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Isolation, characterization, virulence genes, antimicrobial resistant genes, and antibiotic susceptibility pattern of *Vibrio parahaemolyticus* in relation to AHPND from shrimp farms in coastal districts of Tamil Nadu

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Abstract

Vibrio parahaemolyticus is a gram-negative motile bacterium inhabiting marine, estuarine, and coastal environments. It is a critical aquatic pathogen in shrimp farming, and strains possessing pir A and pir B toxins can cause acute hepatopancreatic necrosis disease (AHPND) in shrimps. The study aimed to isolate *V. parahaemolyticus* from the gut and hepatopancreas of shrimps from the selected districts of Tamil Nadu for a duration of 6 months from December 2022 to May 2023 by random sampling, and thirty-two strains were confirmed as *V. parahaemolyticus* from 110 presumptive bacterial isolates. All 32 isolates used in the AP4 PCR protocol failed to detect any isolates carrying AHPND pir toxins (pir A and pir B). Forty-four percent of isolates have shown β-haemolytic activity on blood agar. Of the 32 isolates, two, eleven, and twenty-nine harboured *tdh*, *trh*, and T3SS1 genes, respectively, and none of them possessed T3SS2 gene. Isolates of *V. parahaemolyticus* were resistant to gentamycin, vancomycin, and erythromycin and highly susceptible to ciprofloxacin, ofloxacin, chloramphenicol, amoxyclav, trimethoprim, streptomycin, cefoxitin, and ceftazidime. AMR genes encoding *qnrA*, *tet A*, bla_{SHV}, and *aac-3-IIa* were present in 6%, 16%, 16%, and 22% of the isolates. AMR genes *catA1* and *str B* were negative for all 32 isolates. It is concluded that the prevalence and incidence of *V. parahaemolyticus* were 29% in the studied coastal districts of Tamil Nadu. All isolates were found to be negative for AHPND. Hence, shrimp farms in the studied area were free from infection of AHPND.

Keywords AHPND · *Vibrio parahaemolyticus* · AMR · *tdh* · *trh*

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Highlights
- Isolates of *V. parahaemolyticus* possess virulence genes such as *tdh*, *trh*, and T3SS1.
- All isolates were found to be negative for AHPND.
- Most isolates were resistant to gentamycin, vancomycin, and erythromycin, with AMR genes such as *tet A*, bla_{SHV}, *aac-3-IIa*, and *qnrA*.

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Introduction

In aquaculture, crustaceans are economically significant and monumental in demand for products with high unit value. Crustacean production has risen continuously from 2000 onwards with a growth rate of 9.9%/year and reached 8.4 MT with a value of USD 61.06 billion (FAO 2022). In crustaceans, Pacific white-leg shrimp (*Penaeus vannamei*) is a more popular and demanding product with a unit value of USD 26.7 billion, always making first place on the table in both quality and quantity (Kumar et al. 2021). India contributes significantly to global shrimp aquaculture output, and just one product accounts for more than 70% of the nation's whole value of seafood exports (Navaneeth et al. 2020). In Indian shrimp culture production, *P. vannamei* is a consequential attribute as a noteworthy contributor. With rising demand, the shrimp culture industry intensified, and high scaling growth increased the risk of disease exposure to aquaculture enterprises. It resulted in socioeconomic loss and hindered the shrimp culture industry's growth. Over the past few years, the emergence of acute hepatopancreatic necrosis disease (AHPND) has inflicted severe economic losses on the global shrimp farming industry. AHPND is caused by *Vibrio parahaemolyticus* with a particular strain containing two *Photorhabdus* insect–related (Pir) toxins, pir A and pir B. A bacterial disease known as AHPND has washed out farmed populations of Pacific white-leg shrimp and black tiger shrimp (*P. monodon*) (Tang and Bondad-Reantaso 2019). The first outbreak of AHPND originated in China in 2009, and it gradually spread among other Asian countries, including Vietnam (2010), Malaysia in 2011, Thailand in 2012, the Philippines in 2015, Mexico in 2013, and South America (2016) (Ananda Raja et al. 2017b; Muthukrishnan et al. 2019). Within 25–30 days of stocking PL, mortality started, and occasionally, mass mortality was also observed 10 days after PL stocking. As a result of these characteristics, this disease was previously called "early mortality syndrome" (EMS). Approximately 60% of shrimp production declined in the AHPND-affected areas compared with the 2012 production data and an annual economic loss of USD 1 billion globally (FAO 2013).

V. parahaemolyticus is a gram-negative halophilic, tiny rod and motile bacterium belonging to the *Vibrionaceae* family inhabiting marine, estuarine, and coastal environments (Su and Liu 2007; Wang et al. 2011; Zhang and Orth 2013). It has two major virulence systems: haemolysin and type III secretion system. Haemolysin comprises *tdh*, *trh*, and *tlh*, while T3SS is T3SS1, T3SS2α, and T3SS2β. The virulence factors responsible for *V. parahaemolyticus*' haemolysis and cytotoxicity in host cells are *tdh* and *trh* (Broberg et al. 2011). *V. parahaemolyticus* possesses two distinct sets of type III secretion systems (T3SSs) situated on two separate chromosomes: T3SS1 on chromosome 1 and T3SS2 on chromosome 2 (Calder et al. 2014). T3SS1 primarily plays a role in cytotoxicity, whereas T3SS2 causes enterotoxicity (Hiyoshi et al. 2011). Such virulence genes are responsible for the pathogenicity of *V. parahaemolyticus*.

The study aims to isolate *V. parahaemolyticus* from the gut and hepatopancreas of shrimps from the selected districts of Tamil Nadu and screen the isolates for the occurrence of virulence genes with special reference to APHND pir toxin and the antimicrobial resistance genes.

Materials and methods

Sample collection and isolation of bacteria

Samples were collected from shrimp farms in four coastal districts of Tamil Nadu (Nagapattinam, Pudukkottai, Cuddalore, and Ramanathapuram) from December 2022 to May 2023

(Fig. 1). *P. vannamei* was specifically selected for this study due to its present share as highly cultured shrimp in India. All samples were aseptically collected and transported in chilled conditions using a thermally insulated box within 24 h to the bacteriology laboratory, Department of Fish Pathology and Health Management, Fisheries College and Research Institute Thoothukudi for further analysis.

The hepatopancreas and gut of shrimps were dissected aseptically for all the samples and streaked on thiosulfate citrate bile salt sucrose (TCBS) agar (HiMedia, Mumbai) plates using a sterile loop. Plates were incubated at 32 °C overnight. Presumptive green colonies on TCBS agar plates were again streaked and purified on TSAS (trypticase soy agar with 2% salt) (HiMedia, Mumbai) plates (Canizalez-Roman et al. 2011). Pure bacterial isolates were preserved in TSBS (tryptic soy broth with 2% salt) (HiMedia, Mumbai) containing 20% glycerol at − 80 °C (Fadanka et al. 2022).

Phenotypic characterization of bacteria

Phenotypic characterization and identification of bacterial isolates were performed using the standard protocol (Brenner et al. 2005; Ananda Raja et al. 2017a, c). A series of tests were carried out, such as gram stain, motility, catalase, oxidase, citrate, urease, Voges and Proskauer (VP), methyl red (MR), sugar fermentation (glucose, arabinose, sucrose, lactose, raffinose, galactose, rhamnose), decarboxylase (arginine, ornithine, lysine), and growth at 0, 3, 6, and 8% NaCl concentrations. To identify *V. parahaemolyticus*, all test media were enriched with 2% NaCl, and the experiments were duplicated and then incubated at 32 °C overnight.

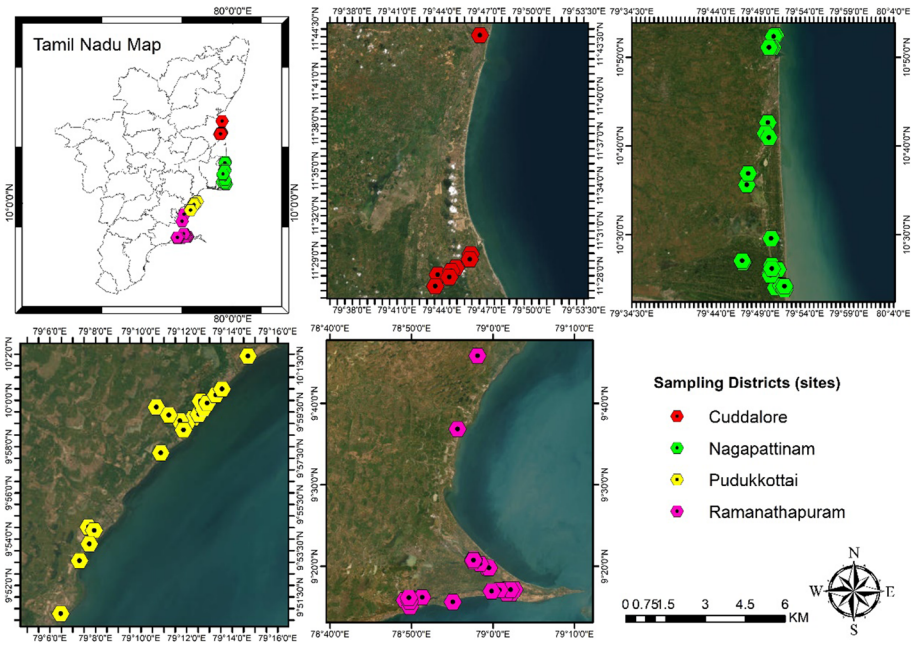

Fig. 1 Location of sampling sites

DNA extraction from bacteria

Pure cultures of bacterial isolates were inoculated into 5 ml of TSBS and incubated for 24 h at 32 °C. After 24 h, 1 ml of culture was taken aseptically and centrifuged at 8000 × *g* for 10 min, and the bacterial pellet was obtained by discarding the supernatant. Two hundred microliters of DNA-XPress Reagent (HiGenoMB, HiMedia, Mumbai) was added to the bacterial pellet. The mixture was thoroughly mixed and incubated at 60 °C for 1 h. It was then centrifuged at 10,000 × *g* for 10 min, and the supernatant was carefully transferred to a fresh sterile tube. An equivalent volume of absolute alcohol (200 µl) was added to the supernatant, followed by centrifugation at 10,000 × *g* for 5 min. The supernatant was discarded, and the DNA pellet was washed twice with 200 µl of 95% alcohol, followed by centrifugation at 8000 × *g* for 5 min each time. After removing the supernatant, the DNA pellet was allowed to air dry for 5 min, and 75 µl of nuclease-free water was added and stored at 4 °C for further use.

Detection of virulence and AMR genes by polymerase chain reaction (PCR)

The PCR amplification of all reactions was carried out using a thermal cycler (Bio-Rad, USA) in a 25 µl reaction mixture with 2 µl each forward and reverse primer, 6.5 µl PCR grade water, 12.5 µl master mix, and 2 µl template DNA. The bacterial isolates were confirmed as *V. parahaemolyticus* following standard protocol and previously published primer *VpM*, *toxR*, and *GyrB* (Bauer and Rørvik 2007; Luan et al. 2007), as in Table 1. The PCR amplification of putative virulence genes of *V. parahaemolyticus* such as thermostable direct haemolysin (*tdh*), tdh-related haemolysin (*trh*), thermolabile haemolysin (*tlh*), type III secretion system one (T3SS1), type III secretion system two alpha (T3SS2α), and type III secretion system two beta (T3SS2β) was carried out using appropriate primers and protocols as mentioned in Table 1. Detection of antimicrobial resistance genes encoding resistance to β-lactams (bla_{SHV}), aminoglycosides (*aac-3-IIa*), chloramphenicol (*catA1*), streptomycins (*str B*), quinolones (*qnrA*), and tetracyclines (*tet A*) was done as per standard primers listed in Table 1. All 32 confirmed *V. parahaemolyticus* isolates were screened for the presence of virulence and AMR genes enlisted above. In a similar way, the confirmed *V. parahaemolyticus* isolates were tested for AHPND using the standard AP4 protocol (Dangtip et al. 2015), cited in Table 1. Shrimps were screened for the presence of WSSV, IHHNV, TSV, IMNV, YHV, and DIV 1 as per the WOAH diagnostic manual to get a specific study on AHPND and *V. parahaemolyticus* without any association of any other bacterial/viral disease.

Determination of haemolytic activity

Blood agar plates (HiMedia, Mumbai) were used for the haemolytic activity. Plates were inoculated with a sterile loop from 16 h young bacterial cultures. After inoculation, plates were incubated for 24 h at 32 °C.

Antibiotic susceptibility test

The standard disc diffusion method (Bauer et al. 1966) was used to study the antibiotic susceptibility of *V. parahaemolyticus* isolates. According to the Kirby-Bauer method, plates were prepared with Mueller Hinton agar (MHA) (HiMedia, Mumbai). The purified bacterial isolates were inoculated in TSBS and incubated at 32 °C for 8 h until light to moderate turbidity

Table 1 Primers used for PCR reactions to identify the presence of virulence genes and, AMR genes of *V. parahaemolyticus*

SN	Gene/primer	Primer Sequence (5′–3′)	Amplicon size (bp)	Annealing temperature	References
1	*VpM*	F-CAGCTACCGAAACAGACGCTA R-TCCTATCGAGGACTCTCTCAAC	675	60	Luan et al. (2007)
2	*GyrB*	F-CGGCGTGGGTGTTTCGGTAGT R-TCCGCTTCGCGCTCATCAATA	285	58	Luan et al. (2007)
3	*toxR*	F-GASTTTGTTTGGCGYGARCAAGGTT R-GGTTCAACGATTGCGTCAGAAG	297	55	Bauer and Rørvik (2007)
4	*tdh*	F-GTAAAGGTCTCTGACTTTTGGAC R-TGGAATAGAACCTTCATCTTCACC	251	55	Tada et al. (1992)
5	*trh*	F-TTGGCTTCGATATTTTCAGTATCT R-CATAACAAACATATGCCCATTTCC	373	55	Tada et al. (1992)
6	*tlh*	F-GTTCTTCGCCAGTTTTGCGT R-GACATTACGTTCTTCGCCGC	354	54	Lee et al. (2015)
7	*vscP* (T3SS1)	F-ACCGATTACTCAAGGCGATG R-TACGTTGTTGGCGTGATTGT	392	60	Noriea et al. (2010)
8	vopB2 (T3SS2α)	F-CTGCAGGTATCGCATCTTCA R-TTAGAACCAACCGACGAAGC	250	60	Noriea et al. (2010)
9	vopB2 (T3SS2β)	F-GAGCCTGTTGCTCTATGGAGCCAGG R-CGACACAGAACGCAATGCTTGCTCG	942	60	Noriea et al. (2010)
10	AP4	F1-ATGAGTAACAATATAAAACATGAAAC R1-ACGATTTCGACGTTCCCCAA	1269	55	Dangtip et al. (2015)
		F2-TTGAGAATACGGGACGTGGG R2-GTTAGTCATGTGAGCACCTTC	230	55	
11	*tetA*	F-GTGAAACCCAACATACCCC R-GAAGGCAAGCAGGATGTAG	888	50	Gow et al. (2008)
12	bla_{SHV}	F-TCGCCTGTGTATTATCTCCC R-CGCAGATAAATCACCACAATG	768	52	Van et al. (2008)

Table 1 (continued)

SN	Gene/primer	Primer Sequence (5′–3′)	Amplicon size (bp)	Annealing temperature	References
13	*aac-3-IIa*	F-CGGAAGGCAATAACGGAG R-TCGAACAGGTAGCACTGAG	740	50	Gow et al. (2008)
14	*str B*	F-TATCTGCGATTGGACCCTCTG R-CATTGCTCATCATTTGATCGGCT	538	60	Sunde and Norström (2005)
15	*qnrA*	F-GGGTATGGATATTATTGATAAAG R-CTAATCCGGCAGCACTATTTA	670	50	Mammeri et al. (2005)
16	*catA1*	F-AGTTGCTCAATGTACCTATAACC R-TTGTAATTCATTAAGCATTCTGCC	547	55	Van et al. (2008)

developed. Inoculum turbidity was compared with standard 0.5 McFarland (0.5 ml 1.175% barium chloride and 99.5 ml 0.36 N sulphuric acid) and adjusted to 1 Optical Density (Ananda Raja et al. 2017a). A sterile, non-toxic cotton swab on a wooden applicator was dipped into an OD-adjusted inoculum containing a young bacterial culture. The entire agar surface of the MHA plate was streaked with a swab three times, and the plate was turned at a 60° angle between each streaking to obtain complete lawn culture. The plates with inoculum were allowed to dry for 10 min with the lid in place. Antibiotic discs (HiMedia, Mumbai) were placed aseptically with at least 30 mm apart centres. The plates were incubated at 32 °C overnight and examined for the size of the inhibition zone (Ananda Raja et al. 2017a). Fourteen antibiotics of 11 different classes were used for this study such as β lactam—amoxyclav (25 µg); second-generation cephalosporin—cefoxitin (30 µg); third-generation cephalosporin—ceftazidime (30 µg); fourth-generation cephalosporins—cefepime (30 µg); aminoglycosides—gentamycin (30 µg), streptomycin (10 µg), and kanamycin (5 µg); quinolones and fluoroquinolones—ciprofloxacin (30 µg) and ofloxacin (5 µg); tetracyclines—oxytetracycline (30 µg); sulphonamides—trimethoprim-sulphamethoxazole (25 µg); phenicol—chloramphenicol (30 µg); glycopeptides—vancomycin (30 µg); and macrolides—erythromycin (10 µg). The inhibition zone diameter (millimeter) was recorded and interpreted according to the Clinical and Laboratory Standard Procedure (CLSI) 2020 guidelines. The multiple antibiotic or drug resistance (MAR) was also calculated based on the procedure (Krumperman 1983). A heatmap showing an antibiogram was prepared using OriginPro 2023b.

Results

Prevalence, identification, and characterization of *V. parahaemolyticus*

Over a period of 6 months from December 2022 to May 2023, a total of 67 shrimp samples were collected from four coastal districts of Tamil Nadu (Nagapattinam, Pudukkottai, Cuddalore, and Ramanathapuram) and processed for the isolation of *V. parahaemolyticus*. One hundred ten bacterial isolates were obtained from 67 shrimp samples using the conventional method (TCBS agar) and subsequently confirmed for *V. parahaemolyticus* using biochemical and molecular methods.

Phenotypic tests demonstrated that all *V. parahaemolyticus* isolates were gram-negative, rod-shaped motile, catalase, oxidase, urease positive and glucose, arabinose, galactose fermentative whereas non-fermentative for sucrose, lactose, and raffinose.

Following biochemical and PCR tests, 32 of 110 isolates were found to be positive for *V. parahaemolyticus* by primer *VpM*, *toxR*, and *GyrB* (Fig. 2). Of the confirmed 32 *V. parahaemolyticus* strains, 9 were isolated from the gut, and the remaining were from the hepatopancreas.

PCR detection of virulence, AMR genes, and AHPND

Results of haemolysin virulence genes showed that 2 (6%) and 11 (34%) of *V. parahaemolyticus* isolates possessed *tdh* and *trh* genes, while all the 32 (100%) isolates harboured *tlh* gene (Fig. 3). Regarding the type III secretion system, the T3SS1 gene was present in 29 (91%) isolates (Fig. 3); none of the 32 possessed the *T3SS2* gene (both T3SS2α and T3SS2β). AMR genes such as tetracycline (*tet A*), beta-lactam (bla_{SHV}), chloramphenicol (*catA1*),

streptomycin (*str B*), aminoglycosides (*aac-3-IIa*), and quinolinones (*qnrA*) were studied and 2 (6%), 5 (16%), 5 (16%), and 7 (22%) isolates carried *qnrA*, *tet A*, bla_{SHV}, and *aac-3-IIa* genes, respectively. However, genes *catA1* and *str B* were negative for all 32 isolates.

All the confirmed *V. parahaemolyticus* isolates were screened for AHPND pir toxins to check the presence of acute hepatopancreatic necrosis disease (AHPND) using AP4 protocol. AHPND pir toxin was absent in all the 32 *V. parahaemolyticus* studied. WOAH-listed diseases such as WSSV, IHHNV, TSV, IMNV, YHV, and DIV 1 were screened for their presence, and all the samples were tested negative for enlisted diseases by PCR.

Determination of haemolytic activity

The haemolytic activity on blood agar was studied according to the protocol of Twedt et al. (1970), and isolates were recorded as β-haemolytic positive with a clear zone around the

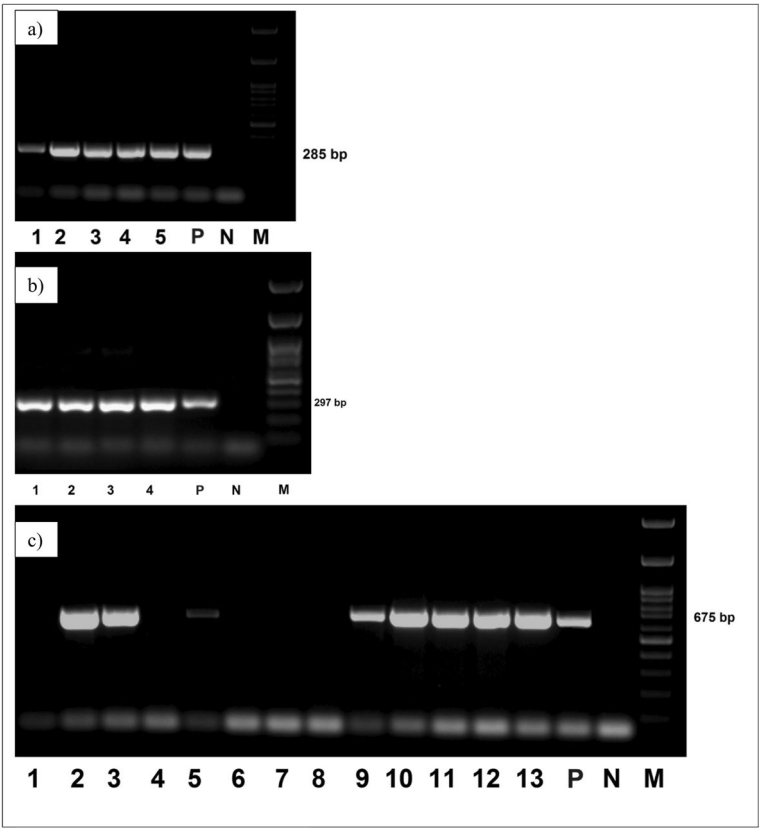

Fig. 2 *V. parahaemolyticus* confirmation by amplification of different gene-specific primers such as *GyrB*, *VpM*, and *toxR*. **a** *GyrB* lanes—M, molecular weight marker; 1–5, representative isolates; P, positive control; N: negative control. **b** *toxR* lanes—M, molecular weight marker, 1–4: representative isolates; P, positive control; N, negative control. **c** *VpM* lanes—M, molecular weight marker, 1–13: representative isolates; P, positive control; N, negative control

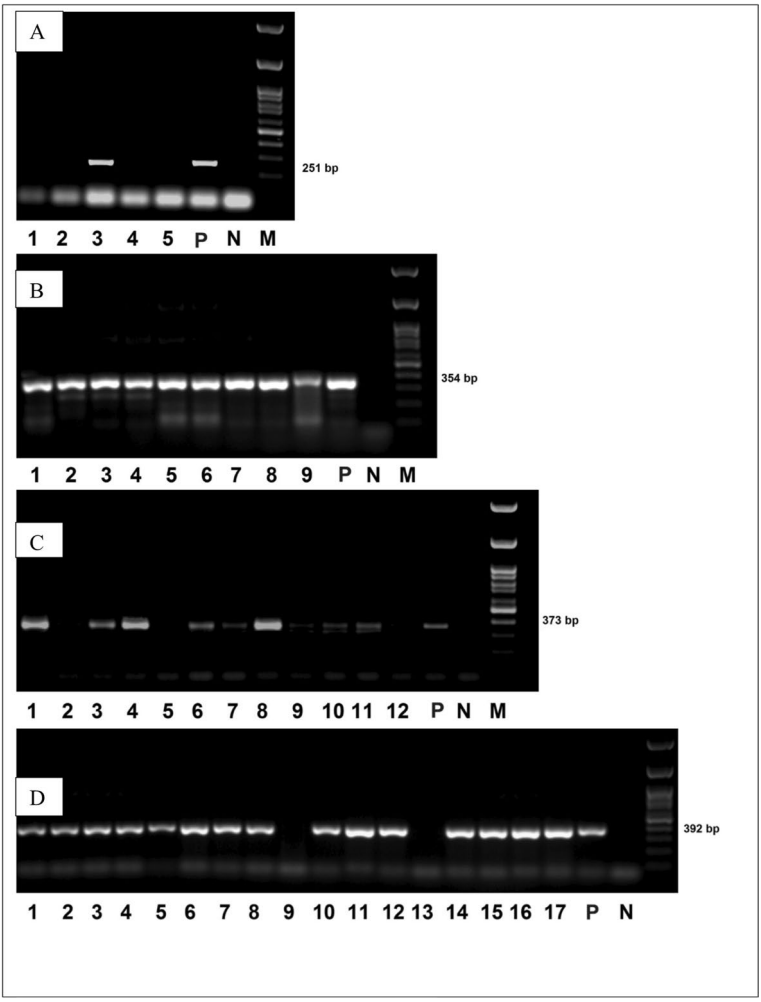

Fig. 3 Amplification of virulence genes of *V. parahaemolyticus* such as *tdh*, *tlh*, *trh,* and T3SS1. **A** *tdh* lanes—M, molecular weight marker; 1–5, representative isolates; P, positive control; N, negative control. **B** *tlh* lanes—M, molecular weight marker; 1–9, representative isolates; P, positive control; N, negative control. **C** *trh* lanes—M, molecular weight marker, 1–12, representative isolates; P, positive control; N, negative control. **D** T3SS1 lanes—M, molecular weight marker; 1–17, representative isolates; P, positive control; N, negative control

colony. β-Haemolysis was observed in 14 (44%) isolates of *V. parahaemolyticus* in the present study.

Antimicrobial susceptibility of *V. parahaemolyticus*

An antibiotic susceptibility test was done for all 32 *V. parahaemolyticus* isolates, for 14 antibiotics belonging to 11 classes such as β-lactams, 2nd-, 3rd-, and 4th-generation cephalosporins, aminoglycosides, quinolones, fluoroquinolones, tetracyclines, sulphonamides,

phenicol, glycopeptides, and macrolides Table 2. The resistance pattern of *V. parahaemolyticus* toward antibiotics given in Table 3. Figure 4 shows a heatmap of the antibiogram depicting resistance of antibiotics in a colour scale from dark blue to dark red and categorized into resistant, susceptible, and intermediate.

MAR index

Multiple antibiotic resistance (MAR) index was estimated for all isolates (Table 3), and the findings revealed that the MAR of 32% of the isolates was less than 0.2. In comparison, a significant portion, 68% of the isolates, had a MAR index range from 0.21 to 0.40. The MAR index was as high as 0.36 in one isolate that was resistant to five antibiotics (VA-CPM-CAZ-E-GEN).

Discussion

V. parahaemolyticus is a gram-negative motile bacterium inhabiting marine, estuarine, and coastal environments (Baffone et al. 2006; Wang et al. 2011). The study results of Caburlotto et al. (2016) and Coly et al. (2013) reported that the prevalence of *V. parahaemolyticus* was 28% and 30% from crustaceans in Italy and seafood in Senegal, respectively. However, other studies have found wide variations in prevalence values, ranging from 2.8% in a survey on crabs and prawns sold in Abidjan, Ivory Coast (Traorã et al. 2012) to 50.5% in frozen shrimp, prawns, and crabs bought in local markets or from import–export businesses in Boulogne-sur-Mer, France (Robert-Pillot et al. 2014). Pathogenic *V. parahaemolyticus* was found in Thailand, the world's leading producer and exporter of farmed shrimps (Yano et al. 2014). In our study, 32 *V. parahaemolyticus* isolates were confirmed with a 29% prevalence from *P. vannamei* shrimp farms of

Table 2 Antimicrobial resistance of *V. parahaemolyticus* ($n=32$)

Antibiotic groups	Antibiotics name	Resistance	Intermediate	Sensitive
β-Lactams	Amoxyclav (AMC)	0 (0%)	3 (9%)	29 (91%)
Second-generation cephalosporins	Cefoxitin (CX)	0 (0%)	8 (25%)	24 (75%)
Third-generation cephalosporins	Ceftazidime (CAZ)	3 (9%)	3 (9%)	26 (82%)
Fourth-generation cephalosporins	Cefepime (CPM)	1 (3%)	19 (59%)	12 (38%)
Aminoglycosides	Gentamycin (G)	23 (72%)	6 (19%)	3 (9%)
	Streptomycin (S)	0 (%)	5 (16%)	27 (84%)
	Kanamycin (K)	1 (3%)	24 (75%)	7 (22%)
Quinolones and fluoroquinolones	Ciprofloxacin (CIP)	0 (%)	0 (%)	32 (100%)
	Ofloxacin (OF)	0 (0%)	0(0%)	32 (100%)
Tetracyclines	Oxytetracycline (O)	0 (0%)	16 (50%)	16 (50%)
Sulphonamides	Trimethoprim (TR)	0 (0%)	2 (6%)	30 (94%)
Phenicol	Chloramphenicol (C)	0 (0%)	0 (0%)	32 (100%)
Glycopeptides	Vancomycin (VA)	28 (88%)	3 (9%)	1 (3%)
Macrolides	Erythromycin (E)	31 (97%)	1 (3%)	0 (0%)

Table 3 Antibiotic resistance pattern and MAR index of *V. parahaemolyticus*

Resistance pattern	Resistance profile	Number of isolates	Number of antibiotics	MAR
I	VA-CPM-CAZ-E-GEN	1	5	0.36
II	VA-K-E-GEN	1	4	0.29
III	VA -CAZ-E-GEN	2	4	0.29
IV	VA-E-GEN	17	3	0.21
V	VA-E	7	2	0.14
VI	E-GEN	2	2	0.14
VII	E	1	1	0.07

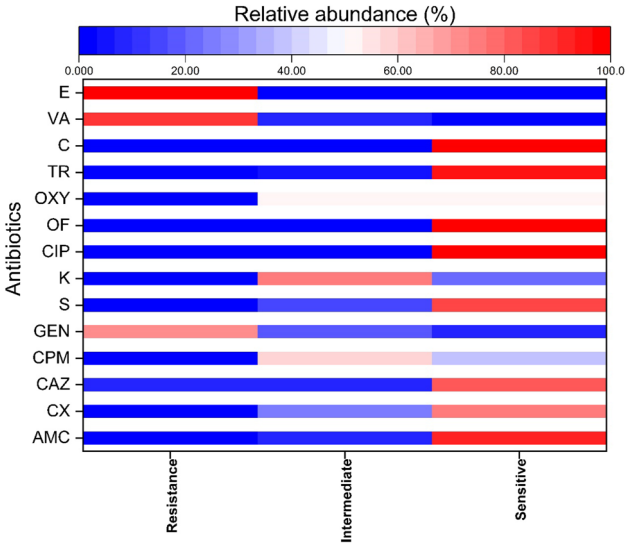

Fig. 4 Heatmap showing the antibiogram profile of *V. parahaemolyticus*

studied coastal districts in Tamil Nadu, which showed relatively same prevalence rate reported by Coly et al. (2013) and Caburlotto et al. (2016).

Because members of the family *Vibrionaceae* belong to the monophyletic group (clades) that are closely related (Sawabe et al. 2013), it becomes challenging to differentiate different species among *Vibrio* spp. The genes (*VpM*, *toxR*, and *GyrB*) targeting *V. parahaemolyticus* were chosen and tested for all isolates, and those that tested positive were confirmed to be *V. parahaemolyticus*.

The virulence factors linked to *V. parahaemolyticus* haemolysis and cytotoxicity activity in the host cell include *tdh* and tdh-related haemolysin (*trh*) (Broberg et al. 2011). The presence of thermostable direct haemolysin (*tdh*) and/or tdh-related haemolysin (*trh*) indicate the human pathogenic nature of *V. parahaemolyticus* isolates (Tada et al. 1992). The pathogenic genes *tdh* and *trh*, which cause diseases in human and marine animals, are often absent from the strains obtained from environmental samples (Deepanjali et al. 2005). However, a previous study showed that environmental sources had a smaller percentage

(1–2%) of human pathogenic strains (Sakazaki et al. 1968). In the present research, out of a total of 32 isolates, 2 (6%) and 11 (34%) isolates harboured the *tdh* gene and *trh* genes, respectively, which showed the pathogenic nature of the isolates obtained from shrimp samples and is a supreme concern for aquatic animals and human safety.

Another haemolysin of *V. parahaemolyticus*, thermolabile haemolysin (*tlh*), is likewise responsible for the lysis of red blood cells and is encoded by the *tlh* gene (Shinoda et al. 1991). All clinical and environmental strains of *V. parahaemolyticus* express *tlh* (Bej et al. 1999). The gene is markedly increased when the intestinal infection is reproduced (Gotoh et al. 2010). All isolates harboured the *tlh* gene in our study, indicating that the *tlh* gene could be considered a biomarker for identifying *V. parahaemolyticus*.

Isolates were further screened for other virulence genes of *V. parahaemolyticus*, such as T3SS genes. Two T3SS genes such as T3SS1 and T3SS2 are present in *V. parahaemolyticus*, on chromosomes 1 and 2, respectively (Calder et al. 2014). T3SS1 primarily causes cytotoxicity, and T3SS2 causes enterotoxicity (Hiyoshi et al. 2011). Ninety-one percent of the total isolates in our study harboured the T3SS1 gene; however, all isolates were negative for T3SS2 genes (both T3SS2α and T3SS2β). Therefore, it could be concluded that *V. parahaemolyticus* isolates were pathogenic but would cause cytotoxicity rather than enterotoxicity.

The bottom line of this study was to conduct a surveillance of AHPND along the southeast coast of India, specifically on the Tamil Nadu coast. All 32 confirmed *V. parahaemolyticus* isolates were investigated for AHPND pir toxins. However, the 32 isolates studied using AP4 PCR failed to detect any isolates carrying pir toxins. Recent studies in India regarding AHPND showed the absence of AHPND in India (Ananda Raja et al. 2017b, 2023; Kumar et al. 2020; Navaneeth et al. 2020). Hence, the current research confirmed that AHPND was not present in the studied locations during the sampling period.

The haemolytic activity is one of the essential virulence factors exhibited by *V. parahaemolyticus* phenotypically (Twedt et al. 1970). Forty-four percent of *V. parahaemolyticus* isolates in our study were β-haemolytic. It pinpointed the virulence nature and potency of the isolates in terms of haemolytic action.

The *V. parahaemolyticus* isolates in the present study were highly resistant to erythromycin and vancomycin, which was consistent with results obtained in the earlier studies (Hamdan et al. 2017; Hu et al. 2020; Elsherif et al. 2023). In our research, isolates showed 72% resistance towards gentamycin among the aminoglycosides group, while previous studies (Lee et al. 2018; Kim et al. 2021; Zhou et al. 2022; Elsherif et al. 2023) showed contrasting results against gentamycin, and were found to be susceptible. The resistance of many antibiotics has been transferred from sensitive to resistant mode with time and uncountable usage. Hence, there are many chances that antibiotics could be sensitive and become resistant one day with continuous use over time.

All the isolates were susceptible to ciprofloxacin, ofloxacin, and chloramphenicol, and it could be understood that these antibiotics are highly effective against *V. parahaemolyticus*. Similarly, previous studies have shown that the susceptibility of *V. parahaemolyticus* isolates against chloramphenicol, ofloxacin, and ciprofloxacin is natural (Xu et al. 2016; Lee et al. 2018; Ryu et al. 2019). Since these are the most frequently used antibiotics to treat infections caused by *Vibrio* spp. (Letchumanan et al. 2014), this finding was satisfactory. Streptomycin resistance was commonly observed in the isolates of *V. parahaemolyticus*, and the resistance which could be due to the everyday use of antibiotics (Kim et al. 2021). This could be the reason why most of the studies showed resistance towards streptomycin (Jiang et al. 2014; Xu et al. 2016; Ryu et al. 2019; Xie et al. 2020; Elsherif et al. 2023). However, in the present study, the results were contrasting regarding streptomycin, and 84% were sensitive. As discussed earlier, resistance to particular antibiotics is variable as the usage and choice

of antibiotics vary from place to place. Some investigations had reported that resistance to trimethoprim-sulphamethoxazole was 75%, 38.2%, and 64.7% in *V. parahaemolyticus* (Ahmed et al. 2018; Zhou et al. 2022; Elsherif et al. 2023). In contrast, some studies showed that *V. parahaemolyticus* was 80%, 78.22%, 97.7%, and 100% susceptible to trimethoprim-sulphamethoxazole (Jiang et al. 2014; Lee et al. 2018; Ryu et al. 2019; Li et al. 2020). In the present study, *V. parahaemolyticus* isolates were sensitive (94%) to trimethoprim-sulphamethoxazole. Hence, our study's findings align with those of earlier investigations. This observed variation might be due to variations in contamination of source water from place to place, since usage of any antibiotics in shrimp aquaculture is strictly prohibited in India with good awareness among the farmers (Ananda Raja et al. 2012). *V. parahaemolyticus* isolates from this study showed 75% and 82% susceptibility to second-generation cephalosporin (cefoxitin) and third-generation cephalosporin (ceftazidime) as well as 59% intermediate levels of resistance and 38% sensitive to a fourth-generation cephalosporin (cefepime). The earlier investigations had reported the same results (Jiang et al. 2014; Lee et al. 2018; Elhadi et al. 2022; Zhou et al. 2022). At the same time, some researchers have shown contradictory findings, such as pathogenic *V. parahaemolyticus* isolated from AHPND-affected *P. vannamei* farmed in the Mekong Delta exhibited resistance against the ceftazidime 100% (Ha et al. 2023). A total of 97.2% resistance of ceftazidime against *V. parahaemolyticus* isolated from shrimp, crab, and gastroenteritis patients was reported in Egypt (Ahmed et al. 2018). In the present research, 75% of *V. parahaemolyticus* showed intermediate resistance towards kanamycin. A similar finding was reported in which 92% and 74.5% of intermediate resistance of *V. parahaemolyticus* to kanamycin were observed, isolates from sea cucumber and aquatic products, respectively (Jiang et al. 2014; Xu et al. 2016). Antibiogram revealed that isolates were 50% sensitive and 50% intermediate resistant to oxytetracycline. Seventy-two percent susceptibility of *V. parahaemolyticus* was reported to oxytetracycline from fish samples (Lee et al. 2018). Isolates of *V. parahaemolyticus* in our study were 91% susceptible to amoxyclav. As per earlier studies, *V. parahaemolyticus* isolates were also demonstrated to be 75%, 95.4%, 95.7%, and 75.83% sensitive against amoxyclav (Jiang et al. 2014; Ryu et al. 2019; Tan et al. 2020; Elhadi et al. 2022).

Multiple antibiotic or drug resistance (MAR) was calculated according to Krumperman (1983). The present study showed that 32% of the isolates had less than 0.2 MAR index value. In the study, 70% and 76.59% of *V. parahaemolyticus* isolates had MAR index values lower than 0.2 (Ryu et al. 2019; Elhadi et al. 2022). A significant section of isolates (65%) in the present study had a MAR index within the range of 0.21 to 0.30. The MAR index was as high as 0.36 in one isolate that was resistant to five antibiotics. The MAR index ranges from 0.07 to 0.50 in *V. parahaemolyticus* isolated in marine and freshwater fish in Selangor (Lee et al. 2018). High values of the MAR index, such as 0.77 and 0.80, were observed in *V. parahaemolyticus* strains isolated from crustaceans and humans in Egypt and retail aquatic products in Nanjing, China, respectively (Ahmed et al. 2018; Zhou et al. 2022). The fluctuation in the MAR index may be due to variations in sample sources, geographic dispersion, and testing procedures.

Conclusion

Determining the virulence potency to cause severe diseases in humans and shrimps and the antimicrobial resistance of *V. parahaemolyticus* isolates such study is foremost essential. The present study highlighted the characterization and prevalence of *V. parahaemolyticus*,

associated virulence genes, AMR genes, and anti-microbial resistance with AHPND surveillance in four coastal districts of Tamil Nadu. The most important aspect of this study proved that India is free from the infection of AHPND in addition to earlier studies.

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Data availability Data will be made available on request.

Declarations

Competing interests The authors declare no competing interests.

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