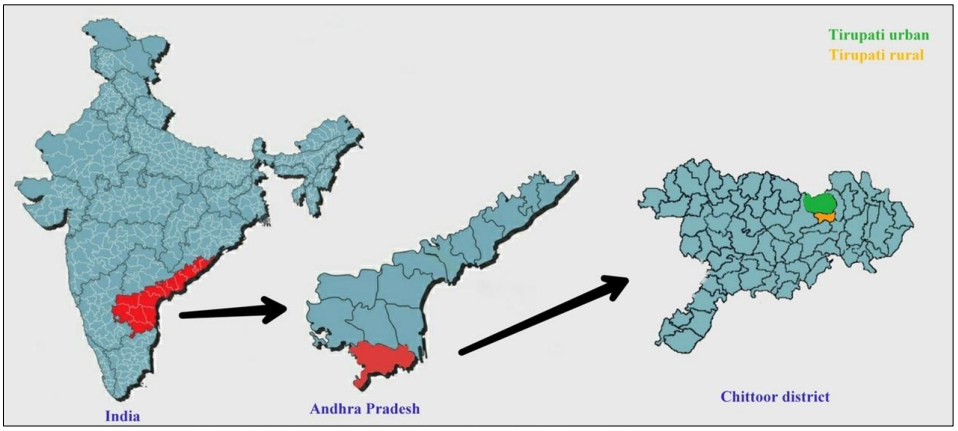

Fig 1: Samples collection area – Tirupati rural and Tirupati urban

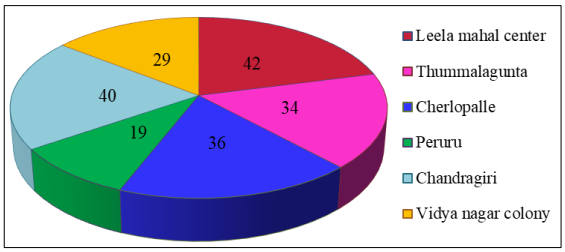

Fig 2: Source and number of raw fish samples collected

Table 1: Biochemical characteristics to differentiate *Aeromonas hydrophila* from other *Aeromonas* spp

S. No	Biochemical test	*A. hydrophila*
1	Oxidase	+
2	Catalase	+
3	Esculin hydrolysis	+
4	Indole production	+
5	Voges – Proskauer test	+
6	Arginine dihydrolase test	+
7	Lysine decarboxylase test	+
8	Gas from glucose	+
9	Sucrose fermentation	+
10	Arabinose fermentation	V
11	Mannitol motility test	+
12	Urease test	-

Molecular confirmation of *Aeromonas* spp. and *Aeromonas hydrophila* isolates

The presumptive isolates were confirmed as *Aeromonas* spp. and *Aeromonas hydrophila* by PCR targeting 16S rRNA and *gyr* B genes which yields amplicon products at 599 bp and 144 bp respectively. The primers used in this study were listed in Table 2. The amplification conditions used for standardization of 16S rRNA gene were -initial denaturation at 94 °C for 5 min followed by 30cycles of denaturation at 94 ^{0}C for 1 min, annealing at 55 ^{0}C for 1 min and extension at 72 ^{0}C for 1 min and a final extension for 5 min at 72 ^{0}C; for standardization of *gyr*B gene - 2-min hot start at 95 °C, 6 cycles of 94 °C for 40 sec, 67 °C for 50 sec, and 72 °C for 40 sec, and 30 cycles of 94 °C for 40 sec, 65 °C for 50 sec, and 72 °C for 40 sec were used.

Virulence gene profiling

Phenotypic characterization by Hemolysis assay

The hemolytic activity of the isolates was determined on 5% Sheep blood agar. *Aeromonas* isolates were streaked onto blood agar plates and the hemolytic activity was determined by observing zone of haemolysis around the colonies after incubation at 37 ^{0}C for 24 hrs. Haemolytic positive isolates were identified by presence of clear (β-haemolysis) or diffuse (α-haemolysis) around the colonies (Reshma *et al.*, 2015) [9].

Table 2: Primers targeting various genes for Genus specific, Species specific and Virulence genes of *Aeromonas hydrophila*

Gene	Primer Sequence 5'-3'	Amplicon size (bp)	Reference
16S rRNA	F: TCATGGCTCAGATTGAACGCT	599	Didugu *et al.* (2015)
	R: CGGGGCTTTCACATCTAACTTATC		
gyrB	F: AGTCTGCCGCCAGTGGC	144	Persson *et al.* (2015)
	R: CRCCCATCGCCTGTTCG		
Aer	F: GCAGAACCCATCTATCCAG	252	Porteen *et al.* (2006)
	R: TTTCTCCGGTAACAGGATTG		
Act	F: AGAAGGTGACCACCACCAAGAACA	232	Nawaz *et al.* (2010)
	R: AACTGACATCGGCCTTGAACTC		

Genotypic characterization of virulence genes

The isolates were screened for virulent genes *viz*., aerolysin (*aer*) and cytotoxic enterotoxin (*act*) by PCR as per the methods described by Porteen *et al.* (2006) [10] and Nawaz *et al.* (2010) [11] respectively with slight modifications. The primers used and the cycling conditions employed to standardize were given in Tables 2 and 3.

Antibiogram

All the *Aeromonas hydrophila* isolates were examined for their antibiotic susceptibility and resistance patterns against ampicillin-sulbactam, cefepime, ceftriaxone, gentamicin, tetracycline, ciprofloxacin, co-trimoxazole and chloramphenicol by using standard disc diffusion method on Mueller – Hinton Agar (MHA) (Bauer *et al.*, 1966) [12]. The resistance or sensitivity patterns of a particular antibiotic were determined by measuring the diameter of the zone of growth of inhibition. The result was interpreted as sensitive, intermediate or resistant based on Clinical and Laboratory Standards Institute interpretative standards (CLSI M45-A2).

Table 3: Cycling conditions for identification of Virulence genes of *Aeromonas hydrophila*

Virulence genes	Cycling conditions				
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
act	94 °C for 5 min	94 ^{0}C for 1 min	55 ^{0}C for 1 min	72 ^{0}C for 1 min	72 ^{0}C at 5 min
		Repeat for 30 cycles			
aer	94 °C for 2 min	94 ^{0}C for 30 sec	55 ^{0}C for 50 sec	72 ^{0}C for 1 min	72 ^{0}C at 10 min
		Repeat for 35 cycles			

Multiple Antibiotic Resistance (MAR index)

MAR index was calculated for all *Aeromonas hydrophila* isolates as per the procedure given by Krumperman (1983) [13] by the formula a/b, where 'a' represents the number of antibiotics to which the isolate is resistant and 'b' the number of antibiotics to which the isolate was tested.

Results

Isolation and identification of *Aeromonas hydrophila*

Out of 200 fish samples screened, 87 (43.5%) isolates were suspected as *Aeromonas* spp. as they produced yellow to honey-colored colonies on SAA agar and round light yellow colonies on ADA agar (Figure 3 and Figure 4). On further confirmation with biochemical tests, it was found that 71 presumptive isolates were identified as *Aeromonas* spp., among which only four isolates (5.7%) were confirmed as *Aeromonas hydrophila.*

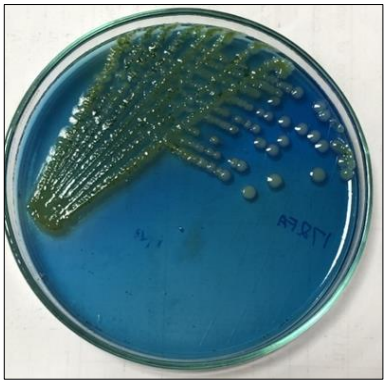

Fig 3: Plate showing growth of light yellow colonies of *Aeromonas* on ampicillin-dextrin agar

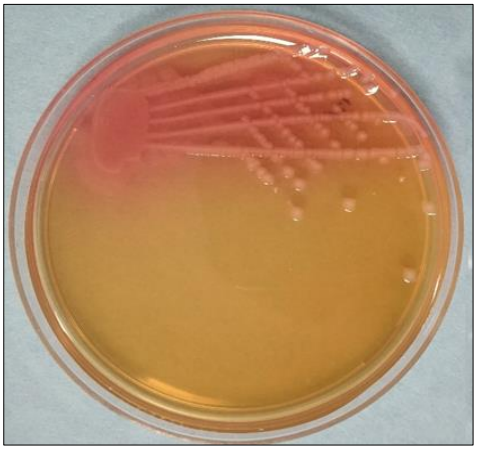

Fig 4: Plate showing growth of yellow to honey coloured colonies of *Aeromonas* on starch ampicillin agar

These culturally confirmed isolates were subjected to PCR for genus specific and species-specific confirmation of *Aeromonas* isolates. It was found that only three isolates (4.47%) among four presumptive isolates were confirmed as *Aeromonas hydrophila* by PCR targeting 16S rRNA gene for genus specific and *gyrB* gene for species specific respectively.

Virulence gene profiling

By phenotypic characterization, all the three *Aeromonas hydrophila* isolates produced β-haemolysis on Sheep blood agar plate showing complete haemolysis surrounding the colonies. On genotypic characterization, it was found that none of the isolate harboured *aer* virulence gene and all the three *Aeromonas hydrophila* isolates shown the presence of *act* virulence gene.

Antibiogram

All the three *Aeromonas hydrophila* isolates have shown different antimicrobial patterns in antimicrobial resistance studies such as sensitive / resistant patterns of the isolates for commercially available antibiotic discs on Muller Hinton agar. Maximum resistance was shown against Ampicillin-Sulbactam (100%) 50% of resistance against Co-trimoxazole, and Ciprofloxacin, 33.3% of resistance against Tetracycline (33.3%), Chloramphenicol, Ceftriaxone and Cefepime. All the three isolates have shown 100% sensitivity against Gentamicin. The MAR index calculated for all the three *Aeromonas hydrophila* isolates in the presents study ranges from 0.3 to 0.5.

Discussion

Isolation and identification of *Aeromonas hydrophila*

In the present study, 71 (35.5%) isolates were confirmed as *Aeromonas* spp. by biochemical characterization which is similar to the findings of Khan *et al*. (2019) [14], who also reported 35% of incidence of Aeromonas spp. in fish samples collected from retail markets of Chhattisgarh state, India.

On contrary to the present study (5.7%), Radu *et al.* (2003) [15], from Kerala reported a slight higher incidence (11.5%) of *A.hydrophila* isolates from fresh water ornamental fish samples. While El- ghareeb *et al.* (2019) [16] from Egypt (54%) and Jeyasanta *et al.* (2018) [17], from India (34.32%) reported higher incidence of *Aeromonas hydrophila* than the present study.

In the present study, only three isolates (4.47%) among 67 *Aeromonas* isolates were confirmed as *A. hydrophila* by PCR targeting *gyrB* gene. These results are in agreement with Nagar *et al.* (2011) [18], from India and Kahraman *et al.* (2017) [19], from Turkey who have reported a 3.85% incidence of *A. hydrophila* from various retail food products and 3.75% from seafood respectively. In contrast, Yogananth *et al.* (2009) [20], and Abd-El-Malek (2017) [4], have reported a higher prevalence of *A. hydrophila* i.e. 40% and 62.9% in fish samples, respectively.

The presence of *Aeromonas* spp. in fish samples may be due to onsite water contamination or secondary contamination during handling, storage and transportation (Nagar *et al.*, 2011) [18]. Sea food is normally left open with little or no ice and fly infestation is common in most of the fish markets which is a reason for cross contamination (Jeyasanta *et al.*, 2018) [17]. The variation in the incidence of *Aeromonas* spp. can be attributed to seasonal variations, geographical differences, regional differences and different isolation methodologies.

Virulence gene profiling

In the present study, all the three *Aeromonas hydrophila* isolates (100%) have shown β- haemolytic activity on sheep blood agar. None of the three *Aeromonas hydrophila* isolates in this study harboured aerolysin (*aer*) which is similar to the findings reported from different parts of India - Bhowmick *et al.* (2009) [21] in surface water samples collected from Kolkata, Kore *et al.* (2014) [22] in chicken and fish washings procured from local markets of Shirwal (Maharashtra), Didugu *et al.* (2015) [23] in bottled water samples collected in and around Greater Hyderabad Municipal Corporation, Hyderabad. In contrast, El-ghareeb *et al.* (2019) [16], detected aerolysin (*aerA*) gene in all (100%) *A. hydrophila* isolates collected from different fish markets distributed in Mansoura city, Egypt.

Similar to the present study, Rather *et al.* (2014) [24], from India also reported 100% incidence *act* gene in *A. hydrophila* isolates recovered from raw meats of all kinds. On contrary, Roges *et al.* (2020) [25], from Brazil has reported a low incidence (44%) of *act* gene in *A. hydrophila* isolates isolated from different animal, food and human sources.

Antimicrobial susceptibility

The results of antimicrobial studies in the present research were in accordance with the findings of Dias *et al.* (2012) [26], and Nagar *et al.* (2011) [18], who also reported variable resistance among A. *hydrophila* isolates against to commonly used antibiotics. From the previous studies, the isolates with MAR index values higher than 0.2 (Joseph *et al*., 2013) [27] indicates that might have originated from high risk sources and have a high risk potential.

The variations in the sensitivity and resistance patterns of the antibiotics in the present study may be attributed to the source of *Aeromonas* isolates and type of antimicrobial agent prescribed in different geographical areas for *Aeromonas* infection. The wide usage of antibiotics in the fish-breeding farms for prevention or treatment of diseased fish as well as their usage as food additives applied through the feed or their solution in water directly implied to increased antibiotic resistance in *Aeromonas* spp.

Conclusion

In conclusion, the occurrence of *Aeromonas* could pose risk to human health through the consumption of raw or undercooked fish. In the field of food microbiology, it seems important to pay more attention to aeromonads as they can develop toxins and survive at low temperatures. Thus, it is recommended to take necessary precautions while handling, transportation, and storage of seafood products to ensure food safety.

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