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Studies of isolation, identification and differentiation of *vibrio* species from *Catla catla* fish by cultural and multiplex PCR methods

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Abstract
The present study was undertaken to standardize Multiplex PCR assay for detection and differentiation of *Vibrio cholera, Vibrio parahaemolyticus* and *Vibrio vulnificus* from freshwater fish *catla catla* and to compare with conventional cultural methods. The samples were collected from local markets of Hyderabad from January to May 2017. The specificity for *Vibrios* was tested using primers from *gyrB* gene which gave amplification product of 493 bp only for *Vibrio* organisms whereas non-*Vibrio* organisms did not yield any products. Primers for *pntA* gene was used for differentiation of *Vibrio* species which gave a specific amplification products of 338, 409 and 656 bp products for *Vibrio cholera*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* respectively. Out of 150 markets raw samples 80 samples were positive for *Vibrio* spp by m-PCR, out of which 47, 21 and 12 were positive for *V. cholerae, V. parahaemolyticus and V. vulnificus* respectively, whereas cultural methods gave 72 positive for *vibrio* species. The mean counts of *V. cholerae, V. parahaemolyticus and V. vulnificus* were 7.1 $x10^5$ cfu, $1.07x10^3$ cfu and $5.8x10^2$ cfu in catla fish.

Keywords: *Catla catla*, *Vibrio*, multiplex PCR, Hyderabad

1. Introduction
Vibriosis is a major economic problem for the seafood industry especially in developing countries and it is responsible for food borne illness in many countries leading to hospitalization and sometimes fatal also [14]. This food borne illness caused by *Vibrios which* belong to the family *Vibrionaciae* and the members of genus *organism* are non-enteric, fermentative, oxidase positive, motile, gram-negative rods that are widely distributed in sea, brackish water as well as freshwater environments [8]. The number of *Vibrio* spp. classified as pathogenic strains is at least 11 including *V. cholerae* as the main cause of diarrhea, *V. parahaemolyticus* as the cause of foodborne gastroenteritis [21] and *V. vulnificus* which is known to cause 95% of all deaths associated with seafood consumption [23].
Vibrio species have also been reported to cause gastrointestinal diseases, skin infections and acute septicemia in humans through ingestion of contaminated seafood or exposure to aquatic environments [20]. Occurrence of *Vibrio* in fish and shellfish has been reported in different countries including Iran [15]. Every year millions of cholera episodes occur throughout the world especially in developing countries and thousands of cases are reported to be fatal [13]. During 2007, a cholera outbreak hit many parts of Orissa in the wake of massive flooding following South Asia's worst monsoon season in living memory, wherein thousands of tribal people affected and hundreds of deaths occurred [17].
V. parahaemolyticus is the leading cause of gastroenteritis or traveller's diarrhea due to the consumption of sea food worldwide, particularly the strains those producing a thermostable direct haemolysin (TDH) and/or a TD Hrelated haemolysin (TRH) [19].
Vibrio vulnificus has been classified into three biotypes. Biotype 1 is found in esturine water and warm marine water, which is opportunistic pathogenic to human beings [6]. Biotype 2 is pathogenic to both eels and human beings and more virulent than biotype1 [6] Biotype 3 is associated with either wound infection or septicemia and is not associated with food consumption [7]. Wound infections and septicemia caused by *V. vulnificus* carry a case fatality rate as high as 50% in healthy patients and severe among immuno-compromised patients or those with liver disease.
The detection of this Organism through cultural method is time consuming, laborious and may not be suitable for VBNC so recent molecular techniques such as polymerase chain reaction

(PCR) offer both rapid reliable and able to detect VBNC state [22]. The multiplex PCR method is recent development, where two or more sets of primers (single pair in simple format of PCR) functioning simultaneously within the same reaction tube. Therefore several gene targets can be amplified to detect the presence of genes or multiple microorganisms at the same time. In brief, *gyrB* is a highly conserved housekeeping gene which is commonly found in *Vibrios* [24] and based on multiple gene sequence comparison of *Vibrios*, *pntA* was found to be the most diverse gene in *V. cholerae, V. parahaemolyticus* and *V. vulnificus*, and could potentially be used for differential detection purpose [26]. Evaluating the risk of *Vibrio organisms* to public health requires determining the potential virulence, hence the present study was designed to undertaken for isolation, identification and differentiatiation common *Vibrio species* in the freshwater fish Catla (*Catla catla*) by conventional as well as multiplex PCR methods.

2. Materials and Methods

The *V. cholerae* (3905-O1); *V. parahaemolyticus* (451) and *V. vulnificus* strains (1145) were obtained from MTCC (Microbial Type Culture Collection), Chandigarh. Other cultures maintained in the Department of Veterinary Public Health, College of Veterinary Science, Rajendranagar were utilized. The fish samples (*Catla catla*) were collected from local markets of Greater Hyderabad Municipal Corporation, Hyderabad. The enrichment broth Alkaline Peptone Water (APW) was used to find out the suitability for multiplex PCR assay. TCBS agar plates were streaked with a loopful of culture from the broth. Identification of Vibrio spp was done as per method of BAM, 1998. The template preparation method heat lysis was done using standard cultures of *Vibrio cholera, Vibrio parahaemolyticus and Vibrio vulnificus.* The primers targeting *gyrB* was used for identification of *Vibrio spp and pntA* genes [26] used for detection and differentiation of *Vibrio cholera, Vibrio parahaemolyticus* and *Vibrio vulnificus* for Multiplex PCR. The details of primers and cyclic condition used in m-PCR are given in Table-1 & 2.

3. Results

In the present study only four *Vibrio spp* carrying *gyrB* yielded specific PCR products of desired length of 493 bp. No specific PCR product was obtained from other organisms tested. Only three *vibrio spp*. carrying *pntA* gene yielded specific products of desired lengths of 338 bp (*V. cholerae*), 409 bp (*V. parahaemolyticus)* and 656 bp (*V. vulnificus*), whereas *V. mimicus* did not yielded specific product. The results are shown in Fig-1.

Out of 150 market raw samples 80(53.33%) samples were positive for *Vibrio* spp by m-PCR, whereas cultural methods gave 72(48%) positive for *vibrio* species. The results are shown in Table-3. Out of 80 positive samples in m-PCR 47 (31.33%), 21(14%) and 12(8%) were positive for *V. cholerae, V. parahaemolyticus and V. vulnificus* respectively, where in cultural methods it was 44(29.33%), 17 (11.33%) and 11 (7.3%).The results are shown in Table-4.

The mean counts of *V. cholerae, V. parahaemolyticus and V. vulnificus* were 7.1 $x10^5$ cfu, $1.07x10^3$ cfu and $5.8x10^2$ cfu in catla fish. The results are shown in Table-5.

4. Discussion

Vibriosis is a major economic problem for the fish industry especially in developing countries and it is responsible for food borne illness in many countries leading to hospitalization and sometimes fatal also[18]. *Vibrio spp*. are the most common and serious pathogen in fish and shellfish marine aquaculture worldwide [9].

In India, *Vibrio species* has been isolated from variety of aqua foods including fish, shrimps, crabs, oysters and canned fish products and also environmental samples collected from different sources [27]. There is wide difference in isolation of *Vibrio species* by various scientists from various sources ranging from 0% [1] to 100% [5]. These differences might be due to geographic, seasonal, salinity, temperature variations and procedures adopted for isolation [16].

Higher incidence of *V. cholerae* of 71.4% in fish by PCR method than the present study (31.33%) was reported earlier [28]. Low incidence (1.9% and 2.5%) by cultural method than the present study was also reported [1]

The incidence of *V. parahaemolyticus* in fish in the present study through m-PCR was 21%, which was less than the incidence 76.37% [3] and 38.8% [10]. The incidence of *V. vulnificus* in fish in the present study was lower than the report earlier [25]

The mean viable count (CFU/g) of *V. cholerae* in fish in the present study was $7.1x10^5$, which was almost similar to the counts ($7.94x10^5$) [12] and lower than the counts of. $4.36x10^7$ during rainy season and $1.77x10^6$ during dry season [11] in cray fish.

The incidence of *V. parahaemolyticus* in total sea food samples in Multiplex PCR assay is another improved technique, where many species/ genes can be identified simultaneously. Multiplex PCR is able to amplify multiplex targets by using several sets of target specific or degenerated primers in a single tube.

To tackle some limitations with the current PCR detection techniques for *Vibrios*, a multiplex PCR method was developed targetting *pntA* and *gyrB* genes simultaneously with time saving and improving the specificity. *gyrB* is a highly conserved housekeeping gene which is commonly found in *Vibrios*), while *pntA* gene encodes transhydrogenase alpha subunit, which is one of the housekeeping genes in most of the *Vibrio* species, *V. parahaemolyticus, V. cholerae, V. vulnificus* and other bacteria.

Table 1: Details of primers used in this study.

Primer	Target	Primer sequence	Amplification product (bp)
pntA1C	*V. cholerae*	5'-CAGTAAAGAAACGACCAAACTC-3'	338
pntA2C		5'-TGCCAGTTTCGATGATGCCG-3'	
pntA1P	*V. parahaemolyticus*	5'-AGCAAGTTTCGATGATGCTG-3'	409
pntA 2P		5'-ACCAGCAACCAAAACTTTCGCT-3'	
pntA 1V	*V. vulnificus*	5'-CTGTAACAAGGCACCGACAA-3'	656
pntA2V		5'-TCACAACCGCACTGATTCCAG-3'	
gyrB1	*Vibrio* isolates	5'-AGCCAAACNAAAGAYAARYT-3'	493
gyrB2		5'-CGYARYTTRTCYGGRTTRTRYTC-3'	

Table 2: Cyclic conditions followed for m-PCR in this study.

S. No.	Name of the Reagent	Quantity (µl)
1.	DNA template	5.0
2.	Buffer	5.0
3.	$MgCl_2$	2.0
4.	dNTP mix	0.5
5.	Taq DNA polymerase	0.3
6.	Primers (gyrB1,2)	1.5 each
7	Primers (pntA 1C,2C,1P,2P,1V,2V)	0.75 each
8	ddH_20	Make to 25
9	Total volume	25

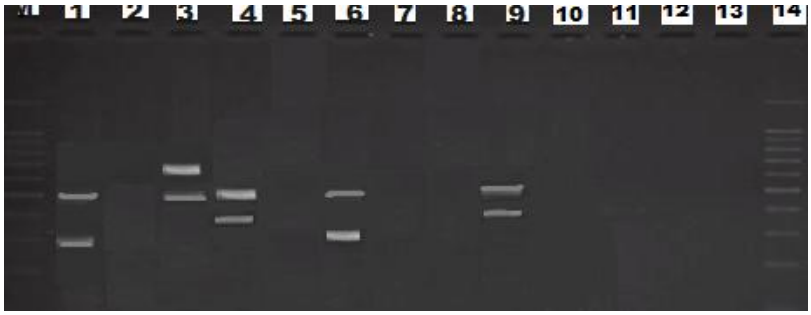

Fig 1: Results for *Catla catla* fish samples for *gyrB/pntA* (*Vibrio* species) through m-PCR
Results for Catla fish samples for *gyrB/pntA* (Vibrio species)
Lane M: 100 bp DNA Ladder, Lane 1 & 6: Sample positive for V.cholerae (493/ 338), Lane 4 & 9: sample positive for V. parahaemolyticus (493/409), Lane 3: Sample positive for V.vulnificus (493/656), Lane 5: Negative control

Table 3: Cultural and PCR results of *Catla catla* fish samples for *Vibrio species*

Type of sample	No. of samples	Positive results for *Vibrio species*				
		Cultural method		m- PCR assay		% of cultural method compared to m-PCR
		No	%	No	%	
Catla Fish	150	72	48	80	53.33	90%

Table 4: m-PCR results of *Catla catla* samples for *V. cholerae, V. parahaemolyticus and V. vulnificus*.

Type of sample	No. of samples positive for *Vibrio spp*	Samples positive for *V. cholerae*		Samples positive for *V. parahaemolyticus*		Samples positive for *V. vulnificus*	
		No.	%	No.	%	No.	%
Catla Fish	80	47	58.75	21	26.25	12	15

Table 5: Mean counts (CFU/g) and Range (CFU/g) of *Vibrio spp.* in *Catla catla* fish.

S.no	Sample	*V. cholerae* (CFU/g)		V. *parahaemolyticus* (CFU/g)		*V. vulnificus* (CFU/g)	
		Mean	Range	Mean	Range	Mean	Range
1.	Catla Fish	$7.1x10^5$	$5.1x10^4$-$3.9x10^6$	$1.07x10^3$	$2.1x10^2$- $5.2x10^4$	$5.8x10^2$	$3.4x10^1$-$2.6x10^3$

5. Conclusion

Detection of *Vibrio* spp in *Catla catla* fish indicates serious public health hazards. The present study revealed that there is serious *Vibrio* species contamination in retail fish market. Proper sanitation and measures should be taken to avoid the food borne ill ness in near future. Proper cold chain maintenance and personel hygiene are required to check the cross contamination.

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