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ANTIBIOTIC SUSCEPTIBILITY PATTERN OF VIBRIO PARAHAEMOLYTICUS ISOLATED FROM RETAIL SEAFOODS IN MYSORE

RAKSHA AJITPRASAD MANIYANKODE, JOSEPH JEYABALAJI KINGSTON, PUJA SHARMA AND HARSH VARDHAN BATRA*

Division of Microbiology, Defence Food Research Laboratory, Siddhartha Nagar, Mysore, Karnataka 570011, India

ABSTRACT

The present work was aimed to evaluate the antimicrobial resistance pattern in *Vibrio parahaemolyticus* isolates from seafoods. A total of 56 *V. parahaemolyticus* isolates could be recovered and identified from various seafoods sold at retail shops of Mysore and they were tested for their sensitivity against 27 antibiotics of varied nature. About 3.6% of these isolates were found to possess the virulent associated genes, *tdh* and *trh.* MAR index analysis illustrated resistance between 0.00- 0.52 and among these 26.8% were multiple drug resistant. The bacterial strains were predominantly resistant to Oxacillin and Pencillin (≥ 72%), while no resistance was observed against Ceftriaxone, Norfloxacin, Ciprofloxacin, oloxacin, Streptomycin, Gentamycin, Neomycin and Amikacin. In conclusion, significant numbers of *V. parahaemolyticus* strains were found to be resistant to antibiotics commonly used as therapeutic agents, feed supplement and growth promoters, indicating a need for supervised use of antibiotics and of frequent surveillance of judicious *V. parahaemolyticus* strains for antimicrobial resistance.

KEY WORDS: V. parahaemolyticus, Antibiotic resistance, Mar index, Multidrug resistance

HARSH VARDHAN BATRA
Division of Microbiology, Defence Food Research Laboratory,
Siddhartha Nagar, Mysore, Karnataka 570011, India

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative, halophilic bacterium ubiquitous in estuarine and coastal marine environments[1]. Since the last few decades, the organism has emerged as a major pathogen of significant concern as it causes seafood-associated outbreaks throughout the world[2]. *V. parahaemolyticus* infection through consumption of contaminated raw or under-cooked seafood results in gastrointestinal illness and exposure of open wound to recreational water lead to severe wound infections and septicaemia[3, 4]. Previous studies have shown that *V. parahaemolyticus* infection is often associated with strains that can produce either of the two haemolysins - Thermostable direct haemolysin (TDH) or TDH-related haemolysin (TRH) encoded by *tdh* and *trh* genes, respectively. The clinical isolates are believed to carry either *tdh*, *trh* or both genes, while low frequencies of the environmental strains are known to possess these genes[5]. Gastrointestinal illness caused by the organism is a self-limiting illness in healthy individuals and in severe cases, fluid rehydration therapy or antibiotics are administered as treatment[6]. Studies have shown that *Vibrio sp.* were highly susceptible to many of the antimicrobial agents previously[7]. But recently, antimicrobial resistance pattern in this organism has extended to a variety of antibiotic groups, especially β-lactams[8]. The reason for the rapid emergence of antimicrobial resistance could be multi-factorial. Waste water from clinical settings, agriculture and aquaculture involving use of antibiotics for effective control of bacteria, eventually reaches the coastal water through sewage systems, which in turn selects the resistant bacterial populations[9]. In some cases movement of a resistant trait could be by horizontal gene transfer from resistant bacterial species to non-resistant ones thereby conferring resistance to latter[10]. On the other hand, indiscriminate use of antibiotics in aquaculture to avoid vibriosis and other bacterial contaminations has also favoured the emergence of resistant strains[11]. Mysore city located at 12.30°N 76.65°E is the second largest city in Karnataka state, India. Though the population of the city is a little less than 0.9 million, pleasant semi-arid climate throughout the year, noted tourist spot locations and festivities, draw huge number of tourists; in 2010 alone, 3.15 million tourists visited Mysore[12]. Inhabited by different ethnic populations, seafood is one of the highly consumed meat products in Mysore. Nonetheless, there are very few intensive seafood markets in the city and many private retail markets instead. The seafood handling in the latter is not continually monitored and thus considered the potential risks of food poisoning. The present study is aimed at isolating and identifying strains of *V. parahaemolyticus* from seafood collected from local retail markets of Mysore and assessing their susceptibility to 27 selected antibiotics. Further their MAR index and then Multiple Drug Resistance were evaluated to analyze the risk imposed by consumption of the implicated food.

MATERIALS AND METHODS

1. Sample collection and isolation

Totally, 78 raw seafood samples (50 fish, 10 shrimp, 8 squid and 10 shell fish) were randomly collected from retail markets in Mysore, India. Only one kind of sample was collected from each retail shop every time. The seafood samples were placed in sterile bags and immediately transported to the laboratory in ice box within 1h of collection. All the samples were analyzed on the day of arrival according to Raghunath and coworkers[13]. Briefly, 25 g of meat of the each sample was homogenized in 225 ml of APW and incubated at 37 °C for 18 h. A loopful of 18 h enriched broth was plated onto TCBS agar (Himedia, India) and incubated overnight at 37 °C. At least 10 typical colonies were picked up, sub-cultured and examined by a series of biochemical tests for identification[14]. For comparative purpose, a standard strain *V. parahaemolyticus* ATCC 17802 was used in the study.

2. PCR based detection of V. parahaemolyticus and their virulence genes

Total DNA of *V. parahaemolyticus* isolates was isolated following protocol described by Tada et al [15]. The presence of the *tlh* gene, a species specific marker for identification of *V. parahaemolyticus,* was determined as described by Nordstrom et al [16], using the primers 5′ ACTCAACACAAGAAGAGATCGACAA 3′ (*tlh* forward) and 5′ GATGAGCGGTTGATGTCCAA 3′ (*tlh* reverse), which produced a 200 bp amplicon. The detection of virulence genes *tdh* and *trh* were verified by using the set of primers *tdh*F: 5' GGTACTAAATGGCTGACATC 3' and *tdh*R: 5' CCACTACCACTCTCATATGC 3' and *trh*F: 5' GGCTCAAAATGGTTAAGCG 3' and *trh*R: 5' CATTTCCGCTCTCATATGC 3', respectively[14]. All the aforementioned primers were synthesized by Eurofins, Bangalore. PCR amplification was performed in Mastercycler Pro thermalcycler (Eppendorf, Germany) in a 20 µl reaction mix containing 50 ng template DNA, 1X PCR buffer (with 1.5 mM $MgCl_2$), 100 µM each dNTP, 1µM each primer and 1 unit *Taq* polymerase (Fermentas, New Delhi, India). The PCR conditions were followed according to Nordstorm et al [16] for *tlh* gene and Tada et al[15] for *tdh* and *trh* genes. The PCR amplicons were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualized under UV transillumination (G-box, Syngene, India).

3. Antimicrobial susceptibility of V. parahaemolytics isolates

Antimicrobial susceptibility of *V. parahaemolyticus* isolates was tested by the disc diffusion method on Muller Hinton agar (Himedia, India) described by Bauer et al[17] against the following antimicrobial agents; β lactam group: Carbenicillin (CB100), Oxacillin (OX1), Ampicillin (A10), Penicillin (P10), Amoxycillin (AM25), Cefuroxime (CU30), Ceftriaxone (CI30); Tetracyclines: Tetracycline (T30), Doxycycline (DO30); Quinolones: Norfloxacin (NX10), Ciprofoxacin (CF5), Nalidixic acid (NA30), Gatifloxacin (GF5), Ofloxacin (OF5); Macrolides: Erythromycin (E15), Azithromycin (AT15); Sulphonamides: Cotrimoxazole (CO25), Sulfafurazole (SF300); Aminoglycosides: Stretomycin (S10), Gentamycin (G10), Amikacin (Ak30), Neomycin (N30); Nitrofurans: Nitrofurantoin (NF300); Amphenicols: Chloramphenicol (C30); Polypeptides: Colistin (CL10), Polymyxin B (PB300); DHFR inhibitors: Trimethoprim (TR5). The isolates in their log phase with turbidity of 0.5 McFarland were spread plated on to Muller Hinton Agar plate using sterile cotton swabs. The discs with concentrations as approved by the Clinical and Laboratory Standards Institute (CLSI) for 27 antibiotics were purchased from HiMedia (Mumbai, India) and placed aseptically on to the plate and inhibition zone was measured after incubation for 24 hrs at 37^{o}C. The isolates were scored as sensitive, resistant or intermediate as per the CLSI guidelines[18]. The multiple antibiotic resistance (MAR) index of every isolate was calculated as per the method described by Krumperman[19] using the formula: a/b, where 'a' represents the number of antibiotics to which a particular isolate was resistant and 'b' the total number of antibiotics tested. All graphical illustrations in this study were depicted using Microsoft Excel 2007 (Microsoft Corp., MD, USA).

RESULTS

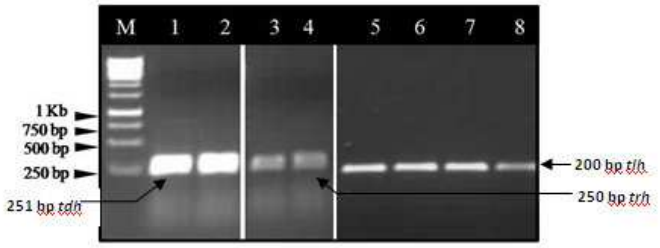
1. Distribution of V. parahaemolyticus in Seafoods

A total of seventy-eight seafood samples were analyzed by conventional procedures for isolation of *V. parahaemolyticus* strains. Although, 59 isolates were biochemically identified to be *V. parahaemolyticus*, only 56 were positive to PCR employing reported primers for the confirmation of the presence of the species specific marker, *tlh* gene[16]. The three *tlh* gene negative isolates were further identified as *V. vulnificus* by an in-house developed PCR (Unpublished data). The prevalence of *V. parahaemolyticus* in the seafood samples from Mysore city retail markets was determined to be 71.8%. The numbers of various types of seafood samples positive for *V. parahaemolyticus* and the percentage recovery of the species from each sample type are shown in Table 1.

Table 1
Detection of V. parahaemolyticus from various seafoods by Conventional method and PCR assay

Seafoods (Number of samples)	*V. parahaemolyticus* positive samples			
	Conventional method (No.)	*tlh*+	*tdh*+	*trh*+
Fish (50)	42	40	0	2
Shrimp (10)	8	7	1	0
Squid (8)	4	4	1	0
Shell fish (10)	4	5	0	0

Figure 1
PCR amplification of V. parahaemolyticus species specific tlh gene and virulence associated tdh and trh genes



Lane-1. Isolate VPF2, 2. Isolate VPF3, 3. Isolate VPSH51, 4. Isolate VPSQ54, 5. V. parahaemolyticus ATCC 17802, 6. Isolate VPF40, 7. Isolate VPF3, 8. Isolate VPSQ54

2. Determination of Virulence potential of V. parahaemolyticus isolates

The *V. parahaemolyticus* strains were assesed for the presence of virulence associated markers like the *tdh* and *trh* genes. The seafood strains were examined by PCR assays to detect the presence of these haemolysin genes. Two fish isolates VPF2 & VPF3 yielded 251 bp *tdh* amplicon and the 250 bp *trh* amplicon could be seen from a shrimp isolate VPSH51 and squid isolate VPSQ54. Overall 3.6 % of the isolates were found to harbor either of the virulence associated genes.

3. Antimicrobial Resistance

The results for antibiotic susceptibility/resistance profiles of 56 *V. parahaemolyticus* isolates are presented in Fig 2 & Supplementary Fig. The isolates were predominantly resistant to Oxacillin (82.1%) and Pencillin (71.4%), while no resistance could be seen for the antibiotics Ceftriazone, Norfloxacin, Ciprofloxacin, Ofloxacin and the aminoglycosides-Streptomycin, Gentamycin, Amikacin and Neomycin. Standard strain ATCC 17802 is the only strain showing resistance to Ciprofloxacin and Ofloxacin (Supplementary Fig). The shell fish isolates showed maximum susceptibility with resistance to only 5 antibiotics- Carbenicillin, Oxacillin, Ampicillin, Pencillin and Amoxycillin belonging to the β- lactam group (Fig 3). The shrimp isolates were either sensitive or showed intermediate resistance to the antibiotic Carbenicillin (Fig 3). The MAR index of all the tested isolates was in the range of 0.00 to 0.52 (Table 2 & 3), the highest being seen in the fish isolate VPF38. The 4 isolates harboring virulent *tdh* (VPF2 and VPF3) and *trh* (VPSH51 and VPSQ54) genes exhibited a MAR index between 0.11-0.19. The Multi-Drug Resistance (MDR) of *V. parahaemolyticus* was identified by observing the resistance pattern of the isolates to different classes of antibiotics. Fifteen out of 56 isolates showed resistance in more than 2 classes of antibiotics (Table 2). Five fish isolates VPF12, VPF14, VPF19, VPF23, VPF24 and a shrimp isolate VPSH50 did not show resistance to any of the antibiotics; they were either sensitive or intermediate. Overall, 10.7% of the isolates were sensitive to 27 antibiotics used in the study.

Table 2
Antibiotic resistant pattern of V. parahaemolyticus isolated from seafoods.

Sl No.	Antibiotic resistant profile*	MDR #	MAR index	No. of isolates
1	OX, P, AM, T, DO, NA, GF, E, TR, CO, SF, PB, CL, NF	+	0.52	1
2	OX, P, T, DO, NA, E, TR, CO, SF, PB, CL	+	0.41	1
3	CB, OX, A, CU, P, AM, TR, CO, PB, CL	+	0.37	1
4	CB, OX, A, CU, P, AM, NA, TR, CO, CL	+	0.37	1
5	OX, P, T, DO, NA, E, PB, CL, NF	+	0.33	1
6	OX, P,T, DO, E, PB, CL, NF	+	0.3	3
7	OX, A, P, NA, E, AT, CL	+	0.26	1
8	OX, P,T DO, E, PB, CL	+	0.26	1
9	OX, T, DO, E, PB, CL, NF	+	0.26	1
10	OX, P, T, DO, PB, CL, NF	+	0.26	2
11	CB, OX, A, P, AM, CL	-	0.22	1
12	OX, A, CU, P, AM, CL	-	0.22	1
13	OX, P, T, DO,NF	+	0.19	1
14	OX, A, P, AM, CL	-	0.19	2
15	CB, OX, A, P, AM	-	0.19	5
16	OX, A, P, AM, NA	-	0.19	1
17	CB, OX, A, P	-	0.15	1
18	OX, P, E, C	+	0.15	1
19	OX, A, P, AM	-	0.15	5
20	OX, A, P	-	0.11	4
21	P, PB, CL	-	0.11	1
22	OX, P	-	0.07	4
23	NA	-	0.04	1
24	P	-	0.04	1
25	AM	-	0.04	1
26	OX	-	0.04	7
27	NIL	-	0	6

Table 3
Multiple Antibiotic Resistance of V. parahaemolyticus isolates from various seafoods.

Seafoods (Number of samples)	MAR index range
Fish (50)	0.00-0.52
Shrimp (10)	0.00-0.30
Squid (8)	0.11-0.26
Shell fish (10)	0.07-0.19

Figure 2
Frequency of antimicrobial patterns of V. parahaemolyticus isolates used in the study

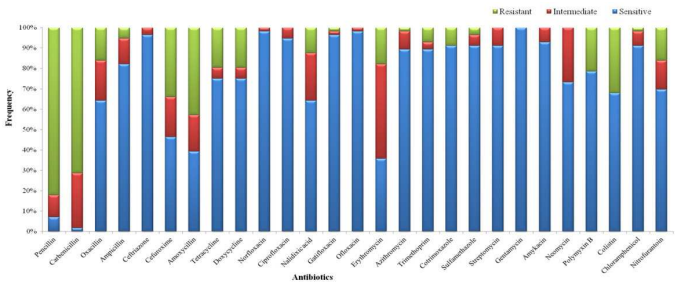
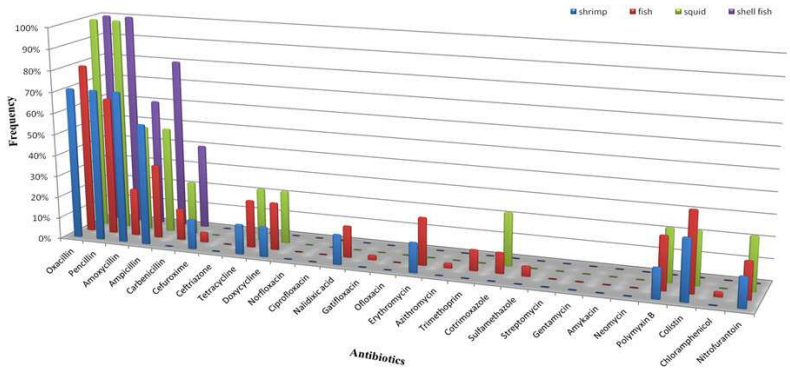


Figure 3
Antibiotic resistance patterns of V. parahaemolyticus isolates from different seafoods



Supplementary Figure: Antibiotic profiles of V. parahaemolyticus isolates used in the study

DISCUSSION

Prevalence of *V. parahaemolyticus* in aquatic environment and marine animals has been increasing worldwide due to a variety of anthropogenic factors like climate change, eutrophication and water contaminantion[20]. The human dependence on the marine environment for seafood and recreation has heightened the potential risk of human infection from marine sources[21]. In the present study, a comprehensive investigation was carried out to determine the prevalence of, *V. parahaemolyticus* in seafoods from local retail shops in Mysore city. Nearly 72% of the seafood samples tested were positive for the organism but only 3.6% of the isolates were found to have the virulence associated markers *tdh* and *trh*. Although the majority of the clinical isolates have these markers, isolates lacking the same have also been recovered from a few patients[22].This emphasises the importance of frequent microbiological monitoring of seafoods. Antibiotic resistance profile of bacterial pathogens provides key information during clinical treatments, epidemiological studies and for effective disease control. The prevalence of antibiotic resistance determinants in *V. parahaemolyticus* isolates recovered worldwide has narrowed the

spectrum of antimicrobials to combat the bacteria thus limiting the therapeutic options to clinicians. About 87.5 % of the isolates recovered from Mysore seafood samples were resistant to the seven β-lactam antibiotics used in this study and this findings correlate with resistance pattern among the isolates recovered elsewhere[8,23]. Further, 20% of the isolates were resistant to Tetracycline, the drug of choice for *V. parahaemolyticus* infections[24]. Therefore, there appears to be a need for an alternate line of antibiotic regimen to be followed in treating *V. parahaemolyticus* infections rather than the frontline antibiotics of β-lactam group and tetracyclines. Our study also revealed that most of the *V. parahaemolyticus* isolates from Mysore city were susceptible to aminoglycosides (73% - 100%), sulfonamides (~90%) and quinolones (64.3% - 98.2%). Indiscriminate usage of antibiotics in aquaculture poses a serious threat for the emergence of multi-drug resistant (MDR) *V. parahaemolyticus,* where the organism is essentially resistant to many of the available antimicrobial agents of same or different classes. The MAR Index analysis (Table 2) revealed that 26.8 % of the isolates had the index value of >0.2, which might indicate that they originated from an environment where several antibiotics were being used. Fish isolate VPF38 has the highest MAR index value of 0.52 and was found resistant to as many as 14 antibiotics belonging to 8 different classes (Table 2). In conclusion, the seafoods from retail markets of Mysore city, India were highly contaminated with *V. parahaemolyticus* (71.2% recovery), though the frequency of virulence markers *trh* and *tdh* in the *V. parahaemolyticus* isolates was low (3.6%). Fifty six isolates recovered from 78 different seafood samples were analysed for their antibiotic susceptibility patterns and the results showed that many isolates were resistant to β-lactams, tetracyclines and polypeptides. Furthermore, 26.8% isolates were resistant to more than two antibiotics of different classes.

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