Iranian Journal of Fisheries Sciences
http:doi.org/ 10.22092/ijfs.2025.133123 2025, 24(2) 435-453

Research Article

Risk assessment of *Vibrio parahaemolyticus* from food contact surfaces in seafood pre-processing plants: A potential threat to value-added processed seafood in India

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Keywords

Antibiotic resistance,
Biofilm formation,
Food contact surface,
MDR,
Seafood safety,
T3SS systems,
Vibrio parahaemolyticus

Article info

Received: June 2023
Accepted: April 2024
Published: March 2025

Abstract

Vibrio parahaemolyticus, autochthonous to marine and estuarine environment, is responsible for foodborne outbreaks associated with seafood. In the present study, a total of 52 *V. parahaemolyticus* strains isolated from various food contact surfaces in seafood pre-processing units located in Cochin were screened for antimicrobial resistance, virulence and biofilm forming capability as part of risk assessment. Multidrug resistance (MDR) was detected in 94.23% of the strains with higher resistance to ampicillin and colistin. Virulence related *trh* and *tdh* genes were present in 30.76% and 3.84% of the isolates, respectively. Among the *T3SS* genes, *T3SS1* and *T3SS2β* were present in 75% and 23.07% of the isolates and none of them harboured *T3SS2α* genes. Among the strains, 86.54% of them were biofilm producers. *V. parahaemolyticus* showed significantly (p<0.05) moderate positive correlations (0.4<r<0.6) between antimicrobial pairs belonging to different antimicrobial classes such as AMP and CL (r=0.57), PI and CL (r=0.54), CB and CL (r=0.48) and S and CL (r=0.48). Our findings highlight the presence of biofilm-forming MDR *V. parahaemolyticus* strains with virulence potential on food contact surfaces, thereby emphasizes the need for continuous monitoring of foodborne pathogens and for improving seafood safety in food processing facilities and retail environments.

Introduction

Vibrio parahaemolyticus is a halophilic foodborne pathogen, which is mostly distributed in aquatic systems worldwide. Among *Vibrio* spp., *V. parahaemolyticus* is the most common shrimp pathogen in aquaculture, responsible for shrimp vibriosis (Zhang *et al*., 2014; Ashrafudoulla *et al*., 2019). It is also associated with foodborne illness in vulnerable individuals including acute gastroenteritis, wound infections, septicemia, and even death due to the consumption of contaminated raw or improperly cooked or mishandled and ready-to-eat seafood particularly shellfishes (Xie *et al*., 2016). Since its discovery in the 1950s in Japan, this organism is associated with seafood-borne illness worldwide (Yang *et al*., 2017). The pathogenicity of *V. parahaemolyticus* is rather complex and most common virulence factors includes thermostable direct hemolysin (tdh), tdh related hemolysin (trh), Type III secretion systems (T3SSs) namely T3SS1 and T3SS2 (Paranjpye *et al*., 2012; Wang *et al*., 2015). Hemolysins are pore-forming proteins that help the pathogen to invade to host tissues. T3SS1 causes cytotoxicity and T3SS2 is especially related to enterotoxicity (Ham and Orth, 2012). *V. parahaemolyticus* are generally susceptible to most clinically used antimicrobials. However, in recent decades, incidence of antibiotic-resistant strains has increased due to its widespread application in clinical and aquaculture sectors (Kang *et al*., 2016). Antibiotic-resistant *V. parahaemolyticus* has been reported recently from various seafoods (Narayanan *et al*., 2020; Ali *et al*., 2021). The prevalence of antibiotic resistance in *V. parahaemolyticus* has led to increasing attention in developing alternative approaches for preventing diseases associated with biofilm.

V. parahaemolyticus is capable of forming biofilm on various biotic or abiotic surfaces such as seafood, stainless steel (SS) by producing distinct adherence factors (Donlan, 2002; Elexson *et al*., 2013). Biofilm is a complex three-dimensional structure enclosed in a gelatinous organic matrix of extracellular biopolymers produced by a heterogenous consortium of bacteria (Mizan *et al*., 2015). Biofilm producers are found to be more resistant to sanitizers and antimicrobial cleaning agents than planktonic cells. As a result, it's difficult to get rid of or neutralize biofilms on food contact surfaces and equipment by normal cleaning and these microorganisms pose a security hazard within the fish processing industry. In the food processing environments, the development of persistent biofilms harboring pathogens is a major problem as bacterial contamination of food-contact surfaces may cause a significant impact on public health (Bridier *et al*., 2015). The Centers for Disease Control and Prevention, national public health institute in the United States, estimate that nearly 65% of all reported infections are caused by bacterial biofilms (Lewis, 2007).

Seafood has been described as one of the fastest-growing sources of food in the world and serves as a major source of income to several developing and developed countries. Approximately 90% of worldwide aquaculture production relies within the Asia region. India is currently the world's second-largest producer of fisheries

after China, with an annual production of 12.59 million tonnes, of which 1.38 million tonnes are exported with a value of 450 billion INR in 2017 - 18 (Handbook on Fisheries Statistics, 2018). However, in earlier times, owing stringent enforcement of quality standards by the importing countries, the seafood exports from India has been banned due to lack of quality. In 1997, the EU banned Indian seafood, citing a lack of hygienic and phytosanitary measures in the industry. In addition, the number of detention and rejection cases is largely reported in Asia because of the detection of *V. parahaemolyticus* (Sujeewa *et al*., 2009). India being a tropical country provides favorable conditions for the rapid dissemination of this pathogenic species. The occurrence of *V. parahaemolyticus* with pandemic characteristics has been reported from seafood in India (Raghunath *et al*., 2008; Pal and Das, 2010; Parthasarathy *et al*., 2016) and was liable for many of the diarrhoeal epidemics, reported in India, Russia, Southeast Asia, Japan, and North America, mainly from hospitalized diarrhoeal cases, saltwater fishes, shellfishes, and coastal environment (Nair *et al*., 2007).

In developing countries, improper sanitary conditions exist within the whole food production chain ranging from primary production to the consumers and therefore the occurrence of a wide range of food-borne diseases create vulnerability in seafood safety. Hence, it's very important to monitor the seafood safety risks associated with seafood production chain. The prevalence of *Vibrio* spp. in shellfish from Cochin estuary and retail markets has been reported earlier (Sudha *et al*., 2012; Silvester *et al*., 2015), however, studies on the incidence of pathogenic *V. parahaemolyticus* on seafood pre-processing environment and biofilm formation ability of the isolates wasn't addressed. As far as we know, no comparative studies can be found in the literature on biofilm formation in the seafood pre-processing environment by *Vibrio* spp. Therefore, in the current study, we analyzed various aspects such as the virulence, antibiogram and biofilm forming potential of *V. parahaemolyticus* isolated from food contact surfaces in a seafood pre-processing environment in Cochin, India. The results of the study are expected to help the industry to recognise this hidden problem and take necessary interventions on urgent basis since seafood products are consumed worldwide.

Materials and methods

Collection of samples

Swab samples from food contact surfaces were collected monthly from two major shrimp pre-processing factories situated in Cochin for two-year period (March 2017 - April 2019) according to the reference method, International Organization for Standardization, 18593 (ISO, 2004). Food contact surfaces such as the processing tables (where peeling and grading were done), plastic crates (used to store the peeled and graded shrimp), and the rubber gloves on the worker's hands, were selected for biofilm bound *V. parahaemolyticus* detection. Sampling was conducted immediately after the cleaning schedule in the pre-processing environment which involves washing the surface with industrial cleaning solutions and scrubber

followed by rinsing with 50 mg/l chlorine water. A sterile stainless-steel template of 25 cm^2 was used to swab the area from food contact surfaces. For worker's hand gloves, swabs from one full hand are used. The swabs were pressed and rolled over the gloves especially between the fingers.

Isolation and identification of V. parahaemolyticus

All swab samples were processed within 2 hrs of collection. Isolation of *V. parahaemolyticus* was performed using the conventional isolation method (FDA, 2004; Sudha *et al*., 2012). Briefly, swab samples of food contact surfaces were enriched in 10 ml of alkaline peptone water (APW) with 3% NaCl and aseptically streaked onto thiosulphate citrate bile salt sucrose (TCBS) agar plates (HiMedia, Mumbai). The presumptive isolates of *V. parahaemolyticus* were picked from the TCBS plates and further confirmed by streaking onto a chromogenic media; HiCrome Vibrio Agar (HiMedia, Mumbai).

PCR screening for species-specific and virulence genes

Genomic DNA of the isolates was extracted by the boiling method (Devi *et al*., 2009). Presumptively identified isolates were further confirmed at molecular level by PCR assay for the *tlh* (450 bp) and *tox R* (368 bp) species-specific genes for *V. parahaemolyticus*, and the presence of virulence genes such as *tdh* and *trh* was detected using multiplex PCR (Bej *et al*., 1999). For detection of *T3SS1* gene, the primer VP1669 previously described by Noriea *et al.* (2010) was used. For presence of *T3SS2α* and *T3SS2β* genes, the primers VPA1346 and VPA1376 as described by Caburlotto *et al.* (2009), were used. PCR was done according to previously described protocol (Silvester *et al*., 2017). Primers used in this study are listed in Table 1.

Table 1: Primers used in the study.

Genes investigated	Primer sequence (5'-3')		Annealing Temperature	References
	Forward	Reverse		
tlh (450 bp)	AAAGCGGATTATGCAGAAGCACTG	GCTACTTTCTAGCATTTTCTCTGC	58°C	Bej *et al.* (1999)
tox R (368 bp)	GTCTTCTGACGCAATCGTTG	ATACGAGTGGTTGCTGTCATG	63°C	
Virulent Genes				
tdh (269 bp)	GTAAAGGTCTCTGACTTTTGGAC	TGGAATAGAACCTTCATCTTCACC	58°C	Bej *et al*. (1999)
trh (500 bp)	TTGGCTTCGATATTTTCAGTATCT	CATAACAAACATATGCCCATTTCCG	58°C	
T3SS1, VP1669 (300 bp)	ACCGAGTTGCCAACGTG	GATTGTTCCGCGATTTCTTG	60°C	Noriea *et al*. (2010)
T3SS2α, VP1346 (393 bp)	GGCTCTGATCTTCGTGAA	GATGTTTCAGGCAACTCTC	60°C	Caburlotto *et al.* (2009); Silvester *et al.* (2017)
T3SS2β, VP1376 (1067 bp)	GCTCTCCTTGGTACCAATCAC	CTGGGATCTTGATGTCAAGGT	50°C	

Antibiotic sensitivity test

Antibiotic susceptibility testing of 52 *V. parahaemolyticus* isolates was done by following the Kirby Bauer disc diffusion method (Bauer *et al*., 1966). A bacterial suspension with turbidity comparable to 0.5 McFarland standards was swabbed onto the mueller-hinton agar (MHA) (Hi-Media, India). Total 25 antibiotic discs (Hi-Media, India) belonged to eleven different classes including aminoglycosides (amikacin, streptomycin, gentamicin), β-lactams (ampicillin, piperacillin, carbenicillin), β-lactams combinations (ampicillin-sulbactam, piperacillin-tazobactam), polypeptide (colistin) quinolones (nalidixic acid), fluoroquinolones (ciprofloxacin, levofloxacin), tetracyclines (tetracycline), nitrofurans (nitrofurantoin), cephalosporins (cefpodoxime, cefotaxime, ceftazidime, ceftriaxone, cefoxitin), carbapenems (ımipenem, meropenem), sulfonamides (co-trimoxazole, trimethoprim), chloramphenicol, and tigecycline were placed on the swabbed agar plates and incubated at 37°C for overnight aerobically. The diameter of zone of inhibition was measured as per the guidelines of Clinical Laboratory Standards Institute. Based on resistance to more than three antibiotics, the isolates were assigned as multi-drug resistant and determined their multiple antibiotic resistance (MAR) index (Krumperman, 1983).

Quantification of biofilm production

The biofilm-forming capacity of the isolates was quantified by microplate assay (Stepanovic *et al*., 2007), with slight modifications. Briefly, the cell suspension was prepared in Tryptone Soy Broth (TSB, Himedia) with turbidity adjusted according to McFarland standard 0.5 (equivalent to 1.5×10^8 cfu/mL). Subsequently, triplicated aliquots of 200 µl of the cell suspension were placed in a 96 well polystyrene microplate and incubated at 37°C for 24 hrs; 200 µL sterile TSB was used as negative control. Each well was washed three times with sterile saline solution (NaCl, 0.85%) to remove loosely adhered cells. The adhered cells were fixed with 200 µL of 99% methanol (15 min), and the microplate was air-dried. The cells were stained with 200 µL of crystal violet solution (0.5%, 5 min). The dye was drained and rinsed with tap water, with subsequent drying of the microplate. Finally, the dye remaining in the wells was resolubilized in 200 µL of glacial acetic acid (33%). The reading of the optical density (OD) was performed using a microplate reader (Varioskan® LUX Microplate Readers, Thermo Scientific) at λ of 570 nm. The mean and standard deviation of the negative control was also calculated (ODc). The isolates with OD≤ODc were considered non-biofilm forming; isolates with ODc<OD≤(ODc×2) were considered weak formers; isolates with (ODc × 2)<OD≤(ODc×4) were considered moderate formers; isolates with (ODc×4)<OD were considered strong formers.

Statistical analysis

Pearson correlation analysis was done in R software (R version 4.0.5 (2021-03-31); https://www.r-project.org), to find the relationship between antimicrobial resistance phenotypes, virulence genes and biofilm formation among *V.*

parahaemolyticus isolates. The correlation was considered strong if the r ≥ 0.6, moderate if the r value was between 0.4 - 0.6, and weak if r<0.4 (Jiang *et al*., 2019).

Results

Prevalence of pathogenic V. parahaemolyticus in biofilm formed on food contact surfaces

In the present study, a qualitative analysis of 144 swab samples collected during 2017 - 2019 from biofilm formed on food contact surfaces in a seafood pre-processing plant was performed for the presence of *Vibrio parahaemolyticus*. Sixty-three swabs (43.75%) were positive for *V. parahaemolyticus*. A total of 75 sucrose non-fermenting colonies that had a green or bluish-green color with a dark blue or green center, presumptive of *V. parahaemolyticus* were selected from the TCBS agar plates. Of the 75 isolates, 52 of them were confirmed as *V. parahaemolyticus* on Hichrome Vibrio agar by producing bluish-green–coloured colonies. All 52 isolates were further confirmed at the molecular level by PCR assay targeting the species-specific *tlh* gene (450 bp) and *tox R* (368 bp) gene (Fig. 1).

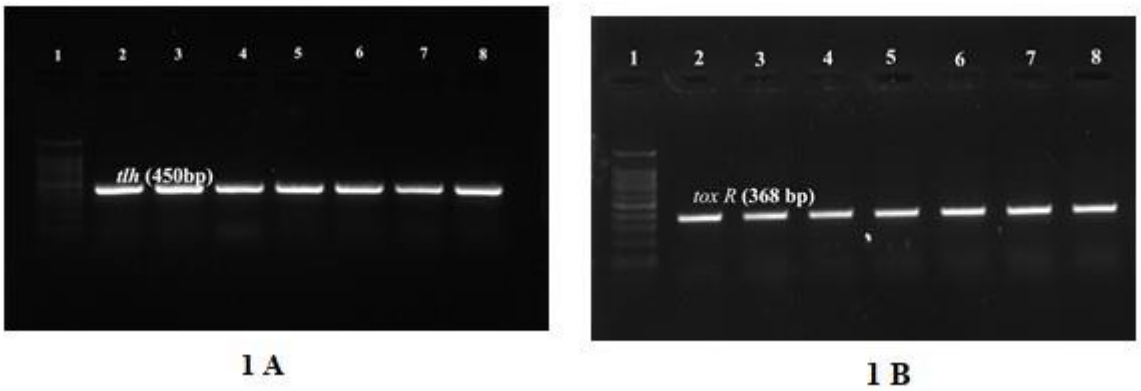


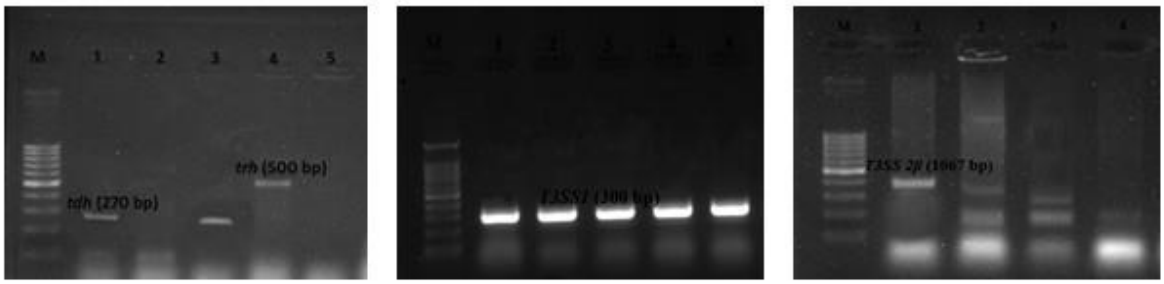
Figure 1: (1A) PCR amplification of *Vibrio parahaemolyticus* specific *tlh* (450 bp) gene products. Lane 1: 100 bp DNA ladder; lanes 2–8: *V. parahaemolyticus* isolates (1B) PCR amplification of *tox R* gene (368 bp). Lane 1: 100 bp DNA ladder; lane 2: *V. parahaemolyticus* ATCC 17802 (positive control); lanes 3–8: *V. parahaemolyticus* isolates.

The highest recovery of the organism was from the processing table (22/52, 42.31%) followed by plastic crates (19/52, 36.54%) and rubber gloves of worker's hands (11/52, 21.15%) (Table 2). The isolates were screened for virulence markers, tdh, trh T3SS1, T3SS2α, and T3SS2β (Fig. 2).

Eighteen (34.62%) isolates harboured either tdh or trh haemolysins (*tdh*/*trh*) and were potentially toxigenic. Among these, the *tdh* gene was present in two isolates (*tdh*+*trh*−) while trh gene (*tdh*−*trh*+) in sixteen isolates (Fig. 3).

Table 2: Prevalence of *V. parahaemolyticus* in biofilm formed on various food contact surfaces.

Food contact Surface	No. of samples analyzed	No. of samples tested +ve	Prevalence of *V. parahaemolyticus* (%)
Processing- Tables	48	32	66.67
Plastic Crates	48	19	39.58
Worker's- hands (Gloves)	48	12	25



2A 2B 2C

Figure 2: (2A) PCR amplification of *Vibrio parahaemolyticus* specific *tdh* (270 bp) and *trh* (500 bp) gene products. Lane M: 100 bp DNA ladder; lanes 1and 3: *tdh* in *V. parahaemolyticus* isolates; lane 4: *trh* in *V. parahaemolyticus* isolate (2B) PCR amplification of *T3SS1* gene VP1669. Lane M: 100 bp DNA ladder; lanes 1-5: *T3SS1* gene in *V. parahaemolyticus* isolates; (2c) PCR amplification of *T3SS2β* gene VPA1376. Lane M: 1Kb DNA ladder; lanes 1: *T3SS2β* gene *in V. parahaemolyticus* isolate.

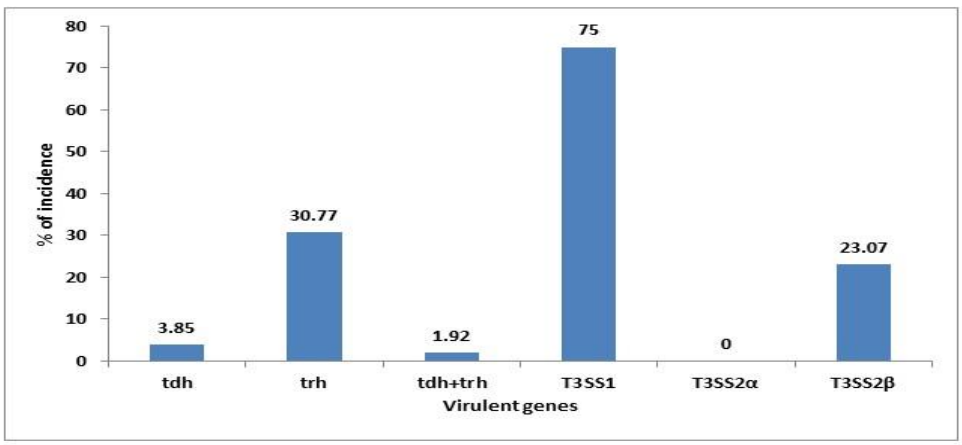


Figure 3: Overall prevalence of virulence genes among *Vibrio parahaemolyticus* in biofilm formed on various food contact surfaces.

Only one isolate possessed both *tdh* and *trh* gene. The isolates were also checked for the presence of T3SS genes such as *T3SS1*, *T3SS2α* and *T3SS2β*. *T3SS1* was the most prevalent virulent gene (41/52, 78.85%) found among *V. parahaemolyticus* strains. *T3SS2α* gene was not detected in any of the strains obtained. Gene encoding the *T3SS2β* was present in 23.08% (12/52) of the strains.

Antibiotic sensitivity of V. parahaemolyticus

The antibiotic resistance profile percentage of *V. parahaemolyticus* is summarized in Figure 4.

Results identified 98.07% of the isolates as ampicillin-resistant. Apart from ampicillin, the majority of them exhibited resistance towards Piperacillin (82.69%), Carbenicillin (78.85%), Colistin (94.23%),

and Streptomycin (78.85%). In the present study, resistance towards the third generation cephalosporins (Cefpodoxime 71.15%, Cefotaxime 71.15%, Ceftazidime 65.39%, Cefoxitin 21.15% and Ceftriaxone 11.53%) was also observed among *V. parahaemolyticus* isolates. All the isolates tested remained susceptible to nalidixic acid (100%), and Chloramphenicol (100%). More than 70% of the isolates were sensitive to Ampicillin-Sulbactam, Gentamicin, Levofloxacin, Co-Trimoxazole, and Tetracycline (Table 3).

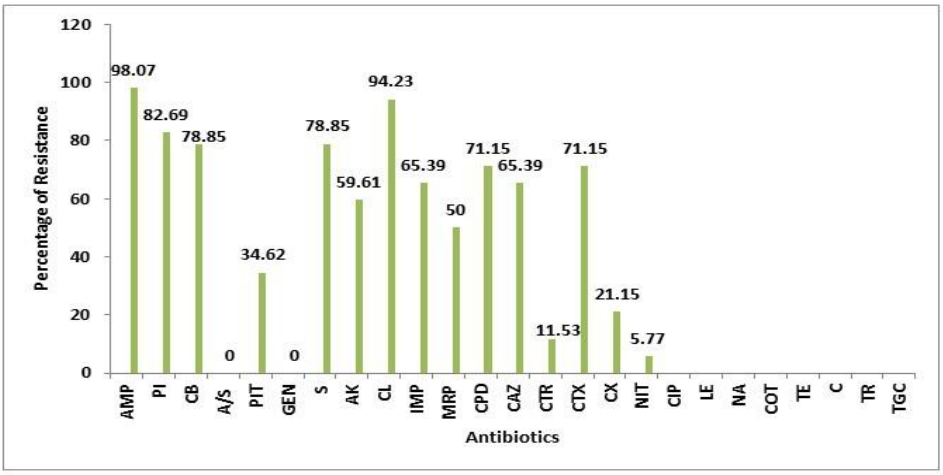


Figure 4: Percentage of antibiotic resistance of *Vibrio parahaemolyticus* isolates from food contact surfaces in seafood processing plants.

Table 3: Antibiotic resistance of *Vibrio parahaemolyticus* isolates from food contact surfaces in seafood processing plants.

Antibiotics	% of Resistance		
	S	I	R
Ampicillin (AMP^{10})	1.92	0	98.07
Piperacillin (PI^{100})	7.69	9.62	82.69
Carbenicillin (CB^{100})	15.38	5.77	78.85
Ampicillin-Sulbactam ($A/S^{10/10}$)	88.46	11.54	0
Piperacillin-Tazobactam ($PIT^{100/10}$)	38.46	26.92	34.62
Gentamicin (GEN^{10})	76.92	23.08	0
Streptomycin (S^{10})	5.77	15.38	78.85
Amikacin (AK^{30})	5.77	34.62	59.61
Colistin (CL^{10})	5.77	0	94.23
Imipenem (IMP^{10})	26.92	7.69	65.39
Meropenem(MRP^{10})	38.46	11.54	50
Cefpodoxime (CPD^{10})	17.31	11.54	71.15
Ceftazidime (CAZ^{30})	7.69	26.92	65.39
Ceftriaxone (CTR^{30})	38.46	50	11.53
Cefotaxime (CTX^{30})	11.54	17.31	71.15
Cefoxitin (CX30)	5.77	73.08	21.15
Nitrofurantoin (NIT^{300})	61.54	32.69	5.77
Ciprofloxacin (CIP^{5})	65.38	34.62	0
Levofloxacin (LE^{5})	76.92	23.08	0
Nalidixic Acid (NA^{30})	100	0	0
Co-Trimoxazole (COT^{25})	80.77	19.23	0
Tetracycline (TE^{30})	76.92	23.08	0
Chloramphenicol (C^{30})	100	0	0
Trimethoprim (TR^{5})	69.23	30.77	0
Tigecycline (TGC^{15})	69.23	30.77	0

The majority of the isolates (94.23%) showed resistance towards multiple antibiotics. The isolates have the MAR index ranged from 0.08 to 0.6 (Table 4).

Table 4: Multiple antibiotic resistance (MAR) index and antibiotic resistance pattern of *Vibrio parahaemolyticus* isolates from food contact surfaces in seafood processing plants.

MAR Index	Resistance Pattern	Percentage of Incidence
0.08	AMP,CL	11.54
0.12	AMP,PI,CL	3.85
0.2	AMP,PI,CB,S,CL	7.69
0.28	AMP,PI,CB,S,CL,CPD,CTX	5.77
0.36	AMP,PI,CB,S,CL,IMP,CPD,CAZ,CTX	5.77
0.4	AMP,PI,CB,S,AK,CL,IMP,CPD,CAZ,CTX	9.62
0.44	AMP,PI,CB,S,AK,CL,IMP,MRP,CPD,CAZ,CTX	15.38
0.48	AMP,PI,CB,PIT,S,AK,CL,IMP,MRP,CPD,CAZ,CTX	13.46
0.52	AMP,PI,CB,PIT,S,AK,CL,IMP,MRP,CPD,CAZ,CTX,CX	9.62
0.56	AMP,PI,CB,PIT,S,AK,CL,IMP,MRP,CPD,CAZ,CTR,CTX,CX	5.77
0.6	AMP,PI,CB,PIT,S,AK,CL,IMP,MRP,CPD,CAZ,CTR,CTX,CX,NIT	5.77

The most repeated resistance pattern was found to be AMP, PI, CB, S, AK, CL, IMP, MRP, CPD, CAZ, CTX. Most of the *trh*+ isolates and the two *tdh*+ isolates have MAR index greater than 0.2. (Detailed description of each isolate such as its source of isolation, MAR index, resistance profile, virulence genes, etc. is provided in Supplementary Table 1).

Biofilm formation capability of V. parahaemolyticus

The biofilm formation capability of *V. parahaemolyticus in vitro* was investigated by microtitre plate assay. In total, the frequency of biofilm formation in *V. parahaemolyticus* was 86.54%; of these, 10 (19.23%) were strong, 19 (36.54%) were moderate and 16 (30.77%) were weak biofilm producers (Fig. 5).

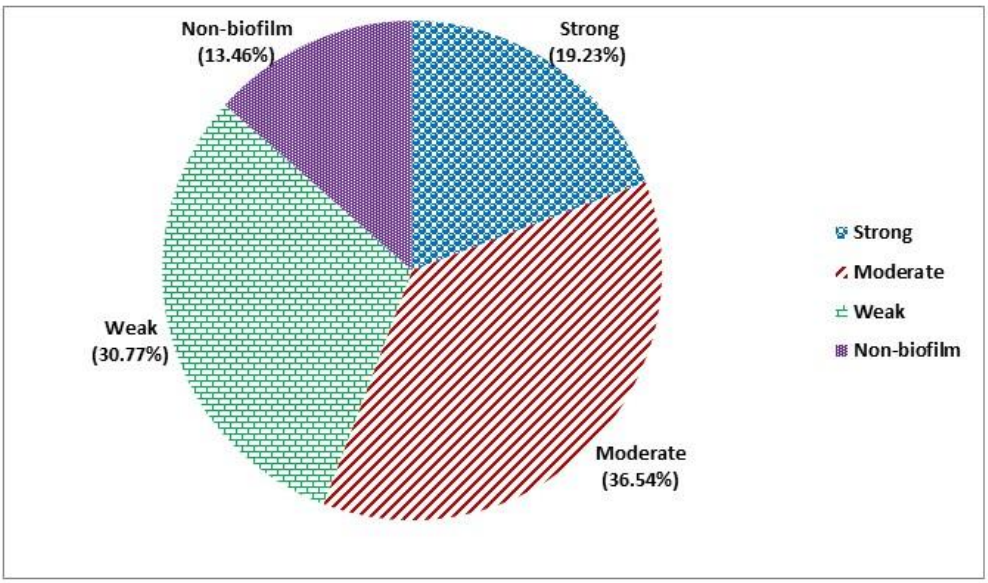


Figure 5: Classification of biofilm producers.

Association of resistance phenotypes, virulence-associated genes and biofilm formation in V. parahaemolyticus

Correlation analysis of antimicrobial resistance phenotypes, biofilm-forming capability and virulence genes for *V. parahaemolyticus* was performed (Fig. 6).

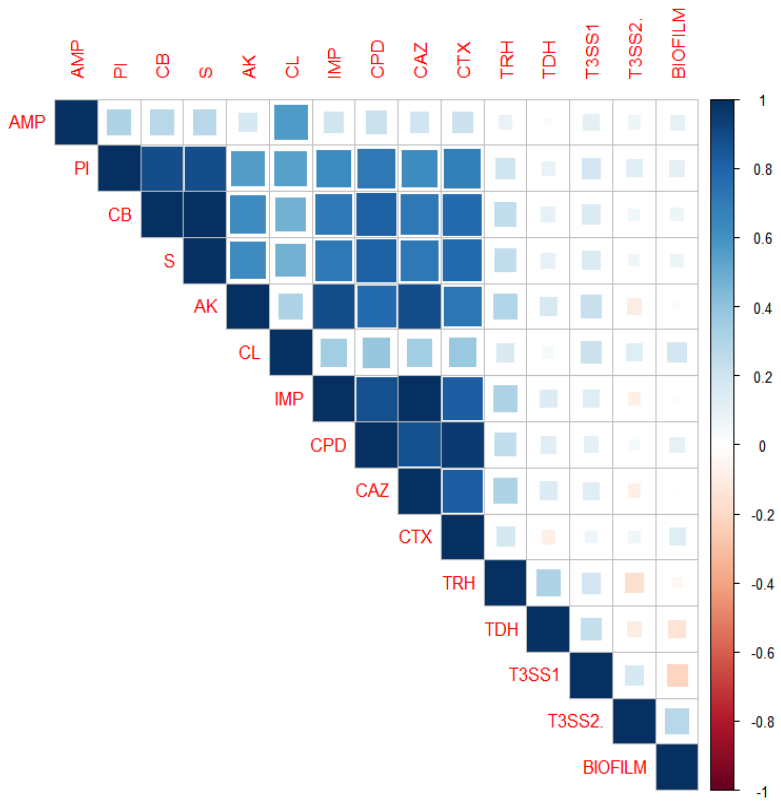


Figure 6: Correlation analysis between resistance phenotypes, virulence determinants and biofilm production among *V. parahaemolyticus* isolates Blue and red colors of boxes indicate positive and negative correlation, respectively. The strength of the colors corresponds to the numerical value of the correlation coefficient (r) and significant correlations (p<0.05).

The results showed significant (p<0.05) moderate positive correlations (0.4<r<0.6) with β- lactam group of antibiotics between antimicrobial pairs belonging to different antimicrobial classes such as those observed for AMP and colistin (r =0.57), PI and CL (r=0.54), CB and CL (r=0.48) and S and CL (r=0.48) among the isolates. Colistin also showed weak positive correlation with other antibiotics such as AK (r=0.3), IMP (r=0.34), CPD(r=0.39), CAZ (r=0.34) and CTX(r= 0.37). PI, CB, S, and AK showed strong positive correlation (r>0.6) with IMP, CPD, CAZ and CTX. AMP showed weak positive correlation (r<0.4) with these antibiotics. There is a weak positive correlation (r<0.4) between antibiotics and virulence gene such as tdh and T3SS1. The virulence gene, tdh showed significantly weak positive correlation (r<0.4) with trh gene. The virulent gene, *trh* was positively correlated (r<0.4) with AK, IMP, CAZ and tdh gene significantly. *T3SS2β* gene was correlated positively either with antimicrobials such as AMP, PI, CB, S, CL, CPD, CTX and negatively with

AK, IMP, CAZ, *trh*, *tdh* and biofilm ability non-significantly. However, biofilm formation was positively correlated significantly (p<0.05) with the antibiotics such as AMP, PI, CB, S, and CL but non-significantly with remaining antibiotics and virulent genes except *tdh*. Weak positive correlation was found between antimicrobials belonging to the same class such as P and AMP (r=0.31; p<0.05).

Discussion

V. parahaemolyticus considered an important seafood-borne pathogen, has been recovered from various marine organisms (Ghenem *et al*., 2017). *V. parahaemolyticus* infections are commonly associated with the consumption of raw or undercooked seafood; however recently, also been found in RTE foods (Jeong *et al*., 2020). Around 30,000 foodborne infections are caused by *V. parahaemolyticus* globally (Roomeela *et al*., 2018). In the present study, a high incidence of the organism was found in seafood processing plants, the highest being from swab samples collected from the processing table (42.31%) as compared to plastic crates (36.54%) and rubber gloves of worker's hands (21.15%). The pre-processing centers analyzed in the present study were maintaining relatively better sanitation and process control. The shrimps pre-processed at these centers were finally processed in higher-end processing factories to value-added shrimps for export markets. Previous study by Kariyawasam and Jayasooriya (2010) had already observed that conditions for biofilm formation were more conducive in shrimp processing factories than that of fish processing. All the food contact surfaces in fish and shrimp processing come into direct contact with either the raw fish or raw shrimp during its processing and they also have rough surfaces, which make them highly favorable for biofilm formation. It was also observed that during daily cleaning, the food contact surfaces were not thoroughly scrubbed off to remove the attached biofilm. They are only subjected to routine washing before and after processing, which removes only the upper detaching layer of the biofilms and biofilm is hydrophobic; it resists being washed away by water (Johnston and Swanson, 2000).

The prevalence of virulent strains (tdh/trh+) in seafood and the environment accounts for less than 2% of isolates (Drake *et al*., 2007), however, most of the clinical *V. parahaemolyticus* isolates carry *tdh* and/or *trh* genes (Letchumanan *et al*., 2015). Earlier literatures report the prevalence of pathogenic *V. parahaemolyticus* possessing *tdh* and *trh* gene from Cochin estuary and tiger shrimp culture environment in Cochin along the south-west coast of India (Devi *et al*., 2009; Silvester *et al*., 2015), and also from retail seafood in India (Pal and Das, 2010; Parthasarathy *et al*., 2016). Most recently, Narayanan *et al*. (2020) described the occurrence of potentially pathogenic *V. parahaemolyticus* possessing the *tdh* and/or *trh* genes in the seafood sold in retail markets of Cochin, Kerala. In the present study, *tdh* and *trh* hemolysin genes, which have a pivotal role in the pathogenesis in human beings, were identified only in 2 (3.84%) and 16 (30.76%) out of 52 isolates, respectively. The potential of *tdh* and *trh* negative isolates to cause disease in humans

could be either due to concurrent infection with virulent strains or loss of virulence genes during infection, and/or uncharacterized and novel virulence factors. The type III secretion systems (T3SSs) also take part in the pathogenicity of *V. parahaemolyticus*. It consists of a needle-like apparatus carrying a large number of effector proteins. When it enters the host cells, it disrupts the host's immune response (Coburn *et al*., 2007; Silvester *et al*., 2017). In the present study, most of the strains produced a 300 bp positive amplification for *T3SS1* gene. This confirmed previous studies stating that *T3SS1* is ubiquitous in *V. parahaemolyticus* (Silvester *et al*., 2015). *T3SS1* gene induces cytotoxicity in the host cells whereas *T3SS2* genes are involved in enterotoxicity (Park *et al*., 2004). *T3SS2* genes are located on the pathogenicity island (Vp-PAI) in Chromosome II of *V. parahaemolyticus* (Ham and Orth, 2012). It promotes the relocation of this distinct DNA segment from a virulent strain to non-virulent indigenous bacteria in the aquatic environment (Caburlotto *et al*., 2009; Silvester *et al*., 2017). The presence of *T3SS* genes in *V. parahaemolyticus* proposes the probability of triggering infection in humans and marine animals by these strains.

In recent decades, antibiotic resistance may be a global public health concern and a food safety problem (Pan *et al*., 2018). The burden of deaths from antimicrobial resistance is predicted to take 10 million lives by 2050, at a cumulative cost to global economic output of 100 trillion USD unless action is taken by the authority (O'Neill, 2016). Multi-drug resistant *V. parahaemolyticus* strains have been reported earlier from South India in seafood from retail markets, Cochin estuary and traditional prawn farms (Sudha *et al*., 2014; Silvester *et al*., 2015). The present study also revealed the MDR strains of *V. parahaemolyticus* from food contact surfaces in seafood pre-processing plant. These MDR potentially pathogenic isolates were resistant to second and third generation cephalosporins. Recently, Narayanan *et al*. (2020) also reported drug resistance towards second, third and fourth generation cephalosporins among *V. parahaemolyticus* in seafood from retail markets of Cochin, Kerala. In consonance with the previous studies, colistin and carbapenems are increasingly becoming ineffective against *V. parahaemolyticus*; both are generally used as a "last resort drug" to treat serious infections caused by multi-drug resistant, Gram-negative pathogens. (Jun *et al*., 2012; Sudha *et al*., 2014; Letchumanan *et al*., 2015; Elmahdi *et al*., 2016). Recently, Lei *et al*. (2019) also reported plasmid mediated colistin resistance gene, *mcr-1* in *V. parahaemolyticus* and highlighted a potential transfer of the *mcr*-1 gene from *E. coli* or other Enterobacteriaceae species to non Enterobacteriaceae Gram-negative bacteria, and suggested the gene is likely to spread rapidly into virulent *V. parahaemolyticus* strains, which can pose a major threat to public health and warrants further investigation. In consistent with these findings, all *tdh*+ and *trh*+ isolates in the present study showed colistin resistance. In agreement with the earlier reports by researchers from various countries regardless of its source from

which they were isolated, a high proportion of the isolates in the current study showed ampicillin resistance (Letchumanan *et al*., 2015; Xie *et al*., 2017; Zhao *et al*., 2018; Jiang *et al*., 2019; Xie *et al*., 2020).

In the present study, most of the isolates (69.23%) from the food contact surfaces had a MAR index greater than 0.2. According to previous reports, this clearly points towards the origin of the strains from a high-risk source of contamination where antibiotics are frequently used (Silvester *et al*., 2015). Previous studies reported the occurrence of multidrug resistant *V. parahaemolyticus* in ready-to-eat (RTE) foods, shrimp, and fish (Letchumanan *et al*., 2015, Xie *et al*., 2020). Therefore, it's crucial to analyze the variations in antimicrobial susceptibility profiles in *V. parahaemolyticus* strains. The high MDR rate is alarming because the emergence of MDR *V. parahaemolyticus* may increase the risk of disease outbreaks from the seafood industry and may be a serious clinical problem in terms of treatment and may cause a rise in fatality rates.

Most seafood-borne pathogens form biofilms on seafood, on food-contact surfaces, and in water (Shikongo-Nambabi *et al*., 2012). *V. parahaemolyticus* isolates were tested for their ability to form biofilms *in vitro* using microtitre plate assay. The results revealed that 86.54% of *V. parahaemolyticus* have ability to form biofilms. Han *et al*. (2016) also reported *V. parahaemolyticus* can form biofilm on food and food contact surfaces during food processing. The preconditioning of the food surfaces with food juice provides a protective matrix for the bacterial cells to impede it (Noyce *et al*., 2006). In shrimp processing units, beheading and degutting operations result in the release of shrimp juices onto these surfaces which are periodically rinsed with chlorinated water. However, the chlorination levels are not monitored regularly to ensure the required concentration of available chlorine. Those *V. parahaemolyticus*, which would probably present on shells or gills of shrimps, then get an ideal environment for proliferation and probably form a biofilm on these surfaces. Mizan *et al*. (2015) reported that fresh fish products may suffer from biofilm formation by *Vibrio* spp. causing significant health and economic issues.

Correlation analysis of antimicrobial resistance phenotypes, biofilm-forming capability and virulence genes among *V. parahaemolyticus* showed that there is a positive association between antibiotics belonging to different classes which indicates that a selective pressure is produced by these antimicrobials due to their indiscriminate use in aquaculture (Okocha *et al*., 2018; Sadat *et al*., 2020). The co-occurrence of the antimicrobial-resistant genes on the chromosome or plasmids may also be another reason for this association (Ramadan *et al*., 2020). Concerning resistance and virulence genes association, a significant positive correlation was only identified between *trh* gene and resistance to amikacin, imipenem and ceftazidime among *V. parahaemolyticus* isolates. Similarly, Sadat *et al*. (2020) reported a positive correlation between *trh* gene and resistance to imipenem (r=0.38) and erythromycin (r=0.45) among *V. parahaemolyticus* isolated from freshwater and marine fish

and shellfish collected from wet markets and supermarkets in Mansoura, Egypt. Nearly similar results have been reported previously in Egypt for *Aeromonas hydrophila* isolates from marketed mullet where resistance and virulence genes were positively correlated (r=0.3) (Ramadan *et al*., 2018). Generally, factors including bacterial species, potential pathogenicity, genetic transmission, and hosts, depict the relationship between antibiotic resistance and virulence (Pan *et al*., 2020). Park *et al.* (2004) and Noriea *et al*. (2010) suggested the presence of a correlation between *tdh* and *T3SS2α* gene and *trh* and *T3SS2 β* gene. However, in the present study, there is any such correlation exists. The present study corroborated with a previous study where environmental strains of *V. parahaemolyticus* which lacked a hemolysin gene revealed the presence of *T3SS2* gene (Paranjpye *et al*., 2012).

Conclusions

The present study is highly significant since the majority of isolated *V. prahaemolyticus* from the food contact surfaces in the seafood processing industry were biofilm-forming MDR *V. parahaemolyticus* strains with virulence potential. Since a baseline study on this aspect is deficient from the processing environment in the seafood industry, it would be worth observing the prevalence of *V. prahaemolyticus* in seafood industry particularly with the occurrence of virulent and drug-resistant groups. There is a possibility of the transfer of these pathogens to human beings through the food chain. It highlights the importance of this current study, as more dominant groups of pathogens establish with changing environments. Cochin is one of the main seafood exporting centers of Kerala; it needs to restrain the outbreak of pathogenic Vibrios to ensure a safe export of seafood. Although the present study is localized in nature, the significance of the study lies in the fact that processed seafood is exported worldwide from Cochin. Aquacultured shrimp produced in India is mostly exported to China, USA, Japan, and countries in the European Union. Therefore, frequent monitoring of the processing environment is necessary to ensure the microbial quality of seafood and public health safety in our study area. Seafood contaminated with pathogenic *V. parahaemolyticus* will lead to export rejection, which in turn affects our economy.

Acknowledgments

The authors are thankful to the Head of the Department of Marine Biology, Microbiology, and Biochemistry, Cochin University of Science and Technology, Cochin, for extending facilities to carry out this work. First author would like to thank Dept. of Science and Technology for providing financial assistance through DST Purse fellowship and also to acknowledge Cochin University of Science and Technology for providing junior and senior research fellowships.

Conflicts of interest

The authors declare that there is no conflict of interest.

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