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Incidence, virulence potential and antibiotic susceptibility of *Vibrio parahaemolyticus* associated with traditional shrimp culture systems of Ernakulam, Kerala

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Original Article

Abstract

Vibrio parahaemolyticus is a versatile, halophilic organism with the potential to act as a shrimp pathogen and a severe human pathogen. The study explores the incidence, virulence potential, and antimicrobial susceptibility of *V. parahaemolyticus* isolates from diseased penaeid shrimp samples obtained from 50 different aquafarms in Ernakulam, Kerala. A total of 121 isolates of *V. parahaemolyticus* were identified by conventional methods and further confirmed by PCR targeting the species-specific markers such as *toxR* and *tlh* genes. All the isolates were confirmed to be in the non-AHPND group and non-virulent to humans without the *tdh* and *trh* genes. Antibiogram analysis of the isolates against a set of 13 commonly used antibiotics revealed the highest resistance towards Amoxyclav in over 71.4% of all the isolates. In contrast, all the isolates showed sensitivity to chloramphenicol (100%). The current study reveals that 82 (68%) of the 121 isolates were showing MAR index greater than 0.2 indicating the potential of these multi-drug resistant isolates to remain as a hub of antimicrobial resistance (AMR) genes in aquatic systems. Continuous evaluation of antimicrobial resistance in aquaculture environments is essential to minimize the flow of clinically important AMR bacteria from culture systems to the outside environment. The region-specific study, therefore, provides information about the existence of non-virulent, non-AHPND, multidrug-resistant *V. parahaemolyticus* in the traditional culture ponds, demanding the need for adopting ideal, eco-friendly alternative measures that could substitute the use of antibiotics in the shrimp culture ecosystems thereby minimizing the chances for detention and rejection of frozen shrimp exported from our country.

Keywords: *Aquaculture, antimicrobial resistance, antibiogram, disease, MAR index*

Introduction

Aquaculture prevails as a dashing and dynamic production sector for high-protein animal food that is palatable and nutritious. *Vibrio parahaemolyticus* is a gram-negative, halophilic bacterium that exists naturally in the marine and coastal environment and is a significant problem in *P. monodon* culture systems, causing 'Red disease', up to 80% mortality. Some strains of *V. parahaemolyticus* that possess a unique 70kb plasmid (pVA1) can cause a baffling problem in marine shrimp known as Acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, EMS) all along Southeast Asia and Latin America, (Lee *et al.*, 2015). In India, shrimp aquaculture has witnessed several cases of widespread mortality within 40 - 50 days of stocking due to *V. parahaemolyticus* infection in *Penaeus vannamei* (Pacific white shrimp) grow-out farms (Kattapuni *et al.*, 2021). Thus *V. parahaemolyticus* is considered an emerging pathogen in the aquaculture industry, affecting our country's overall economic development with a serious impact on food productivity, animal welfare and human health. Pathogenic *V. parahaemolyticus* can have potential gastrointestinal diseases in humans resulting from consuming raw or uncooked seafood. This microorganism has gained more attention as a human pathogen in recent years, due to the continued outbreak of seafood poisoning in different parts of the world (Karunasagar *et al.*, 2016). The prominent virulence

factors of *V. parahaemolyticus* in terms of pathogenicity are thermostable direct hemolysin (TDH) and thermostable direct-related hemolysin (TRH) (Tada *et al.*, 1992). PCR targeted to the *toxR* gene or *tlh* (thermostable labile hemolysin, another species-specific marker) is exploited as a method for species-level identification as *toxR* is a regulatory gene present in all the strains regardless of their capacity to produce TDH or TRH (Kim *et al.*, 1999; Dileep *et al.*, 2003). Many antibiotics, sanitisers and chemicals are being used to control diseases in aqua farms. This brings about terrible environmental effects, the inception of antibiotic resistance and the prevalence of chemical residues in animal tissues. Several scientific reports show that *Vibrio* isolates have resisted regularly used antibiotics such as enrofloxacin, florfenicol, trimethoprim, and oxytetracycline in shrimp rearing farms which pose a major threat in its control (Roque *et al.*, 2001; Molina *et al.*, 2002).

The autochthonous behaviour and the versatile nature of *V. parahaemolyticus* contribute to its global distribution, thereby making it a model organism for the "one health" concept, realizing the interconnection of human health with animal health and its environment (Karunasagar *et al.,* 2016). The high metabolic diversity exhibited by pathogenic and non-pathogenic *V. parahaemolyticus* strains is proof of their wide tolerance to temperature, salinity and pH. It is this facilitating them to adapt to almost all marine, and estuarine environments (shrimp farms), leading to swift disease dispersion into new zones (Soto-Rodriguez *et al.*, 2018). The study thus evaluates the current status of occurrence, virulence potential and AMR in *V. parahaemolyticus* isolates associated with diseased penaeid shrimps collected from different aquaculture systems in Ernakulam.

Material and methods

Sample collection and processing

The study used the diseased shrimp samples collected from 50 different aqua farms of Ernakulam. Samples were transported to the lab on cool gel packs and processed on the same day. The diseased shrimps showed signs of necrosis, discolouration of gills, loose cuticle, white gut and lethargy. Shrimp samples (25 g) were weighed aseptically and homogenized with 225 ml of sterile Alkaline Peptone Water (APW) with 3% salt to isolate *V. parahaemolyticus*. Then the samples were incubated at 37 °C. After 18h of incubation, an inoculum of culture was streaked upon thiosulphate citrate bile salt sucrose agar (TCBS) with 3% NaCl and VP medium (Kaper *et al.*, 1980). Subsequently, the plates were incubated at 37 ºC for 24 h. The round bluish-green suspective colonies of *V. parahaemolyticus* were then subcultured on nutrient agar slants with 3% NaCl.

Biochemical identification of obtained isolates of V. parahaemolyticus

Isolated green colonies were subjected to a battery of biochemical tests for phenotypic characterization. 3% NaCl was included in the preparation of all the biochemical media. Alsina's scheme-based biochemical identification and grouping into keys were also done (Alsina and Blanch, 1994), optimized by Ottaviani *et al.*, 2003. Different biochemical tests *viz.* arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, acetoin production, N-acetyl-glucosamine assays, utilization of citrate and D-glucosamine responses were recorded from API.

Detection of total and pathogenic V. parahaemolyticus by PCR

Overnight grown culture (1.5 ml) of the organism in LB broth was used for the genomic DNA extraction by CTAB/NaCl method. (Sambrook and Russell, 2001). The quantity and quality of DNA at an absorbance of 260 nm and 280 nm were assessed using a UV spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). For the molecular characterization of the isolates, Polymerase Chain Reaction (PCR) was performed to analyse the presence of species-specific genes such as *toxR* and *tlh* in *V. parahaemolyticus* isolates from the shrimp samples. The genes coding for virulence such as *tdh*, *trh* and *pir*A^{vp} were also screened by PCR. DNA extraction and PCR protocol for detecting AHPND are based on AP3 method according to the protocol of (Sirikharin *et al.*, 2015). The details of the primer used for the PCR amplification for molecular detection of each *V. parahaemolyticus* isolate are shown in Table 1. The reaction mixtures were amplified in a thermal cycler (BIORAD T100, USA) and the details of PCR conditions are given in Table 2. The products were resolved on 1.2% agarose gels, at constant 100V in 1 X TBE buffer using Ethidium bromide (0.5 mg/ml) as the intercalating dye, imaged and analysed using a gel documentation system (BIO-RAD, GEL DOC EZ IMAGER, USA).

Table 1. Primers with their nucleotide sequence used in the PCR for detection of species specific and pathogenic genes of *V. parahaemolyticus*

Primer	Sequence 5'-3'	Amplicon size (bp)	Reference
*tox*RVP (F)	GTCTTCTGACGCAATCGTTG	368	Kim *et al.*, 1999
*tox*RVP (R)	ATACGAGTGGTTGCTGTCATG		
tlh VP (F)	AAAGCGGATTATGCAGAAGCACTG	450	Bej *et al.*,1999
tlh VP (R)	GCTACTTTCTAGCATTTTCTCTGC		
tdh VP (F)	CCACTACCACTCTCATATGC	251	Tada *et al.*, 1992
tdh VP (R)	GGTACTAAATGGCTGACATC		
trh VP (F)	GGCTCAAAATGGTTAAGCG	250	Tada *et al.*, 1992
trh VP (R)	CATTTCCGCTCTCATATGC		
*pir*A^{vp} VP(F)	ATGAGTAACAATATAAAACATGAAAC	333	Sirikharin *et al.*, 2015
*pir*A^{vp} VP(R)	GTGGTAATAGATTGTACAGAA		

Table 2. Thermo cycling conditions for the amplification of the genes

Primer	Number of cycles	Denaturation	Primer annealing	Primer extension
ToxR	35	94˚Cfor 1 min	63˚C for 1 min	72˚C for 1 min
Tlh	35	94˚Cfor 1 min	65˚C for 1 min	72˚C for 1 min
Trh	35	94˚Cfor 1 min	55˚C for 1 min	72˚C for 1 min
Tdh	35	94˚Cfor 1 min	55˚C for 1 min	72˚C for 1 min
pirA^{vp}	30	94˚Cfor 5 min	53˚C for 30 sec	72˚C for 5 min

Antimicrobial susceptibility screening

The antibiotic susceptibility model shown by the recovered bacterial isolates was determined using commercial antibiotic disks (HI MEDIA, MUMBAI) following the Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines by the Kirby Bauer disc diffusion method (Bauer *et al.*, 1996). A total of 13 antibiotics, *viz.*, Amoxyclav (AMC 30 mcg/disc), Nitrofurantoin (NIT 300 mcg/disc), Gentamicin (GEN 10 mcg/disc), Norfloxacin (NX 10 mcg/disc), Chloramphenicol (C 30 mcg/disc), Ciprofloxacin (CIP 5 mcg/disc), Erythromycin (E 15 mcg/disc), Polymyxin-B (PB 300 units/disc), Streptomycin (S 10 mcg/disc), Tetracycline (TE 30 mcg/disc), Neomycin(N 10 mcg/disc), Oxytetracycline (O 30 mcg/disc) and Nitrofurazone (NR 100 mcg/disc) were used for the present study. A bacterial inoculum, taken from TSB (with 3% salt) after overnight culture, meeting the turbidity equivalent standards of 0.5 McFarland, was used for the performance of susceptibility testing. The quality control organism used for this antibiotic susceptibility test was *Escherichia coli* ATCC 25922.

Multiple Antibiotic Resistance Index

Multiple Antibiotic resistances (MAR) index was checked for those isolates which expressed resistance to more than three antibiotics (Krumperman, 1985). MAR index = a/b where 'a' is the number of antibiotics to which the isolate shows resistance and 'b' is the number of antibiotics to which the isolate was exposed. MAR index value greater than 0.2 is considered to have originated from massive risk sources of contamination (Preena *et al.,* 2020). MAR index value of less than or equal to 0.2 is regarded to have originated from the least contaminated sources.

Results and discussion

Resolution, phenotypic and molecular confirmation of isolates

The characteristic bluish-green colonies of *V. parahaemolyticus* in TCBS agar and VP medium were subjected to morphological and biochemical characterization to confirm the presence of *V. parahaemolyticus*. Out of the 126 presumptive isolates obtained from various shrimp samples, only 121 isolates showed typical biochemical characteristics of *V. parahaemolyticus*. *Vibrio species* are heterogenous gram negative, comma-shaped bacteria responsible for causing vibriosis in aquaculture systems. Isolates showed characteristic positive results for indole, oxidase, catalase and gelatinase test. Arginine and ornithine decarboxylase tests were also positive. Regardless of their ability to produce TDH or TRH, *toxR* is a regulatory gene possessed by all strains of *V. parahaemolyticus,* which is considered a species-specific marker for the confirmation of the organism (Kim *et al.*, 1999). The thermolabile hemolysin gene *tlh* was previously used as species specific marker to identify *V. parahaemolyticus* (Bej *et al.*, 1999). In agreement with this, all the strains of *V. parahaemolyticus* obtained were screened for the same, and most were found positive. The PCR results admit that among the 126 isolates screened, 121 showed positive results for the presence of characteristic species-specific genes of *toxR* and *tlh,* indicating the presence of *V. parahaemolyticus* (Table 3). The representative gel pictures showing PCR amplification of *toxR* and *tlh* genes are depicted in Fig. 1&2.

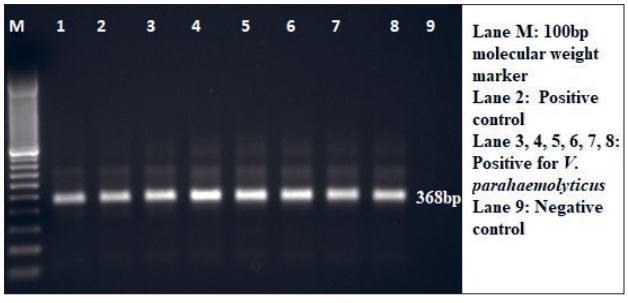

Fig. 1. Representative gel picture showing PCR amplification for *toxR* gene of *V. parahaemolyticus*

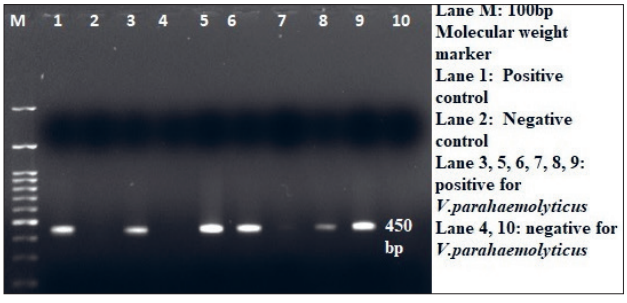

Fig. 2. Representative gel picture showing PCR amplification of *tlh* gene of *V. parahaemolyticus*

Table 3. PCR results of the *V. parahemolyticus* isolates screened for the presence of *toxR* and *tlh* genes

Isolates code	toxR	tlh	Isolates code	toxR	tlh
V1	+	+	V44	+	+
V2	+	+	V45	+	+
V3	+	+	V46	+	+
V4	+	+	V47	+	+
V5	+	+	V48	+	+
V6	+	+	V49	+	+
V7	+	+	V50	+	+
V8	+	+	V51	+	+
V9	+	+	V52	+	+
V10	+	+	V53	+	+
V11	+	+	V54	+	+
V12	+	+	V55	+	+
V13	+	+	V56	+	+
V14	+	+	V57	+	+
V15	+	+	V58	+	+
V16	+	+	V59	+	+
V17	+	+	V60	+	+
V18	+	+	V61	+	+
V19	+	+	V62	+	+
V20	+	+	V63	+	+
V21	+	+	V64	+	+
V22	+	+	V65	+	+
V23	+	+	V66	+	+
V24	+	+	V67	+	+
V25	+	+	V68	+	+
V26	+	+	V69	+	+
V27	+	+	V70	+	+
V28	+	+	V71	+	+
V29	+	+	V72	+	+
V30	+	+	V73	+	+
V31	+	+	V74	+	+
V32	+	+	V75	+	+
V33	+	+	V76	+	+
V34	+	+	V77	+	+
V35	+	+	V78	+	+
V36	+	+	V79	+	+
V37	+	+	V80	+	+
V38	+	+	V81	+	+
V39	+	+	V82	+	+
V40	+	+	V83	+	+
V41	+	+	V84	+	+
V42	+	+	V85	+	+
V43	+	+	V86	+	+
			V87	+	+

Isolates code	toxR	tlh
V88	+	+
V89	+	+
V90	+	+
V91	+	+
V92	+	+
V93	+	+
V94	+	+
V95	+	+
V96	+	+
V97	+	+
V98	+	+
V99	+	+
V100	+	+
V101	+	+
V102	+	+
V103	+	+
V104	+	+
V105	+	+
V106	+	+
V107	+	+
V108	+	+
V109	+	+
V110	+	+
V111	+	+
V112	+	+
V113	+	+
V114	+	+
V115	+	+
V116	+	+
V117	+	+
V118	+	+
V119	+	+
V120	+	+
V121	+	+
V122	-	-
V123	-	-
V124	-	-
V125	-	-
V126	-	-

* note: (+) = present ; (-) = absent

Pathogenicity detection

The virulence factors owing to the pathogenicity of *V. parahaemolyticus* are TDH and TRH, encoded by the *tdh* and *trh* genes, respectively. Considering the pathogenic potential of this organism in causing gastroenteritis in humans, pathogenic genes contributing to this, like *tdh* and *trh* were also screened. Owing to the frequent rejection of seafood exported to EU countries, this bacterium has gained much public attention, which made us screen the pathogenicity in the samples obtained. Such pathogenic strains were almost nil in Ernakulam's shrimp culture environment as found in this study. This finding is in close agreement with the report that the environmental sample-arrived strains lack *tdh* and *trh* genes which are responsible for illness in humans and marine animals (Deepanjali *et al.*, 2005; Canizalez-Roman *et al.*, 2011; Gutierrez West *et al.*, 2013). This supports the earlier reports of Letchumanan *et al.* (2015), where only 6.5% (13/200) of the *V. parahaemolyticus* isolates collected from shellfish samples were *trh*-positive, and none of the samples was *tdh*-positive. Still, the pathogenicity of VP is complex and interactive (Sun *et al.*, 2019). In marine shrimp aquaculture, some strains of *V. parahaemolyticus* can cause Acute hepatopancreatic necrosis disease (AHPND), resulting in 100% mortality (Flegel, 2012; Burge *et al.*, 2014). AHPND strains of *V. parahaemolyticus* harbour a 70 kb conjugative plasmid carrying *pirA* and *pirB* genes encoding a binary Photorhabdus insect-related toxin A and B (*PirAB*) (Sirikharin *et al.*, 2015). However, the present study found that the strains of *V. parahemolyticus* present in the shrimp culture environment of Ernakulam do not belong to the AHPND type strain. This supports the report of (Navneeth *et al.*, 2020), where *V. parahemolyticus* isolates recovered from the aquaculture ponds of the Southeast coast of India belong to non-AHPND group. The study is a line with the findings of Das, *et al.* (2017) depicted that AHPND positive strains are not detected in *V. parahemolyticus* isolates of Sundarban, India. Ernakulam District of Kerala belongs to the southwest coast of India and our study, gives proof for the non-AHPND status of this area. Mortalities occurred in farmed shrimp in 2013 due to *V. parahaemolyticus* strains lacking *pirA* and *pirB* genes (Kumar *et al.*, 2014) highlights the significance of host-pathogen interactions of this non-AHPND and non-virulent *V. parahaemolyticus* in causing infection in shrimps and its risk for human infections is yet another big question of concern.

Antibiotic resistance study

Antibiotic resistance development in pathogenic bacteria due to the indiscriminate use of antibiotics in aqua farms gives rise to serious problems in the treatment of infectious diseases (Preena *et al.*, 2020). Being a versatile halophilic organism in terms of host and habitat, *V. parahaemolyicus* can acquire genes, which can improve its fitness according to the environment (Karunasagar

et al., 2016). The acquisition of genomic islands and mobile genetic elements in isolates tested even negative for the pVA plasmid-encoded PirAB toxin(associated with AHPND) and shrimp virulence can lead to a possible transition of an environmental *V. parahaemolyticus* to emerge as pathogens of aquaculture species (Kattapuni *et al*., 2021). Antibiogram analysis of the isolated *V. parahaemolyticus* strains from different penaeid shrimps of various aquaculture systems of Ernakulam in the current study revealed the emergence of multidrug-resistant isolates in the aquatic environment (Table 4). The present study observed that the obtained non-pathogenic strains exhibited maximum resistance to the antibiotics tested. This, in one way, closely agrees with the findings of Soto-Rodriguez *et al*., 2018, which state that irrespective of the phenotypic similarity of the pathogenic and non-pathogenic strains, of *V. parahaemolyticus* strains, the pathogenic strains exhibited more sensitivity to tested antibiotics than non-pathogenic strains. Antimicrobials are mostly used as a prophylactic and therapeutic measure, not as a growth promoter in aquaculture (Cabello *et al*., 2013). The commonly allowed and used antibiotics in the Asian aquaculture industry to ensure continuous production of seafood are oxytetracycline, tetracycline, quinolone, sulphonamides, and trimethoprim (Rico *et al*., 2012; Yano *et al*., 2014). There are reports of isolation and detection of antimicrobial-resistant *V. parahaemolyticus* in Asian countries like Thailand (Yano *et al*., 2014), Malaysia (Al-Othrubi *et al*., 2011) and China (Xu *et al*., 2014). This occurrence has raised concern because of the increased amount of antibiotic-resistant pathogenic strains of *V. parahaemolyticus* in the environment towards clinically employed antibiotics.

The Fig. 3. describes the percentage of strains resistant to individual antibiotic types. Among the 121 isolates tested for antibiotic susceptibility, 82 isolates (68%) showed resistance to most of the antibiotics used in the study. Resistance to Amoxyclav was observed in 71% of the isolates. Amoxyclav (beta-lactams) were found as the most resistant antibiotic strains, which supports one latest finding in Malaysia (Tan *et al*., 2020), where *V. parahaemolyticus* isolated from different seafood samples exhibited high resistance to beta-lactam antibiotics, including penicillins and cephalosporins. Even this beta-lactamase inhibitor combination (Amoxicillin / clavulanate) was less effective in treating *V. parahaemolyticus* infections in our study. Many previous reports revealed high beta-lactam resistance exhibited by *V. parahaemolyticus* isolates (Molina-Aja *et al*., 2002; Manjusha *et al*., 2005; Devi *et al*., 2009; Silvester *et al*., 2015). In contrast, 100% susceptibility was observed towards chloramphenicol, norfloxacin and ciprofloxacin (91%), polymixin B and erythromycin (86%), gentamycin and nitrofurantoin (81%) (Xu *et al*., 2016; Tan *et al*., 2020). Our findings are in line with these previous reports of antibiotic susceptibility of *V. parahaemolyticus* towards chloramphenicol, ciprofloxacin, gentamycin, erythromycin and nitrofurantoin. A prior study along the southwest coast of India also reported the sensitivity of *V. parahaemolyticus* towards nitrofurantoin and trimethoprim (Devi *et al*., 2009).

Table 4. Antibiotic resistance profile of 121 isolates of *V. parahaemolyticus* from shrimp farms of Ernakulam, India

Isolates code	Antibiotics (mcg/ml)												
	CIP	AMC	S	N	NIT	E	PB	TE	O	NX	NR	C	GEN
V1	_	R	R	_	_	_	R	_	_	_	_	_	_
V2	_	R	R	R	_	_	_	_	R	_	R	_	R
V3	_	R	R	R	_	_	_	_	R	_	_	_	_
V4	_	R	R	_	_	_	_	_	_	_	_	_	_
V5	_	R	_	_	_	_	_	_	_	_	R	_	_
V6	R	R	R	R	R	_	R	_	_	_	_	_	_
V7	_	R	_	_	_	_	_	_	_	_	R	_	_
V8	_	R	R	_	_	_	_	_	_	_	_	_	_
V9	_	_	_	_	R	_	_	R	_	_	_	_	_
V10	_	_	_	_	_	_	_	_	_	_	_	_	_
V11	_	_	_	_	R	_	_	R	R	_	_	_	_
V12	_	R	_	_	_	_	_	_	_	_	_	_	_
V13	_	R	_	_	_	_	_	R	R	_	R	_	_
V14	R	R	_	_	_	_	_	R	R	_	R	_	R
V15	_	R	_	R	_	R	_	R	R	_	R	_	_
V16	_	R	R	_	_	R	R	R	R	R	R	_	_

Isolates code	Antibiotics (mcg/ml)												
	CIP	AMC	S	N	NIT	E	PB	TE	O	NX	NR	C	GEN
V17	_	_	_	_	R	_	_	R	R	_	R	_	_
V18	_	_	_	_	_	_	_	R	R	_	R	_	_
V19	_	_	R	_	_	_	_	_	_	_	_	_	_
V20	_	R	R	R	_	R	_	_	_	R	R	_	R
V21	R	R	R	R	_	_	_	_	_	_	_	_	R
V22	_	R	_	_	_	R	_	_	R	_	_	_	_
V23	_	_	R	_	_	_	R	_	R	_	R	_	_
V24	_	R	_	R	_	_	_	R	_	_	R	_	_
V25	_	_	R	R	_	_	_	R	_	R	R	_	R
V26	_	R	_	R	_	R	_	_	R	_	_	_	_
V27	_	R	R	_	R	_	_	R	_	_	_	_	_
V28	R	_	R	R	_	_	_	R	_	_	_	_	_
V29	_	_	R	_	_	_	_	R	R	_	_	_	R
V30	_	R	_	R	_	_	_	_	_	_	R	_	_
V31	_	_	_	_	R	R	_	_	R	R	_	_	_
V32	_	R	R	_	_	_	R	_	_	_	R	_	_
V33	R	_	R	R	_	_	_	_	R	_	R	_	_
V34	_	R	R	_	_	_	_	R	_	_	_	_	R
V35	_	_	R	_	R	R	_	_	R	_	R	_	_
V36	R	_	_	_	_	_	_	_	R	R	R	_	_
V37	_	R	R	_	_	R	_	_	R	_	_	_	_
V38	_	_	R	_	_	_	R	_	R	_	R	_	_
V39	_	_	R	_	R	_	_	_	_	_	R	_	R
V40	_	R	R	R	_	_	_	R	_	_	_	_	_
V41	_	R	_	_	_	_	_	_	R	R	_	_	_
V42	_	R	R	_	R	_	_	_	R	_	_	_	_
V43	_	R	R	_	_	R	_	_	_	_	R	_	_
V44	_	_	R	R	_	_	_	_	R	_	R	_	R
V45	R	_	R	_	_	_	R	_	_	_	R	_	_
V46	_	R	_	_	R	_	_	_	R	_	R	_	_
V47	_	R	_	_	_	_	R	_	_	_	R	_	_
V48	R	_	_	R	_	_	_	R	_	_	R	_	R
V49	_	R	_	_	R	_	_	_	_	R	R	_	_
V50	_	R	R	_	_	R	_	_	R	_	_	_	_
V51	_	R	_	_	_	R	_	R	R	_	_	_	_
V52	_	_	R	R	R	R	_	_	R	_	R	_	_
V53	_	R	_	_	_	_	R	_	_	_	R	_	_
V54	R	R	R	_	_	_	_	_	_	R	_	_	_
V55	_	R	R	_	_	_	R	_	R	_	_	_	_
V56	_	R	R	_	_	_	R	_	_	_	R	_	_
V57	_	_	R	_	_	_	_	R	_	_	R	_	R
V58	_	_	R	R	_	_	_	R	_	_	R	_	_
V59	_	R	R	_	R	_	_	_	R	_	_	_	_

Isolates code	Antibiotics (mcg/ml)												
	CIP	AMC	S	N	NIT	E	PB	TE	O	NX	NR	C	GEN
V60	_	R	_	_	_	_	_	_	R	R	_	_	_
V61	_	R	_	R	_	_	_	_	_	_	R	_	R
V62	_	_	_	R	R	R	_	R	_	_	R	_	_
V63	_	R	_	R	_	_	_	R	_	_	R	_	_
V64	_	R	R	_	_	_	R	_	_	_	R	_	_
V65	_	R	_	_	_	R	_	_	_	_	R	_	R
V66	_	R	R	R	_	R	_	_	_	_	_	_	_
V67	_	R	R	_	R	_	_	_	R	_	_	_	_
V68	_	R	R	_	_	_	R	_	R	_	_	_	_
V69	_	R	_	R	_	_	_	R	_	_	R	_	_
V70	_	R	_	_	R	_	_	_	R	_	R	_	_
V71	_	R	_	_	_	_	R	_	R	_	_	_	_
V72	_	R	R	_	_	_	R	_	_	_	R	_	_
V73	_	R	R	R	_	_	_	R	_	_	_	_	_
V74	R	R	R	_	_	_	_	R	_	_	_	_	_
V75	_	R	_	_	R	_	_	R	_	_	R	_	_
V76	_	R	R	_	R	_	_	_	_	_	R	_	_
V77	_	_	R	_	_	_	_	R	_	_	R	_	R
V78	_	R	_	R	_	_	_	_	R	_	_	_	_
V79	_	R	R	_	_	R	_	_	R	_	_	_	_
V80	_	R	_	_	_	R	_	_	R	_	_	_	_
V81	R	R	_	_	_	_	_	R	_	_	R	_	_
V82	_	_	R	_	_	_	_	R	_	_	R	_	R
V83	_	R	_	R	_	_	_	_	R	_	R	_	_
V84	_	R	_	R	R	_	_	_	_	_	R	_	_
V85	_	R	_	R	_	_	_	R	_	_	R	_	_
V86	_	R	R	_	_	_	_	R	_	_	_	_	R
V87	_	R	R	_	R	_	_	_	R	_	_	_	_
V88	_	R	_	_	_	_	R	_	R	_	_	_	_
V89	_	R	_	_	_	_	_	R	R	_	R	_	_
V90	_	R	_	_	_	_	_	R	R	_	R	_	_
V91	_	R	_	_	_	_	_	R	_	_	R	_	_
V92	_	R	R	_	_	_	_	R	_	_	_	_	R
V93	_	R	_	R	R	_	_	_	_	_	_	_	_
V94	_	R	R	_	_	_	_	_	_	_	R	_	_
V95	_	_	R	R	R	_	_	R	_	_	_	_	_
V96	_	R	R	_	_	_	_	_	_	_	R	_	R
V97	_	R	R	_	_	_	_	R	_	_	R	_	_
V98	_	R	_	R	_	_	_	_	R	_	_	_	_
V99	_	R	_	_	_	_	_	_	R	_	R	_	R
V100	_	R	R	_	_	_	_	R	_	_	R	_	_
V101	_	R	_	R	_	_	_	R	_	_	R	_	_
V102	_	R	_	_	_	_	_	R	_	R	_	_	R

Isolates code	Antibiotics (mcg/ml)												
	CIP	AMC	S	N	NIT	E	PB	TE	O	NX	NR	C	GEN
V103	_	R	R	_	_	_	_	R	_	_	_	_	R
V104	_	R	_	_	R	_	_	_	R	R	_	_	_
V105	_	_	_	R	_	_	_	_	R	_	_	_	_
V106	_	R	_	R	_	_	_	R	R	_	_	_	_
V107	_	R	_	R	_	_	_	_	R	_	_	_	_
V108	_	R	_	_	_	_	_	R	R	_	_	_	R
V109	_	_	_	_	_	_	_	R	R	_	_	_	_
V110	_	R	_	_	_	_	_	R	R	_	_	_	_
V111	_	_	_	_	_	_	_	R	R	_	_	_	R
V112	_	_	_	_	_	_	R	_	R	_	_	_	_
V113	_	R	_	_	R	_	_	_	_	_	_	_	_
V114	_	R	_	_	_	_	_	_	_	_	_	_	_
V115	_	R	_	_	_	_	_	_	_	_	_	_	_
V116	_	_	R	_	_	_	_	_	_	_	_	_	_
V117	_	R	R	_	_	_	_	_	_	_	_	_	_
V118	_	_	_	_	_	_	_	_	_	_	_	_	_
V119	_	_	_	_	_	_	_	_	_	_	_	_	_
V120	_	_	_	_	_	_	_	_	_	_	_	_	_
V121	_	_	_	_	_	_	_	R	_	_	_	_	_

*note: (R) = resistant; (-) = sensitive/ intermediate

According to our research, Chloramphenicol is highly effective in treating *V. parahaemolyticus* infections, as 100% susceptibility is exhibited by almost all the isolates studied.

The multiple antibiotic resistances (MAR) pattern of isolates was measured, and the index is presented in Fig 4. MAR index value of more than 0.2 is believed to have originated from high-risk sources of contamination (Paul *et al.*, 1997). The MAR index of most isolates was in the range of 0.15-0.46, but was as high as 0.61 in one isolate resistant to 8 antimicrobials. However, depending on the number of antibiotics tested and the area of study, MAR indices vary and are unsuitable for comparison between two locations (Tan *et al.*, 2020). In this study, the MAR index value of almost half of the isolates was greater than or equal to 0.2, suggesting that most shrimps were extensively exposed to antimicrobials. The study area is Ernakulam District of Kerala, India, where the shrimp culture environment is greatly influenced by urban, industrial, human

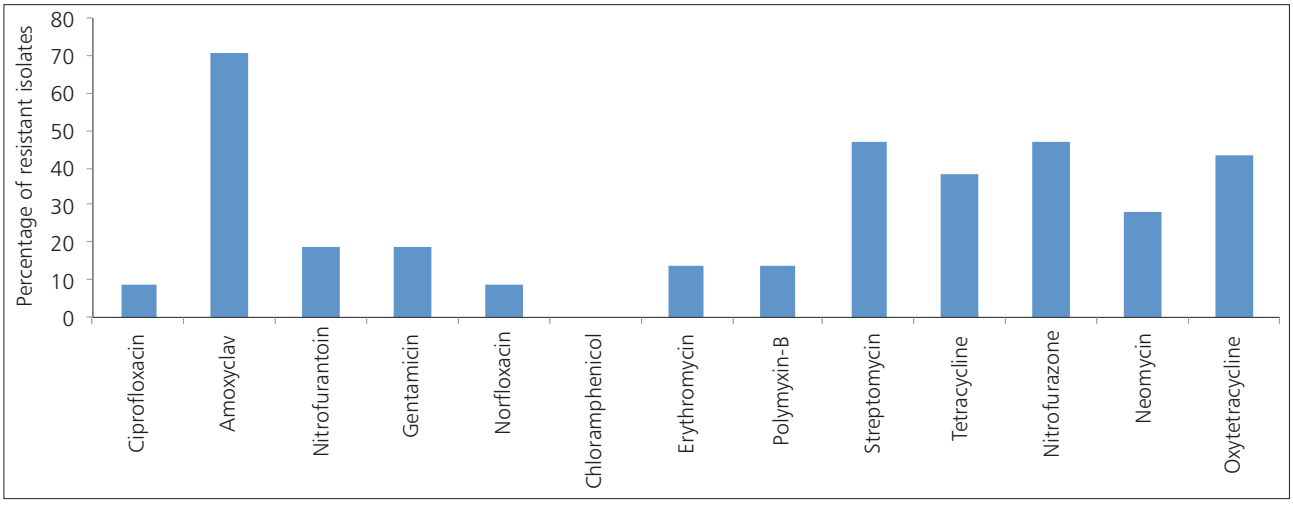

Fig. 3. Percentage of *V. parahaemolyticus* strains resistant to antibiotics tested in this study

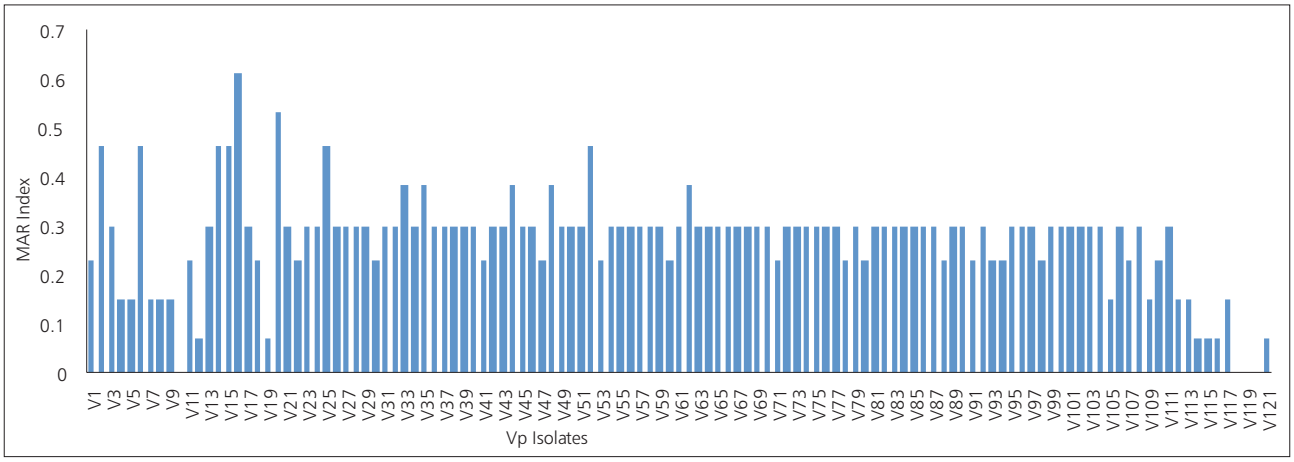

Fig. 4. MAR index of all the 121 isolates of *V. parahemolyticus* obtained from shrimp farms

and hospital wastewater. Most of the shrimps cultured here are taken to the export market; thus utmost care is needed to avoid contamination of the samples with these multidrug-resistant isolates.- There is always a risk for the multidrug-resistant genes to get transferred to other bacteria, including those which are pathogenic to humans due to the horizontal spread of the plasmids. Therefore the progressive increase in antibiotic resistance in the shrimp culture environment is a critical area of concern. AMR's status needs to be monitored well in a continuous manner.

In conclusion, the present study is supporting evidence that the tropical shrimp culture environment greatly favours the growth of *V. parahaemolyticus* and is autochthonous in the shrimp culture environment of India, especially Kerala. The study also confirmed the non-prevalence of AHPND strains in the traditional shrimp farms of Ernakulam, India and the absence of human pathogenic strains of *V. parahemolyticus* in the aquaculture environment of the study area. Even though the ability to acquire virulent genes to improve the host adaptation fitness of *V. parahaemolyticus* cannot be ruled out. As the pathogenesis of *tdh* and *trh* negative strains of *V. parahaemolyticus* remains a big question, the topic of this study is very relevant considering the risk of infection caused by these non-AHPND and non-virulent strains of VP in the shrimp culture environment. Additionally, the analysis is critical from the point of view of antimicrobial resistance, a critical area of concern. Multidrug resistance of 68% of the VP strains obtained in this study indicated a significant threat to human health and the risk of cultured penaeid shrimps to acting as vehicles of *V. parahaemolyticus* resistant to beta-lactams. Though the present study reports the existence of non-virulent but multidrug-resistant *V. parahaemolyticus*, the possible transition of this autochthonous organism to a virulent pathogen by acquiring genomic islands and mobile genetic elements cannot be excluded. The study demands extended surveillance in this region and continuous monitoring of *V. parahaemolyticus* strains, pathogenicity mechanisms and their susceptibility to antibiotics to ensure human health and food safety.

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