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Antibiotic resistant profile of *Pseudomonas* spp. *Aeromonas* spp. and *Pseudoalteromonas* spp. isolated from cultured whiteleg shrimps

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Abstract
Antimicrobial resistance has become a global threat to both animal and human health, with its threat being doubled by increased usage of antibiotics in aquaculture sector. The present study was aimed in revealing the antimicrobial resistance pattern associated with selected digestive tract microbial community in cultured *Penaeus vannamei*. A total of 173 isolates were collected and characterised from the digestive tract of cultured shrimps, collected from six different regions of Maharashtra and Gujarat. Of which, 31 (21.5%) were biochemically identified as *Pseudomonas spp*, 30 (20.8%) were identified as *Aeromonas spp* and 26 (18.2%) were identified as *Pseudoalteromonas spp* and tested against 12 different antibiotics for the detection of antibiotic resistance. Results revealed that a high percentage of the isolates were resistant to Ampicillin, Cephalothin, Aztreonam and Erythromycin, along with high prevalence of multidrug resistance.

Keywords: Antibiotics, AMR, *Pseudomonas* spp. *Aeromonas* spp. and *Pseudoalteromonas* spp

Introduction
In the present world scenario, whiteleg shrimp i.e. *Penaeus vannamei* has become one of the important cultured crustacean species. Several studies have been done on this crustacean, but still further research is needed on the microbial community of the shrimp, to understand the physiology, metabolism and their interaction with this micro biome. The microbial flora and fauna of cultured organisms play a crucial role in aquaculture because it can affect host growth and survival, of which the digestive tract microbial community is the most concerned. The development of digestive tract micro biota in an organism is a steady process from the beginning of their birth. In case of aquatic organisms, the micro biota is highly influenced by the surrounding water environment, their feeding habits as well as their hormones and enzyme activity.

Antimicrobial resistance (AMR) has become one of the three major health threats in the world, for both animal and public health sectors, impacted by both human and non-human antimicrobial usage (OIE,FAO,WHO 2004) [14]. The spread of AMR proceeds mainly due to the rampant use of antibiotics in humans and animals which broadly includes fishes which carries a characteristic risk of selection of Antimicrobial resistant genes (ARGs). In shrimp industry, the use and abuse of aquaculture has led to an increase in antibiotic resistance in fish pathogens, which have the potential to transfer of these resistant determinants to and from the animals and human microbial community (Cabello, 2006 [2], Cabello *et al*., 2013) [3]. Gut microbial community of the shrimps have high potential for the development of antimicrobial resistance, since most of the antibiotics are administered orally through feed. However, only little data is available on the gut microbial community of shrimps as compared to the studies on fishes and the antimicrobial resistance associated with those bacterial communities. This study is focused on characterisation of the digestive tract microbial community of the cultured *Penaeus vannamei*, for the better understanding of the microbial fauna and fauna.

Materials and Methods
The study has been carried out in the Department of Aquatic Animal Health Management, ICAR-CIFE, Mumbai.

Sample material

One-hundred and twenty individuals of *Penaeus vannamei* were collected from the culture areas of Maharashtra and Gujarat and transported live to the laboratory. These animals were anaesthetised in ice slurry and dissected immediately under sterile conditions. Organs such as hepatopancreas, intestine and stomach were removed aseptically and each organ was progressed through a series of dilutions from $1x10^{-1}$ to $1x10^{-4}$ using sterile phosphate buffered saline.

Isolation of total bacteria

Each dilution was inoculated into sterile Zobell marine agar plates using standard plate extension, with two replicates per dilution. After inoculation, the plates were incubated at $28^0C+ 2^0C$ for 24 hrs and colonies were selected based on their morphological characteristics. For each selected colony, additional two colonies were selected with similar morphology, because similar morphology could be of different strains. Each selected colony was inoculated into sterile ZMA agar plates and incubated for 24 hours at 28°C ± 2. Re-inoculation of the selected colonies continued using the plate streak method, until pure strains were obtained. These isolated colonies were streaked onto ZMA slants and stored at 4^0C for further biochemical characterisation.

Biochemical characterisation of the isolates

Each isolate was run through a series of biochemical tests for the characterisation of the bacteria, based on their biochemical properties. In the initial stage of characterisation bacterial colonies were distinguished into Gram positive and Gram negative based on their gram staining characteristics. Under 100X magnifications, gram positive were indicated by purple colour and gram negative as red/pink colour. In the second stage of characterisation, bacterial colonies were subjected to 12 different biochemical tests such as oxidase and catalase tests, Triple sugar iron agar, in dole, methyl red, Vogues - Proskauer and citrate tests, Salt tolerance test, ONPG test and Moeller decarboxylation tests and subjected to growth on Glutamate Starch Phenol red (GSP) agar were performed as previously described by Oliver (1983) and Ottaviani *et al* (2001) [16].

Antibiotic susceptibility testing

A total of twelve different antibiotics such as Ampicillin (10mcg), Cephalothin (30mcg), Aztreonam (30mcg), chloramphenicol (30mcg), Erythromycin (15mcg), Furazolidone (30mg), Nalidixic acid (30mcg), Neomycin (30mcg), Nitrofurantoin (300mcg), Norfloxacin (10mcg), Sulphamethizole (300mcg) and Tetracycline (30mcg) were used for antimicrobial susceptibility testing by disk diffusion method. Overnight bacterial cultures were prepared in sterile nutrient broth (2% salt) and streaked on surface of sterile Mueller-Hinton agar plates under aseptic condition. The antibiotic disks were placed on the surface of agar plates and incubated at 30^0C for 24 hrs and after incubation the results were determined by measuring the diameter of zone of inhibition followed by interpretation according to CLSI standards.

Result and Discussion

Total bacterial isolates

The understanding of the micro biota of *P. vannamei* remains insufficient compared to vertebrates, including terrestrial livestock and finfish species (Colston *et al*., 2015) [5]. Throughout the life of an organism, its micro biota provides metabolic and protective functions which are aimed at assisting in digestive processes and nutrient absorption to provide energy to the organism. Their trophic functions promote cell growth and differentiation, as well as stimulating of the immune system. Their protective functions are present from birth since they act as the first line of defense against pathogenic, exogenous or opportunist microorganisms, creating a barrier effect (Isolauri *et al*. 2001) [11]. The modulation of micro biota as an alternative approach to maximize production and disease control using prebiotics, probiotics, and synbiotics requires a comprehensive knowledge of the diversity of the micro biota in the host organism. Understanding the bacterial ecology in the crustacean gut can help to improve both the management of hatcheries for higher productivity and the safety of shrimps as food (Gainza *et al*., 2018) [8].

In the present study, the bacterial isolates were characterised based on their biochemical characteristics. A total of 173 bacterial isolates were collected and characterised according to the biochemical tests proposed by Alsina *et al.* (1994) [1]. Oxley *et al*. 2002 [17], that reported *Aeromonas, Pleisomonas, Photobacterium, Pseudomonas, Pseudoalteromonas* and *Vibrio* from different parts of the *Peneaus merguiensis* digestive tract. Gomez-Gil *et al*. 1998 [9] Moss *et al*. 2000 [13] Oxley *et al*. 2002 [17] Esiobu and Yamazaki 2003 [7] Liu *et al*. 2011 [12] have reported these as normal flora in the shrimp digestive system. Biochemical characterisation of the 173 isolates revealed the presence of 31(21.5%) *Pseudomonas spp,* 30(20.8%) *Aeromonas spp* and 26(18.2%) *Pseudoalteromonas spp,* which were found in comparison with the study by Alsina *et al*. (1994) [1]. *Pseudoalteromonas spp* was found in abundance in the digestive tract which according to Ivanova *et al*., 2002; Holmstrom *et al*., 2002 was a common genus in the marine environment with about 30 species being present in seawater, algae and marine invertebrates. The genera *Vibrio spp, Pseudomonas spp and Aeromonas spp* were predominant in the intestine of *P. vannamei*, which were in comparison with the study by Zhang *et al*., 2014 [21].

All the selected isolates of *Pseudomonas spp, Aeromonas spp* and *Pseudoalteromonas spp* were subjected to antimicrobial testing against 12 different antibiotics using disk diffusion method. Of the 26 *Pseudoalteromonas* isolates, 19 (73.07%) were resistant to AMP, 8 (30.8%) were resistant to AZT, 6 (23.07%) were resistant to CEP and 4 (15.4%) were resistant to ERY. Based on the previous reports, most of the gram negative isolates were found resistant to beta-lactam class of antibiotics (Costa *et al*., 2015) [6]. Stratev *et al*., 2014 [19] reported that resistance of *Aeromonas spp* from shrimp source were shown resistance against a wide range b-lactams including Ampicillin, Cephalothin and Aztreonam along with Erythromycin and sensitive to Chloramphenicol. The results of present study showed that, of the 30 *Aeromonas* isolates, 21 (70%) isolates were resistant to AMP, 14 (46.6%) were resistant to CEP, 5 (16.6%) were resistant to AZT and 2 (6.6%) were resistant to ERY. Moreover, of the 31 *Pseudomonas* isolates, 24 (77.4%) were resistant to AMP, 9 (29.03%) were resistant to AZT, 16 (51.6%) were resistant to CEP and 5 (16.12%) were resistant to ERY. This infers that most of the isolates of *Pseudomonas spp* were found resistant against Beta-lactams and also showed intermediate resistance against them which were previously reported by Olasumbo *et al*., 2011 from *Pseudomonas spp* isolated from fish and

shrimp sources. Multidrug resistance were observed in 51 isolates against AMP, CEP, AZT and ERY, which has been tabulated in Table 2. All the isolates showed sensitivity towards chloramphenicol, sulphamethizole, tetracycline, and Nalidixic acid, Norfloxacin, Nitrofurantoin and Furazolidone.

Table 1: Biochemical test results

Bacterial Isolate	BIOCHEMICAL TESTS											
	Oxidase	**Catalase**	**Indole**	**MR**	**VP**	**Citrate**	**Ornithine**	**lysine**	**ONPG**	**Arg. dihy**	**0%NaCl**	**6%NaCl**
Pseudoalteromonas spp	+	+	-	ND	ND	-	*	*	*	-	-	+
Aeromonas spp	+	+	+	+	+	+	*	*	*	+	*	*
Pseudomonas spp	+	+	+	-	-	+	*	*	*	+	*	*

Table 2: Antibiotic susceptibility profile

Code	Bacterial Isolate	AMP	CEP	AZT	CHL	SUL	NAL	NOR	NEO	ERY	TET	NIT	FUR
H1B	*Pseudoalteromonas* spp	R	S	R	S	S	S	S	S	R	S	S	S
H1E	*Pseudomonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
H1F	*Pseudomonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
G1A	*Pseudomonas* spp	R	I	R	S	S	S	S	S	I	S	S	S
G1B	*Aeromonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
G1C	*Pseudoalteromonas* spp	R	I	R	S	S	S	S	S	I	S	S	S
G1G	*Aeromonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
H2B	*Pseudoalteromonas* spp	R	R	R	S	S	S	S	S	R	S	S	S
H2D	*Pseudoalteromonas* spp	R	R	R	S	S	S	S	S	I	S	S	S
H3A	*Pseudoalteromonas* spp	R	R	R	S	S	S	S	I	R	S	S	S
H3C	*Pseudoalteromonas* spp	R	R	R	S	S	S	S	I	R	S	S	S
H3I	*Pseudomonas* spp	R	R	I	S	S	S	S	S	R	S	S	S
H3J	*Pseudomonas* spp	R	R	I	S	S	S	S	S	R	S	S	S
H3K	*Aeromonas* spp	R	S	S	S	S	S	S	S	R	S	S	S
H3L	*Aeromonas* spp	R	R	I	S	S	S	S	I	I	S	S	S
G3C	*Pseudoalteromonas* spp	R	R	I	S	S	S	S	I	I	S	S	S
G3K	*Pseudomonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
G3M	*Aeromonas* spp	R	R	I	S	S	S	S	S	R	S	S	S
G3N	*Aeromonas* spp	R	R	I	S	S	S	S	I	I	S	S	S
G3P	*Pseudomonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
H4A	*Pseudoalteromonas* spp	R	I	R	S	S	S	S	S	I	S	S	S
H4B	*Pseudoalteromonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
H4I	*Pseudomonas* spp	R	I	R	S	S	S	S	S	R	S	S	S
H4K	*Pseudomonas* spp	R	I	R	S	S	S	S	S	I	S	S	S
H4L	*Pseudomonas* spp	R	R	I	S	S	S	S	S	R	S	S	S
H4M	*Aeromonas* spp	R	I	R	S	S	S	S	S	I	S	S	S
H4O	*Aeromonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
H4P	*Aeromonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
G4A	*Pseudoalteromonas* spp	R	I	R	S	S	S	S	S	I	S	S	S
G4K	*Pseudomonas* spp	R	I	R	S	S	S	S	S	S	S	S	S
G4L	*Pseudomonas* spp	R	R	I	S	S	S	S	S	S	S	S	S
G4M	*Aeromonas* spp	S	R	R	S	S	S	S	S	S	S	S	S
G4N	*Aeromonas* spp	S	R	R	S	S	S	S	S	S	S	S	S
G4O	*Pseudomonas* spp	S	R	R	S	S	S	S	S	S	S	S	S
G4P	*Pseudomonas* spp	R	R	R	S	S	S	S	S	S	S	S	S
H5M	*Pseudomonas* spp	R	R	R	S	S	S	S	S	R	S	S	S
H5N	*Pseudomonas* spp	R	R	I	S	S	S	S	S	S	S	S	S
H5O	*Aeromonas* spp	R	R	R	S	S	S	S	I	I	S	S	S
H5P	*Aeromonas* spp	R	R	R	S	S	S	S	S	I	S	S	S
G5A	*Pseudomonas* spp	R	R	R	S	S	S	S	S	I	S	S	S
G5B	*Pseudomonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
G5C	*Aeromonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
G5D	*Pseudomonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
H6M	*Aeromonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
H6N	*Aeromonas* spp	R	R	I	S	S	S	S	S	I	S	S	S
H6P	*Pseudomonas* spp	R	R	R	S	S	S	S	S	I	S	S	S
G6L	*Aeromonas spp*	R	R	R	S	S	S	S	S	I	S	S	S
G6M	*Aeromonas spp*	S	R	R	S	S	S	S	S	I	S	S	S
G6N	*Aeromonas spp*	R	R	R	S	S	S	S	S	I	S	S	S
G6O	*Aeromonas spp*	R	R	R	S	S	S	S	S	I	S	S	S
G6P	*Aeromonas spp*	R	R	R	S	S	S	S	I	I	S	S	S

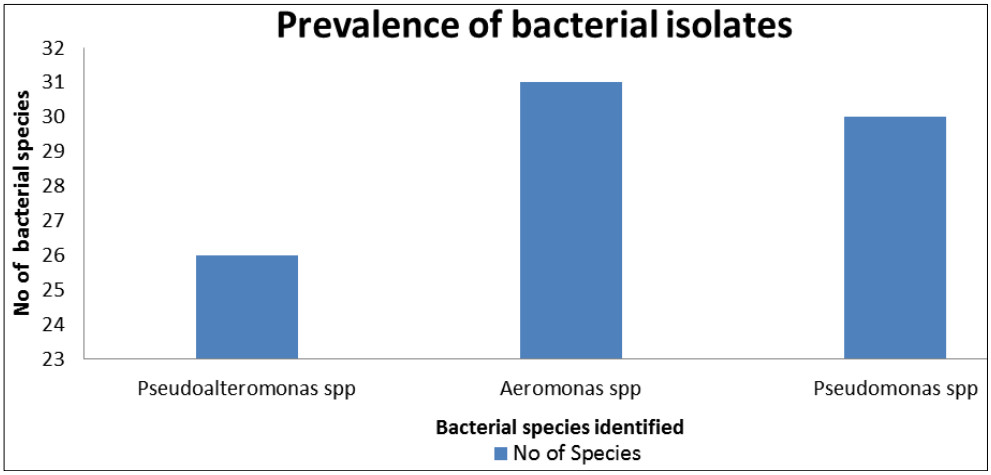

Fig 1: Prevalence of bacterial isolates

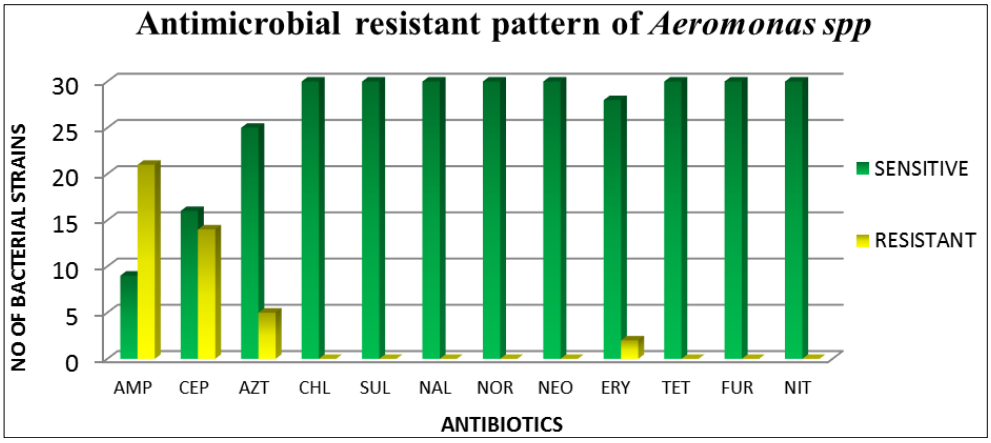

Fig 2: Antimicrobial resistant pattern of *Aeromonas spp*

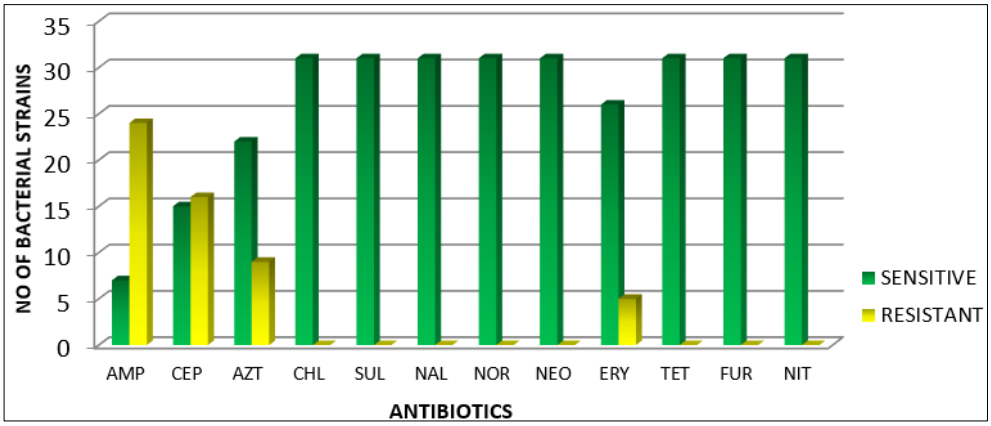

Fig 3: Antimicrobial resistant pattern *of Pseudomonas* spp.

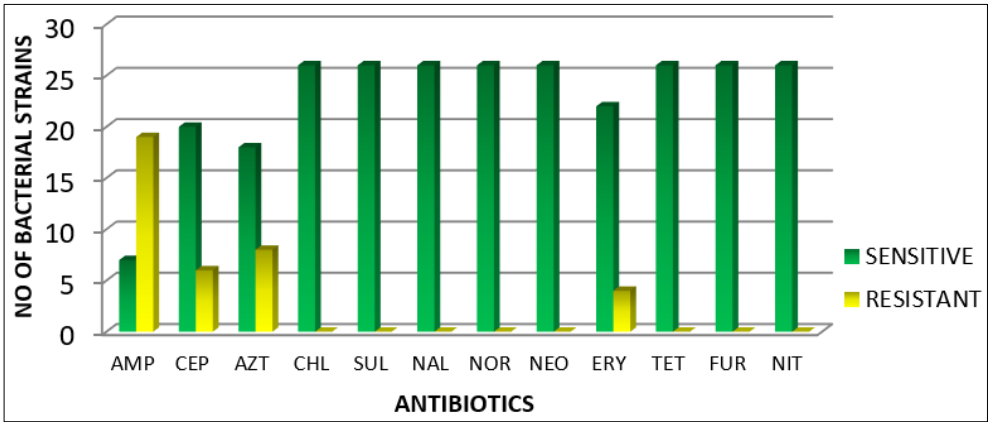

Fig 4: Antimicrobial resistant pattern of *Pseudoalteromonas* spp.

Conclusion

The microbial community of a cultured species can play an important role in aquaculture because it can affect host growth and survival. Shrimp is being threatened by newly emerging diseases globally; thus, understanding the driving factors that govern its gut micro biota would facilitate an initial step to re-establish and maintain a "healthy" gut micro biota. The data reported here on the prevalence of microbial community of shrimp gut bacteria can form the basis for future research aimed at optimizing shrimp culture practices. However, more investigations will be needed to analyse the roles of this microbial community in healthy and diseased shrimp.

Conflict of interest

The authors declare no conflict of interest.

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