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Prevalence of Antimicrobial Resistance Among *Vibrio spp.* Isolated from the Digestive Tract of Cultured *Penaeus vannamei*

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ABSTRACT

Shrimp digestive tract microflora has been considered important, as it provides several protective and metabolic functions. Misuse of antibiotics could lead to AMR in the gut microbial community, which could be transferred to humans. The present study aimed in determining the prevalence of the digestive tract microbial community and AMR associated with them. A total of 173 isolates were collected and characterised from the digestive tract of 120 shrimps, collected from six different regions of Maharashtra and Gujarat. A total of 144 gram negative isolates comprised predominantly of *Vibrio spp* were isolated and characterised. Antimicrobial susceptibility pattern of the isolates against 12 different antibiotics was carried out using disk diffusion method. Most of the isolates showed resistant against beta-lactam class of antibiotics and macrolide antibiotics. Among the 144 G negative isolates, 61 (39.5%) isolates were presumptively identified as *Vibrio spp*, based on their growth on the specific agar plates. Biochemical characterisation of the 61 *Vibrio* isolates revealed the presence of 31.14% of *Vibrio cholerae*, 31.14% of *V. parahaemolyticus*, 19.67% of *V. vulnificus*, 9.8% of *V. harveyi* and 8.2% of *V. alginolyticus*. The antibiogram profile showed that the 40 (74.07%) isolates were resistant to ampicillin, 24 (44.4%) were resistant to cephalothin, 21 (38.8%) were to aztreonam and 17 (31.5%) were resistant against erythromycin. Prevalence of multi-drug resistance was also observed among the bacterial isolates.

Keywords: *Penaeus vannamei*, *Vibrio spp*, Antibiotics, AMR

Aquaculture has become the fastest growing food sector in the world by a continuous process of expansion, intensification, and diversification of culture practices (Bostock *et al.*, 2010). Due to the increased production of exotic species and the expansion of the production of indigenous species to new geographical locations, there has been an increase in the risk of diseases and their treatment alike. These diseases are often treated by the use of antimicrobials as therapeutics, but there has been an increasing usage of these antimicrobials as prophylactic, prevails in the aquaculture sector (Tirado *et al.*, 2010). The major concern revolving the use of antimicrobials in aquaculture is the threat to favour the emergence of a reservoir of antimicrobial resistance genes (ARGs) that may be eventually transferred to clinically relevant bacteria (World Health Organization, 2006). On a global scale, several different classes of antimicrobials such as sulphonamides, penicillins, macrolides, quinolones, phenicols, and tetracyclines (Sapkota *et al*., 2008) have been used in aquaculture, all of which are being listed as critically or highly important antimicrobials in human medicine (WHO, 2011). However, the controlled use of antimicrobials in global aquaculture industry is highly important to treat bacterial diseases effectively and to maintain fish health and human welfare.

Shrimp farming in the global scenario has been increased dramatically due to high consumer demand and a need for large-scale, reliable shellfish supply. But, bacterial diseases and infections that can spread rapidly among these shrimps impose a great threat to the industry and so, farmers relies

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on the extensive use of antibiotics, either as a prophylactic or as a therapeutic to protect their stocks. However, the usage of antibiotics for controlling bacterial diseases in aquaculture has been proven unsustainable and barren due to development of antibiotic resistance in pathogens one of the greatest human health challenges of the 21st century. Bacterial population such as *Vibrio vulnificus, Aeromonas hydrophila, Mycobacterium marinum, Streptococcus iniae* and *Photobacterium damselae* were considered as zoonotic pathogen reservoirs as they were reported to cause infections among the fish handlers in aquaculture facilities and these accompanied with AMR can pose a high threat to human health. Digestive tract microbial community of shrimps are reported to be high in bacteria of class Gamma-proteobacteria, which includes commercially important members of family enterobacteriaceae, vibrionaceae and pseudomonadaceae. *Vibrio spp* are found to be the most abundant bacterial community in the digestive tract of cultured shrimps(Gomez-Gil *et al*., 1998) and several reports of antimicrobial resistance among these bacteria have been found to produce valuable information on the usage of antimicrobials in the shrimp farming. The present study aims in the prevalence of antimicrobial resistance among *Vibrio spp* isolated from the digestive tract of cultured *Penaeus vannamei* from Maharashtra and Gujarat.

MATERIALS AND METHODS

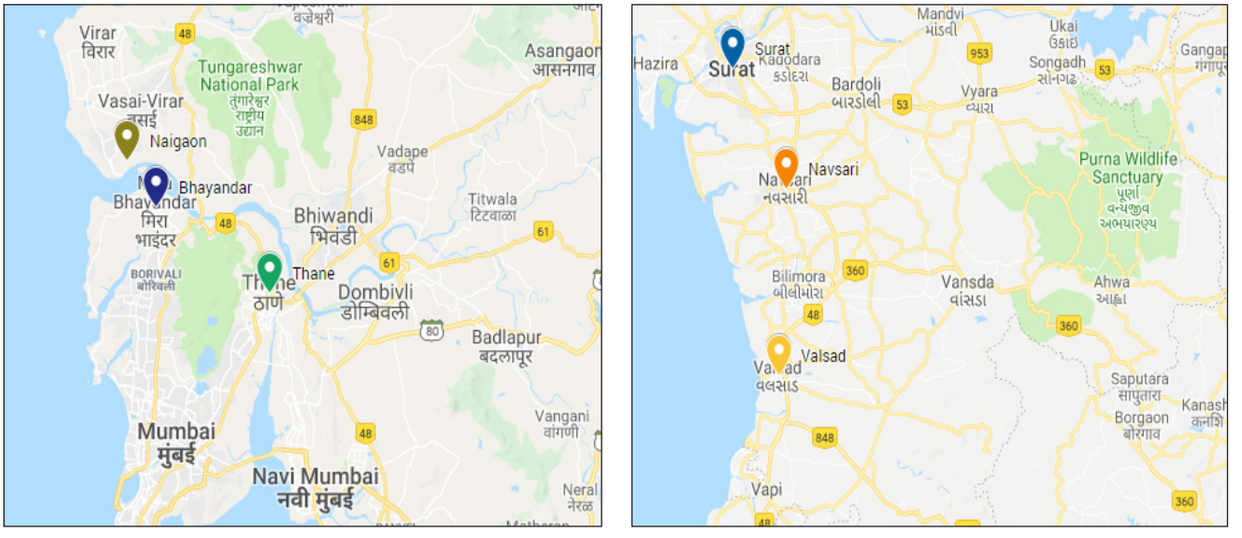
The present study has been conducted at the Department of Aquatic Animal Health Management, ICAR-CIFE, Mumbai.

Sample collection and processing

A total of 120 live shrimps were collected from six commercial shrimp farms of Maharashtra and Gujarat (Fig. 1). Shrimps were killed by dipping in ice slurry for 5-10 mins and dissected aseptically for the retrieval of digestive tract organs such as hepatopancreas, stomach and intestine. The organs were homogenised with 90ml of Phosphate buffered saline for the uniform release of microbiome. These homogenates were then proceeded for the isolation of total bacteria, followed by the isolation of *Vibrio spp*.

Isolation of total bacteria

Each organ homogenate was run through a series of dilution from 10^{-1} to 10^{-4} using phosphate buffered saline and the dilutions were inoculated on sterile Zobell marine agar (ZMA) plates using standard plate extension techniques, with two replicates per dilution. The plates were then incubated at 30°C + 2°C for 24hrs, followed by selection of



(A) **(B)**

Fig. 1: Showing sampling areas **(A)** Maharashtra **(B)** Gujarat

bacterial colonies based on morphological characteristics. These colonies were cultured continuously for obtaining pure bacterial isolates, which were then, stored in sterile ZMA agar slants for further characterisation.

Isolation and characterisation of *Vibrio spp*

Pure bacterial isolates were characterised biochemically for the identification of *Vibrio spp*. The isolates were primarily inoculated on sterile Thiosulfate-citrate-bile salts-sucrose (TCBS) agar and HiChrome Vibrio agar plates (Hi-media, Mumbai) for the specific isolation of *Vibrio* isolates. Colony characteristics were noted followed by staining and biochemical techniques for the identification and characterisation of the *Vibrio* isolates that have grown over the specific agar plates. These *Vibrio* isolates were then proceeded for antimicrobial susceptibility testing.

Antimicrobial susceptibility testing

Bacterial isolates were subjected to antimicrobial testing by disc diffusion method proposed by Baeur *et al*. (1996). Overnight bacterial cultures were prepared using Trypticase soy broth and the cultures were streaked oversterile Mueller-Hinton agar using cotton swabs. Antibiotic discs were then placed over the surface of the agar and incubated at 28°C for 18-24hrs. After incubation, the antibiogram was determined by measuring the diameter of zone of inhibition following CLSI standards (Clinical and Laboratory Standards Institute, 29th Ed).

RESULTS AND DISCUSSION

Isolation of total bacteria

Bacterial colonies with distinct morphological characteristics were selected in triplicates from ZMA plates and streaked continuously for obtaining pure colonies. A total of 173 isolates were obtained and initially distinguished into gram positive and gram negative, based on their staining characteristics. A total of 144 (83.2%) bacterial isolates were identified as gram negative and 29 (16.8%) grampositive isolates were obtained.

Identification and characterisation of *Vibrio spp*

Obtained gram negative isolates (144) were inoculated on TCBS and HiChrome Vibrio agar plates for the specific isolation of *Vibrio spp*. Among the 144 Gram negative isolates, 61 (39.5%) isolates were identified as *Vibrio spp*, based on their growth on the specific agar plates. The prevalence of high percentage of gram-negative bacteria (83.2 %) than the gram-positive isolates (16.8 %) was found in comparison with 78% and 22% as reported by Oxley *et al*. (2002). Among these isolates, *Vibrio spp* were found abundant and this was supported by Gomez-Gil *et al*. (1998), in which *Vibrio spp* were reported common in the shrimp digestive tract. Among the *Vibrio spp*, different strains of *V. cholerae, V. parahaemolyticus, V. alginolyticus, V. Harveyi* and *V. vulnificus* were isolated and characterised, which were previously isolated and characterised from the digestive tract of *P. vannamei* by Tzuc *et al*. (2014). Gopal *et al*. (2005) reported the predominance of *vibrio* species from shrimp samples as 3–19% of *Vibrio alginolyticus*, 2-13% of *V. parahaemolyticus*, 1-7% of *V. harveyi* and 1-4 % of *V. vulnificus* from 47 isolates. Biochemical characterisation of the 61 *Vibrio* isolates in the present study revealed that the presence of 19(31.14%) *Vibrio cholerae*, 19 (31.14%) *V. parahaemolyticus*, 12 (19.67%) *V. vulnificus*, 6 (9.8%) *V. harveyi* and 5 (8.2%) *V. alginolyticus* (Fig. 2) which seems to be higher predominance comparing to the results obtained by Gopal *et al*. (2005). This observed predominance of *V. alginolyticus, V. Parahemolyticus* and *V. harveyi* is explained by their significant role in the degradation of accumulated feed, shrimp exuviae, etc., in shrimp farming which confirms the important role in recycling of insoluble, carbon containing material, mainly chitin played by the members of the family vibrionaceae (Svitil *et al*., 1997; Keyhani and Roseman, 1999; Meibom *et al*., 2004).

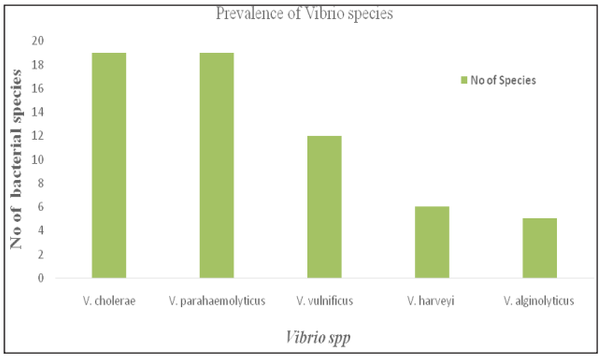


Fig. 2: Prevalence of *Vibrio spp* from the isolates

Antibiogram

All the 61 *Vibrio* isolates were tested against 12 antibiotics using disc diffusion method, of which, 54 (88.5%) isolates were found resistant to one or more antibiotics. Of the 61 isolates, 40 (74.07%) isolates were resistant to ampicillin, 24 (44.4%) were resistant to cephalothin, 21 (38.8%) were to aztreonam and 17 (31.5%) were resistant against erythromycin (Fig. 3).

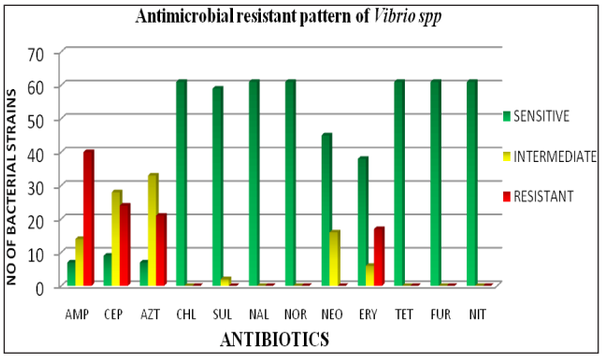


Fig. 3: Antimicrobial resistant pattern of *Vibrio spp*

High levels of intermediate resistance were also observed against neomycin and sulphamethoxazole and multi-drug resistance was observed in 36 (66.6%) isolates. The genera *Vibrio spp, Pseudomonas spp and Aeromonas spp* were predominant in the intestine of *P.vannamei*, which were previously studied and reported by Zhang *et al*. (2014). Antibiotics used for the study were Ampicillin, Cephalothin, Aztreonam, Chloramphenicol, Erythromycin, Furazolidone, Nalidixic acid, Neomycin, Nitrofurantoin, Norfloxacin, Sulphamethizole and Tetracycline. These antibiotics were reported as commonly used antibiotics in shrimp and fish aquaculture by Costa *et al*. (2015), Holmstrom *et al*. (2003), Bermdez *et al*. (2012). Most of the gram-negative isolates were found resistant to beta-lactam class of antibiotics and this was supported by the study by Costa *et al*. (2015). Most of the *Vibrio spp.* isolated from the digestive tract were found resistant to Ampicillin and Erythromycin which was *at par* with the report by Costa *et al*. (2015). Almost all the isolates of *Vibrio spp.* were resistant to Ampicillin and ampicillin was reported to be less effective against *Vibrio spp.* by Costa *et al*. (2015). Resistance of *Vibrio spp.* isolated from *Penaeus monodon*

Table 1: Antibiotic Susceptibility Profile

Code	Bacterial Isolate	AMP	CEP	AZT	CHL	SUL	NAL	NOR	NEO	ERY	TET	NIT	FUR
H1G	*Vibrio parahaemolyticus*	**R**	S	I	S	S	S	S	S	I	S	S	S
H1H	*Vibrio alginolyticus*	**R**	I	I	S	S	S	S	S	**R**	S	S	S
G1E	*Vibrio cholerae*	**R**	I	S	S	S	S	S	S	I	S	S	S
G1F	*Vibrio cholerae*	S	**R**	I	S	S	S	S	S	I	S	S	S
H2E	*Vibrio alginolyticus*	**R**	**R**	I	S	S	S	S	S	I	S	S	S
H2F	*Vibrio vulnificus*	**R**	**R**	**R**	S	I	S	S	I	**R**	S	S	S
H2G	*Vibrio parahaemolyticus*	S	**R**	I	S	S	S	S	S	I	S	S	S
H2H	*Vibrio cholerae*	**R**	**R**	I	S	S	S	S	S	I	S	S	S
H2I	*Vibrio cholerae*	**R**	**R**	I	S	S	S	S	I	I	S	S	S
H2J	*Vibrio parahaemolyticus*	**R**	**R**	**R**	S	S	S	S	S	I	S	S	S
G2I	*Vibrio alginolyticus*	**R**	I	S	S	S	S	S	S	**R**	S	S	S
G2J	*Vibrio cholerae*	**R**	**R**	I	S	S	S	S	S	**R**	S	S	S
G2K	*Vibrio harveyi*	S	I	S	S	S	S	S	S	**R**	S	S	S
G2L	*Vibrio parahaemolyticus*	S	S	S	S	S	S	S	S	I	S	S	S
H3E	*Vibrio parahaemolyticus*	**R**	**R**	**R**	S	S	S	S	S	**R**	S	S	S
H3F	*Vibrio vulnificus*	S	I	**R**	S	I	S	S	I	I	S	S	S

H3G	*Vibrio vulnificus*	S	S	S	S	S	S	S	S	**R**	S	S	S
H3H	*Vibrio parahaemolyticus*	**R**	**R**	**R**	S	S	S	S	I	**R**	S	S	S
G3E	*Vibrio cholerae*	**R**	I	**R**	S	S	S	S	S	**R**	S	S	S
G3F	*Vibrio cholerae*	**R**	I	**R**	S	S	S	S	S	**R**	S	S	S
G3G	*Vibrio cholerae*	**R**	I	**R**	S	S	S	S	S	**R**	S	S	S
G3H	*Vibrio cholerae*	**R**	I	**R**	S	S	S	S	S	**R**	S	S	S
H4E	*Vibrio cholerae*	**R**	**R**	I	S	S	S	S	S	I	S	S	S
H4F	*Vibrio parahaemolyticus*	S	S	I	S	S	S	S	S	I	S	S	S
H4G	*Vibrio cholerae*	**R**	**R**	I	S	S	S	S	S	I	S	S	S
H4H	*Vibrio parahaemolyticus*	**R**	I	**R**	S	S	S	S	S	I	S	S	S
G4E	*Vibrio vulnificus*	**R**	**R**	I	S	S	S	S	S	S	S	S	S
G4F	*Vibrio cholerae*	S	I	S	S	S	S	S	**I**	S	S	S	S
G4G	*Vibrio cholerae*	S	I	I	S	S	S	S	S	S	S	S	S
G4H	*Vibrio cholerae*	**R**	S	**R**	S	S	S	S	S	S	S	S	S
G4I	*Vibrio parahaemolyticus*	**R**	I	I	S	S	S	S	S	S	S	S	S
G4J	*Vibrio parahaemolyticus*	**R**	I	I	S	S	S	S	I	S	S	S	S
H5E	*Vibrio parahaemolyticus*	**R**	I	**R**	S	S	S	S	I	I	S	S	S
H5F	*Vibrio parahaemolyticus*	**R**	**R**	**R**	S	S	S	S	S	I	S	S	S
H5G	*Vibrio harveyi*	**I**	**R**	I	S	S	S	S	I	I	S	S	S
H5H	*Vibrio harveyi*	I	**R**	I	S	S	S	S	I	I	S	S	S
H5I	*Vibrio cholerae*	S	I	I	S	S	S	S	S	I	S	S	S
H5J	*Vibrio vulnificus*	**R**	I	I	S	S	S	S	S	**R**	S	S	S
H5K	*Vibrio vulnificus*	I	**R**	**R**	S	S	S	S	S	I	S	S	S
H5L	*Vibrio vulnificus*	**R**	I	I	S	S	S	S	I	I	S	S	S
G5E	*Vibrio parahaemolyticus*	**R**	I	I	S	S	S	S	S	I	S	S	S
G5F	*Vibrio parahaemolyticus*	**R**	I	I	S	S	S	S	S	I	S	S	S
G5G	*Vibrio cholerae*	I	I	**R**	S	S	S	S	I	**R**	S	S	S
G5H	*Vibrio harveyi*	**I**	**R**	**I**	S	S	S	S	I	I	S	S	S
G5I	*Vibrio cholerae*	I	I	**R**	S	S	S	S	I	**R**	S	S	S
G5J	*Vibrio vulnificus*	**R**	**R**	I	S	S	S	S	I	I	S	S	S
G5K	*Vibrio vulnificus*	**R**	**R**	I	S	S	S	S	I	I	S	S	S
G5L	*Vibrio vulnificus*	**R**	**R**	I	S	S	S	S	I	I	S	S	S
H6E	*Vibrio alginolyticus*	**R**	I	**R**	S	S	S	S	S	I	S	S	S
H6F	*Vibrio alginolyticus*	**R**	I	**R**	S	S	S	S	S	I	S	S	S
H6G	*Vibrio parahemolyticus*	**R**	**R**	I	S	S	S	S	S	I	S	S	S
H6H	*Vibrio vulnificus*	**R**	I	**R**	S	S	S	S	S	I	S	S	S

H6I	*Vibrio cholerae*	S	S	I	S	S	S	S	S	I	S	I	I
H6J	*Vibrio vulnificus*	**R**	I	**R**	S	S	S	S	S	**R**	S	S	S
H6K	*Vibrio parahemolyticus*	R	**R**	I	S	S	S	S	S	**R**	S	S	S
H6L	*Vibrio parahemolyticus*	**R**	**R**	I	S	S	S	S	S	I	S	S	S
G6E	*Vibrio parahemolyticus*	S	S	I	S	S	S	S	S	I	S	S	S
G6F	*Vibrio parahemolyticus*	**R**	I	**R**	S	S	S	S	S	I	S	S	S
G6G	*Vibrio harveyi*	S	S	S	S	S	S	S	I	I	S	S	I
G6H	*Vibrio harveyi*	S	S	I	S	S	S	S	S	I	S	S	S
G6I	*Vibrio cholerae*	**R**	I	**R**	S	S	S	S	I	I	S	S	S

AMP = Ampicillin; CEP = Cephalothin; AZT = Aztreonam; CHL= chloramphenicol; SUL; Sulphamethaxozole; NAL= Nalidixic acid; NOR= Norfloxacin; NEO = Neomycin; ERY = Erythromycin; TET = Tetracycline; NIT = Nitrofurazone; FUR = Furazolidone

Table 2: Biochemical characterization of the isolates

Bacterial Isolate	**BIOCHEMICAL TESTS**											
	Oxi	*Cat*	*Ind*	*MR*	*VP*	*Cit*	*Orn*	*lys*	*ONPG*	*Arg.*	*0% S*	*6% S*
Vibrio cholerae	+	+	+	-	V	V	+	+	+	-	+	-
V. parahaemolyticus	+	+	+	+	-	+	+	+	-	-	-	+
Vibrio alginolyticus	+	+	+	-	+	+	+	+	-	-	-	+
Vibrio harveyi	+	+	+	+	-	-	+	+	-	-	-	+
Vibrio vulnificus	+	+	+	+	-	+	+	+	+	-	-	+

Oxi = Oxidase; Cat = Catalase; Ind = Indole; MR = Methyl Red; VP = Voges Prokuer; Cit = Citrate; Orn = Ornithine; Lys = Lysine; ONGP = Ortho Nitro Phenyl β Galactosidase; Arg = Arginine; 0% S = 0% NaCl; 6% S = 6% NaCl

showed resistance to Ampicillin (61%) and Erythromycin (20%) and susceptible to Chloramphenicol was reported by Kitiyodom *et al*. (2010) which was in accordance with this study. All the isolates showed sensitivity towards chloramphenicol, sulphamethizole, tetracycline, nalidixic acid, norfloxacin, nitrofurantoin and furazolidone. But in the study by Kim *et al*. (1999), *V. parahaemolyticus* isolates from *Penaeus monodon* were found resistant to nalidixic acid and tetracycline with 60% and 21% resistance, which was at contrast with this study. But complete sensitivity towards nalidixic acid by *Vibrio spp.* were reported from *Penaeus vannamei* by Rebouças *et al*. (2010) was *at par* with the study.

CONCLUSION

Antimicrobials formed an integral part of shrimp farming because of their usage as prophylactic as well as feed additives which results in the emergence of antimicrobial resistance among bacteria. Dueto increase in shrimp consumption, there is a great chance of transfer of these antimicrobial resistance bacteria or the genes responsible for the resistance to humans as well as other terrestrial animals.

This study focused on the antimicrobial resistance among the shrimp digestive tract microflora and the factors responsible for the resistance. Hence, in this study, the bacterial microflora of the digestive tract was characterised, which could be important for creating a data baseline from which further research could explore their possible application as probiotics. Prevalence and characterisation of antimicrobial resistance among the isolates could be helpful in understanding the propagation of AMR and in formulating mitigating measures.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author declares that there is no conflict of interest.

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