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Prevalence, Antibiotic-Resistance and Virulence Characteristics of *Vibrio parahaemolyticus* strains isolated from fish farms on the East Coast of Andhra Pradesh

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ABSTRACT

The aim of this study is to investigate the Antimicrobial drug resistance (AMR) of Vibrio parahemolyticus from gut samples of farm fishes on the East coast of Andhra Pradesh. A total of 342 fresh fish samples were collected from culture farm (n=158) and uncultured farm/natural ponds (n=184) farms of 4 places on the Eastcoast of A.P, state viz., Kakinada, Narsapur, Machilipatnam and Nagayalanka regions. Throughout the study period, samples were collected in 2019. The materials were processed for V. parahemolyticus isolation and polymerase chain reaction identification of their putative virulence genes (PCR).There were 1164 isolates of V. parahemolyticus. Thirty-six isolates (3.09%) tested positive for the tdh gene, 21 (1.54%) tested positive for the trh gene, and 18 (1.89%) tested positive for both the tdh and trh genes. The resistance patterns of the 1164 isolates tested were quite diverse, ranging from 0.34 percent to 79.15 percent, against the 15 antimicrobial drugs used. Resistance to ampicillin (74.61%), amoxicillin (75.15 percent), and cefalexin (76.13%) has increased. Streptomycin (91.05%), ciprofloxacin (90.70%), ceftriaxone (92.91%), and imipenem (99.46%) were the isolates with the highest levels of susceptibility. A higher frequency of AMR to multiple antimicrobials in this investigation was seen in cultured farm isolates compared to natural ponds. The current study shows variation in AMR in different places on the East coast of A.P. state and different farming systems, indicating the prevalence of antimicrobial resistance is related due to virulence gene characteristics in V. parahemolyticus.

Keywords: *Antimicrobial resistance (AMR),V. parahemolyticus, cultured Fish farms,Natural Ponds, East coast of A.P.*

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INTRODUCTION

Aquaculture is the major occupation and promisingly growing sector that allows marine and inland fishing due to its global demand and economic value [1]. Aquaculture in India is expanding at more than 7% per year. Freshwater aquaculture accounts for over 95% of annual total aquaculture production [2]. Andhra Pradesh is India's second largest coastline state, with a length of 972km and 1140.7km^2 coastal wetlands. Due to extensive cultivation, affected fishes exhibit skin and appendage necrosis, which causes malformations in the body, slow growth, and internal organ liquefaction. Excessive mortality is a challenge in aquaculture farming [3].

India consumes 3% of the world's aquaculture antibiotics, which is expected to treble by 2030, making India the fourth-largest consumer of aquaculture antibiotics in food animal production, behind China, the United States, and Brazil. The enforcement of prudent antibiotic usage across the nation is seriously hampered by the lack of enforcement on the use of antibiotics in aquaculture [4]. AMR is expected to cause 10 million deaths annually and $100 trillion in lost output worldwide by 2050 [5]. Antibiotic resistance (ABR) is one of the most significant issues in aquaculture by the development of antimicrobial resistance (AMR). Antibiotic resistance has increased due to the widespread use of antibiotics to treat bacterial infections in fish and the persistence of these drugs in aquatic environments [6].

One of the most common pathogenic bacteria, *Vibrio parahemolyticus*, is a leading cause of disease in farming settings [7]. The tdh and trh genes are major virulence factors in *V. parahemolyticus*. They thus are thought to be the most critical genetic attributes for enterotoxin,cytotoxic and hemolyticactions in host cells [8].Although *V.parahemolyticus* is often sensitive to a wide range of antimicrobials, particularly

third-generation antibiotics such as cephalosporins like ceftriaxone, ceftazidime, and cefotaxime, are gaining higher antibiotic resistance in aquaculture [9]. Understanding the AMR profiles of *V. parahemolyticus*, a target bacteria for AMR monitoring in aquaculture, will help us better understand the spread of AMR in aquatic environments. Unfortunately, no information is available regarding the AMR profiling of *V. parahaemolyticus* strains in farm fishes of coastal A.P. Therefore, the current study exemplifies the prevalence AMR profiles presence of virulence genes in *V. parahaemolyticus* isolates.

MATERIAL AND METHODS

Fish samples collection

A total of 342 fish samples were collected from cultured farms and (n = 158) and uncultured (Natural ponds) (n = 184) of the East coast of Andhra Pradesh in the following four sampling stations viz. Kakinada (n = 92) Narsapur (n = 84), Machilipatnam (n = 78)and Nagayalanka(n = 88).

Isolation and identification

Fish homogenates were processed in 250 mL sterile alkaline peptone water at pH 8.5 for 1 minute to isolate *V. parahemolyticus* strains. For selective enrichment, the specimens were incubated at 37°C for 8 and 16 hours. TCBS Agar plates were infected with a loopful of enriched homogenized cultures, and the plates were then incubated at 37°C for 24 hours. On cultured plates, bluish-green colonies of 2- 5 mm diameter were picked up and incubated in 3% NaCl Tryptic soy broth at 37°C for 24 h[10]. Colonies with characteristic yellow color were considered for biochemical identification assays [11].

Molecular detection of *tdh* and *trh*genes

Template DNA for PCR amplification of the tdh and trh genes was extracted from V. parahemolyticus using the conventional boiling and snap cooling procedure [12]. Multiplex PCR was used to characterize *V. parahemolyticus* isolates at the molecular level. Specific forward and reverse primers were used to identify the isolated strains' tdh and trh genes. The total volume of the PCR reaction mixture was 25 L. In addition to the template DNA and the 'F' and 'R' primers, the PCR reaction mixture contains 1X PCR buffer, 3mM $MgCl_2$, 100mM dNTP, 2 L Tag DNA Polymerase, and 10 L of primers. Denaturation at 95 degrees Celsius for 4 minutes was continued by 40 cycles of annealing at 61 degrees Celsius for 45 seconds and extension at 72 degrees Celsius for 2 min for tdh and trh genes, respectively. PCR amplicons were separated on a 1% Agarose gel by electrophoresis with EthBR in Tris Borate buffer at 100 volts for 30 minutes. 100 bp DNA ladder was employed as a standard marker. A gel documentation method was used to observe and photograph the PCR amplicons[10,13]

Antimicrobial Susceptibility Assay

In vitro antimicrobial susceptibility assay by disc diffusion was performed to all the isolates on Mueller Hinton agar (MHA)[14]using 14 commercially available antibiotic discs viz., streptomycin (10 µg), piperacillin (5 µg), imipenem (5 µg), gentamicin (5 µg), nalidixic acid (5 µg),amoxycillin (30 µg), ampicillin (30 µg), cefotaxime (30 µg), aztreonam (30 µg), ceftazidime (30 µg), cefalexin (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), and cefixime (5 µg). Type strain *V. parahemolyticus* MTCC 451 was used as the control strain for the AMR assay. A pure colony of each isolate was inoculated into 10 mL Tryptic Soy Broth (TSB) at 37°C overnight under constant shaking. The suspension density of 1 mL was spread over MHAevenly with 'L' spreader before the antibiotic discs were placed. The plates with the antibiotic discs were incubated at 37°C for 24 h for observation and measurement of a zone of inhibition [10].

Statistical analysis

Using the 'Z' test of two proportions with normal approximation, we compared the antimicrobial resistance patterns of *V. parahemolyticus* isolates from the four coastal regions of Andhra Pradesh. The level of statistical significance (Chi-square test) was set at 0.05.

RESULTS AND DISCUSSION

There were a total of 1164 *V. parahemolyticus* isolates from 342 fish gut samples, with the highest rates emerging from cultured fish farms (n = 606) and uncultured fish farms (n = 632). Infected fish gut samples yielded a significantly higher number of large isolates (n = 1021) than samples from fish that appeared to be uninfected (n = 143). The tdh and trh genes in 1164 *V. parahemolyticus* strains were examined using a PCR amplification. The tdh gene was found in 36 of the 1164 fish isolates (3.09%) that were gut infected. It was discovered that 21 isolates (21/1164; 1.8%) had the trh gene, with 18 isolates (18/1164; 1.54%) testing positive for both the tdh and trh genes, respectively (Fig 2).

The antimicrobial susceptibility of all 1,164 *V. parahemolyticus* isolates was evaluated against 14 different antibiotics. All of them showed resistance to at least three antimicrobials, with imipenem (0.34%) showing the lowest level of resistance, followed by ciprofloxacin (8.79%), streptomycin (9.36%), cefotaxime (8.41%), aztreonam (10.56%), ceftazidime (11.40%), gentamicin (13.41%), nalidixic acid

(29.72%), and cefixime (17.01%) Ceftazidime (84.37%), aztreonam (88.62%), cefotaxime (91.58%), streptomycin (90.63%), ciprofloxacin (90.78%), ceftriaxone (92.90%), and imipenem (99.46%) were the antibiotics against which isolates showed increasing susceptibility. and Table 1 shows the findings.

There was no statistically significant difference in ampicillin resistance between isolates from Narsapur and Kakinada, but there was a significant difference between Machilipatnam and Nagayalanka. Isolates from Narsapur had the highest (89.29%) and lowest (58.59%) percentages of resistance, respectively. In the case of amoxicillin, all isolates showed a significant difference in resistance in Nagayalanka, with the highest (86.28%) and lowest (64.92%) recorded, respectively. In terms of aztreonam resistance, there was no discernible difference between Narsapur (22.0%) and the lowest prevalence (6.27%). The highest rate (87.07%) and lowest rate (75.58%) of cefalexin resistance were found in Nagayalanka and Machilipatnam, respectively.

The lowest (3.51% and 1.70%) and highest (19.73% and 17.34%) resistance to streptomycin and ceftazidime was found in isolates from Narsapur and Nagayalanka, respectively. In contrast, isolates from Narsapur and Kakinada had the lowest (1.39%) and highest (16.38%) rates of ciprofloxacin resistance, respectively. The highest cefixime resistance profiles were found in Narsapur (26.76%), while the lowest were found in Nagayalanka (7.03%). The four sampling sites showed significant differences in antimicrobial drug resistance to nalidixic acid, ceftriaxone, and cefotaxime. Isolates from Narsapur showed the highest rates of resistance to cefotaxime and ceftriaxone, at 15.73 and 12.71 percent, respectively, while those from Nagayalanka state showed the lowest rates of resistance, at 1.95% and 3.92 percent, respectively.

AMR was found in isolates from Machilipatnam and Nagayalanka. There was a significant difference in AMR between Kakinada (36.74%) and Nagayalanka (61.32%) isolates when it came to resistance to piperacillin. There was a notable difference between isolates from Narsapur (6.52%) and Machilipatnam (14.74%) in terms of gentamicin resistance. With the exception of imipenem, antibiotic resistance varied significantly across all four sampling sites (P value 0.05).

Comparing the drug resistance profiles of *V. parahemolyticus* isolates showed an increased prevalence of AMR for a variety of antimicrobials originating in cultured fish farming than in natural ponds (Figure 3). The significant proportion of the 606 isolates from cultured fish farms were resistant to ampicillin (89.40%), followed by cefalexin (82.41%) and amoxicillin (81.02%). Antibiotics like streptomycin (10.39%), ciprofloxacin (8.36%), cefotaxime (7.58%), and ceftriaxone (7.06%) were also found to be the least resistant in the cultured farming system. Impenem (0.34%), ceftriaxone (7.44%), and ciprofloxacin (8.51%) showed the lowest levels of AMR of V. parahemolyticus among isolates (n = 558) from uncultured fish farms, while cefalexin (79.64%) and amoxicillin (76.21%) showed the highest levels of resistance. There was a statistically significant difference in ampicillin and nalidixic acid resistance between the cultured and uncultured fish farms of the four sampling stations. In contrast, there was no noticeable difference in AMR between ceftriaxone and imipenem.

Antimicrobial resistance in intestinal bacteria is critical in human and veterinary medicine. Fish, shrimp, oysters, and other aquatic organisms may serve as reservoirs and possible sources for dispersing antibiotic-resistant microbial diversity in the ecosystem [15]. Many gram-negative bacteria, most notably *V. parahemolyticus*, have evolved mechanisms of resistance to beta-lactam antibiotics by producing extended-spectrum beta-lactamases, which metabolize most penicillins, extended-spectrum cephalosporins, and aztreonam [16].

This study found a huge variation in the sensitivity of *V. parahemolyticus* isolates to several antimicrobial drugs, with antimicrobial resistance ranging from 0.34 to 79.13%. Cefalexin, amoxicillin and ampicillin, were the antibiotics that showed the highest level of resistance in our study. In contrast, imipenem, ceftriaxone, cefotaxime, ciprofloxacin, and streptomycin showed the highest level of susceptibility. Although most farmers originally used the medications above often to diagnose gastroenteritis and certain other diseases, the significant development of resistance in the coastal regions of A.P state is concerning.

We observed the isolates' high susceptibility to ciprofloxacin (91.6%) and gentamicin (88.02%), which is in accordance with the results of previous authors [17]and other scientists [18, 19]. In contrast to the findings of [20], resistance to streptomycin, ceftriaxone, ceftaxime, cefzilime, and ampicillin varies greatly In contrast to previous reports that indicated the prevalence of gentamicin resistance in sub-Saharan African nations at up to 20% for *V. parahemolyticus* [21]but also 39.1% in India [22], our investigation detected only a small percentage of AMR to gentamicin. Similar findings of significant levels of AMR despite ciprofloxacin in *V. parahemolyticus* infections in aquaculture were reported in India [23]. Perhaps surprisingly, a similar susceptibility pattern has been noted before by other researchers [24, 25]. Our results confirming the low prevalence of imipenem resistance among clinical isolates[26,27]are in line with those of other researchers.

According to researchers, irrational antibiotic use is the leading cause of antibiotic resistance [13]. The antibiotics amoxicillin, cefalexin, and ampicillin are widely used in farming in three of the stations we sampled because they are inexpensive and readily available. The widespread application of all these antibiotics in aquaculture has assisted select for resistant strains. In addition, mutational changes in genes essential for establishing resistance patterns in *V. parahemolyticus* [28] may account for this phenomenon. Moreover, our results showed that lateral gene transfer contributed to increasing in antibiotic resistance among *V. parahemolyticus* isolates [29].

There may be a critical relationship between the management practices in place and the increased incidence of AMR in cultured farming systems compared to uncultured farming systems. The use of antibiotics for long periods of time for treatment and control of infection may lead to greater AMR nevertheless, in both resistance and virulence characteristics case of many cultured fish farms. In *V. parahemolyticus* strains, existence of pathogenic (tdh and trh) and conferring resistance genes can lead to the creation of hybrid plasmids including both resistance and virulence, which therefore pose a greater public health threat [30]. In this study, we found that all *V. parahemolyticus* isolates both virulent and resistant were resistant to two or more antimicrobial drugs, which suggests the existence of MDR strains with virulence traits [24, 31]. Antibiotic overuse is likely to blame for the proliferation of multidrug-resistant strains of *V. parahemolyticus*.

Our findings called for a more careful evaluation of pathogenic strains of *V. parahemolyticus* focusing on antimicrobial drug resistance due to the high consumption of farmed fish in coastal areas. Simultaneously identifying *V. parahemolyticus* isolates exhibiting virulence and multidrug resistance is a public health issue. Evolutionary trends favoring hybrids of pathogenic and resistant strains result from this persistent selective pressure. Thus, it is important to conduct proper antimicrobial susceptibility testing, including molecular characterization of these strains frequently in fish farming. Multiple antibiotic resistance was seen in some of the *V. parahemolyticus* isolates tested here. Effective prevention or treatment of *V. parahemolyticus*-induced illness requires an accurate and clear understanding of antibiotic-resistance trends in aquaculture.

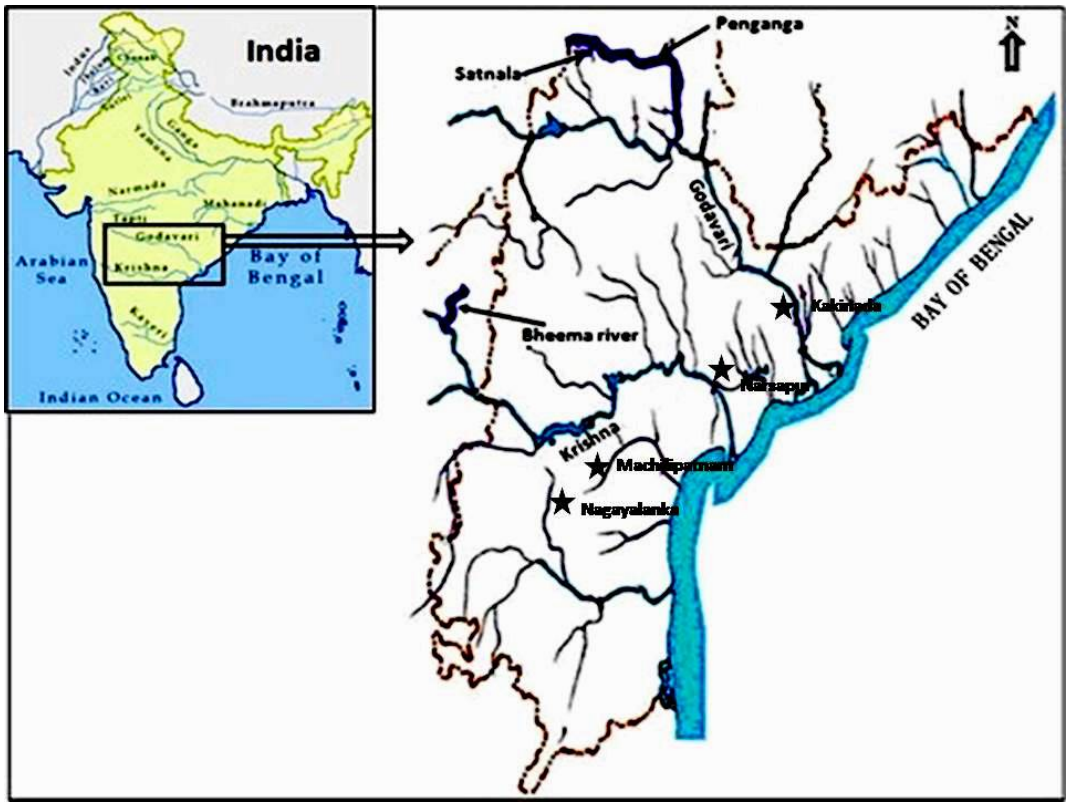

Fig. 1.A map representing the four sampling sites (marked with star) in coastal Andhra Pradesh within Krishna and Godavari delta which is a representative sampling map used for the study.

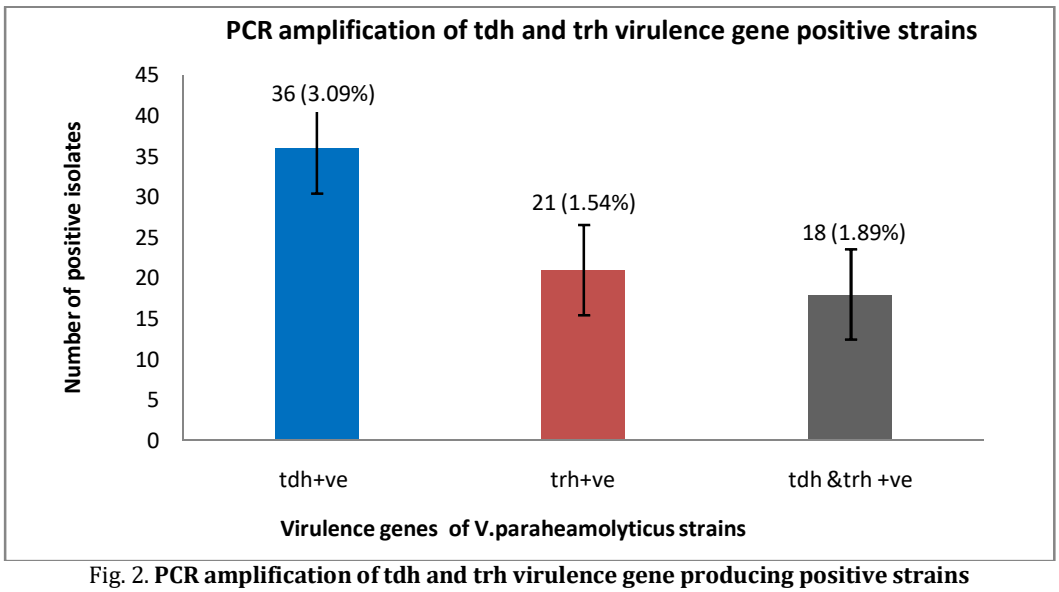

Fig. 2. **PCR amplification of tdh and trh virulence gene producing positive strains**

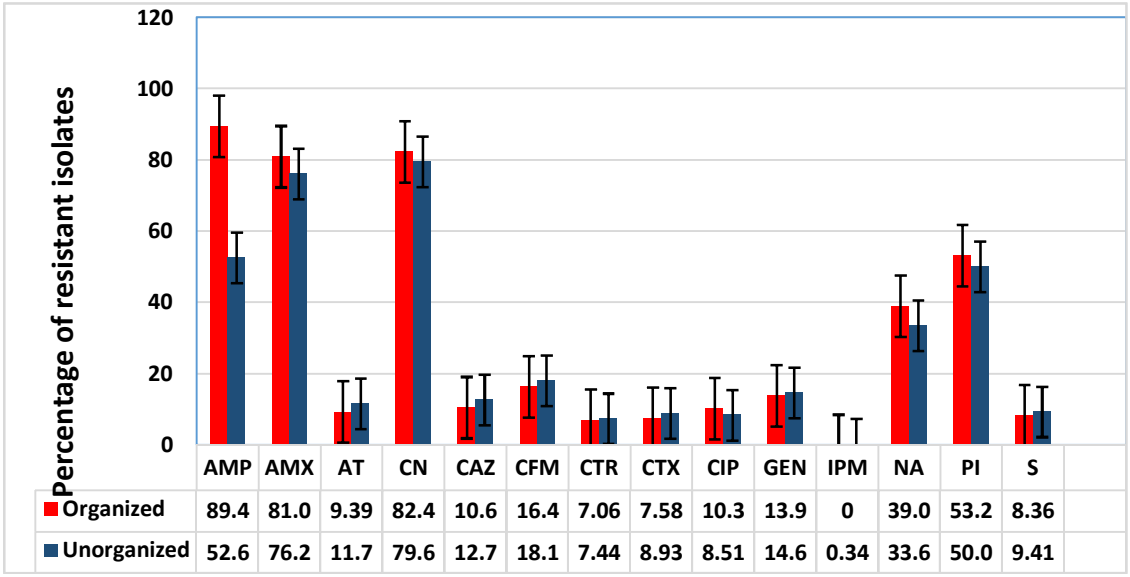

Fig. 3. Antimicrobial resistance profiles of *Vibrio parahemolyticus* isolates from cultured fish farms and natural ponds in Andhra Pradesh's east coast region

Table 1. Antimicrobial resistance of *Vibrio parahemolyticus* isolates in the four east coast regions of Andhra Pradesh. Note: Percentage (%) is given in parentheses

Antimicrobial		Kakinada (n=287)	Machilipatnam (n=322)	Narsapur (n=299)	Nagayalanka (n=256)	Total (n=1164)
Amp	S	46(16.02)	112 (34.78)	32(10.70)	106 (41.40)	296 (25.42)
	R	241 (83.97)	210 (65.21)	267 (89.29)	150 (58.59)	868 (74.57)
Amx	S	52 (18.11)	56(17.39)	41(13.71)	77 (30.07)	226 (19.41)
	R	235 (81.88)	256 (82.60)	258 (86.28)	179 (69.92)	938 (80.58)
At	S	269 (93.72)	301 (93.47)	233 (77.92)	238 (92.96)	1041 (88.43)
	R	18 (6.27)	21 (6.52)	66(22.07)	18 (7.03)	123(10.56)
Cn	S	53 (18.46)	40(12.42)	73(24.41)	61(23.82)	227(19.50)
	R	234 (81.53)	282 (87.57)	226 (75.58)	195 (76.17)	937 (80.49)
Caz	S	265 (92.33)	271 (84.16)	248(82.94)	247 (96.40)	1031 (88.57)
	R	22 (7.66)	51(15.83)	51(17.05)	9 (3.51)	133(11.40)
Cfm	S	239 (83.27)	276 (85.71)	213 (71.23)	238 (92.90)	966 (82.98)
	R	48 (16.72)	46(14.28)	86(38.76)	18 (7.03)	198 (17.01)
Ctr	S	273 (95.12)	297(92.23)	261 (87.29)	251 (98.04)	1082 (92.90)
	R	14 (4.87)	25(7.76)	38(12.70)	5 (1.95)	82 (7.04)
Ctx	S	271 (94.42)	295(91.61)	252 (84.28)	248 (96.80)	1066 (91.58)

	R	16 (5.57)	27(8.38)	47(15.71)	8(3.12)	98 (8.41)
Cip	S	282 (98.25)	277(86.02)	250 (83.61)	247 (96.41)	1056 (90.72)
	R	5 (1.39)	45(13.97)	49(16.38)	9(3.51)	107 (9.19)
Gen	S	253 (88.15)	301 (93.47)	225 (74.91)	229 (89.04)	1007 (86.01)
	R	34 (11.84)	21 (6.52)	74(24.74)	27 (10.50)	156(13.40)
Ipm	S	286 (99.56)	320 (99.37)	299 (100)	255 (99.2)	1159 (99.57)
	R	1 (0.34)	2 (0.62)	0 (0.0)	01(0.39)	4 (0.34)
Na	S	256 (89.19)	208 (64.59)	158(52.80)	197 (76.91)	809 (69.50)
	R	31 (10.80)	1114 (35.04)	141(47.15)	59 (23.04)	345 (29.63)
Pi	S	109 (37.97)	163 (50.62)	149(49.80)	162 (63.20)	583 (50.08)
	R	176 (61.32)	159(49.3)	150(50.16)	94 (36.70)	579 (49.71)
S	S	276 (96.16)	280(66.97)	247(82.60)	252 (98.30)	1055 (89.63)
	R	11 (3.83)	42(13.04s)	52(17.30)	4(1.56)	109 (9.36)

S = Sensitive; R = Resistant
Amp = Ampicillin, Amx = Amoxycillin, At= Aztreonam, Cn = Cephalexin,
Caz = Ceftazidime, Cfm = Cefixime, Ctr = Ceftriaxone, Ctx = Cefotaxime,
Cip = Ciprofloxacin, Gen = Gentamicin, Ipm = Imipenem, Na = Nalidixic acid,
Pi = Piperacillin, S = streptomycin.

CONCLUSIONS

Pathogenic *Vibrio* species are the primary risk for seafood safety, particularly farm fishes that harbor *V. parahaemolyticus.* Anthropogenic impacts along coastal water may also increase the growth of *Vibrio* spp. in AP state and might have facilitated the emergence of new pathogenic lineages by lateral transfers and recombination. Fish from both cultured and uncultured farms in four distinct sample stations were tested positive for *V. parahemolyticus* exhibiting AMR traits, with the prevalence of AMR being higher in the former Increased scrutiny on the usage of antimicrobials in cultured fish farms became necessary due to the occurrence of elevated levels of resistance to most antimicrobials and the predominance of MDR traits viz. tdh and trh positive strains.In A.P. state, there is a lack of data on the prevalence of AMR in *V. parahemolyticus* isolates, especially with fish farming. In order to assess the prevalence of antibiotic resistance and use, a state wide surveillance system is required.

CONFLICT OF INTEREST DECLARATION

The authors disclaim any potential conflicts of interest.

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