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Research Article

In-Vitro and *In-Vivo* Pathogenicity of Mar, Biofilm Forming Non-Cholera *Vibrios* (NCV) From Asian Tiger Shrimp (*Penaeus monodon*): Implications for Food Safety and Sanitation

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Abstract

Contamination of aquatic environment with antibiotic resistant bacteria is a multi-factorial global threat with deleterious effects on human and animal health. In this preliminary study, we examined the *in-vitro* susceptibility of 14 antibiotics against 4 biofilm forming non-cholera *Vibrios*, (*Vibrio alginolyticus* and *Vibrio parahemolyticus*) isolated from fresh and healthy Asian Tiger Shrimp (*Penaeus monodon*), sourced from retail fish markets in Cochin during a three-month period. Biofilm forming capacities were evaluated by qualitative and quantitative assays as well as by characterization of biofilms under different food related stress conditions. Growth temperature and NaCl concentration influenced biofilm formation profoundly. Antibiotic resistance/susceptibility profiles of 4 biofilm formers assessed by Kirby-Bauer disc diffusion method, showed resistance to 13 of the 14 antibiotics tested. The pathogenicity profile of the isolates was elucidated by *in-vitro* assays like exo-enzyme profiling, auto-aggregation and surface hydrophobicity. In addition, a *Caenorhabditis elegans* based *in-vivo* pathogenicity testing by survival score analysis was also included. All these findings reveal that shrimp or related seafood harbors antibiotic resistant, biofilm forming *Vibrios*, indicating high-risk of food-related illnesses in humans. Further understanding of these processes will provide novel insights into the therapeutics and prevention of biofilm-related *Vibrio* infections in the aquaculture/seafood industry.

Keywords: *Vibrios*; Shrimp; Antibiotic resistance; Pathogenicity; Biofilms; Food contamination; *C.elegans*

Introduction

Bacterial adherence to food products or contact surfaces is a significant source of contamination, causing hygiene/health issues as well as economic losses in the seafood industry. Biofilms being a predominant and successful mode of microbial life, can implicitly develop on natural as well as man-made [1]. These heterogeneous microbial communities enclosed in a self-synthesized layer of complex polysaccharides, proteins, lipids and extracellular DNA, collectively called the extracellular polymeric substance or EPS [2,3], can colonize different sea foods like cockles, shrimp, crabs etc raising food safety concerns on a global scale.

Farmed shrimp are administered antibiotics like gentamicin, sulphonamides, tetracyclines, chloramphenicol, trimethoprim, fluoroquinolones etc., on a regular basis to prevent or treat bacterial diseases [4]. Even as tetracycline is recommended in shrimp farming [5], the last decade saw several reports on the acquisition of tetracycline resistance *via* plasmids or other mobile genetic elements [6]. Antibiotic resistant halophilic `Non-Cholera *Vibrios*' (NCV's) associated with shrimp farming in India have been documented [7,8]. However, they do not satisfactory correlate (if any) biofilm formation, antibiotic resistance and virulence potential of *Vibrios*. The dangerous spread of biofilm related bacterial infections augmented the demand to study 'host pathogen interactions' using *Caenorhabditis elegans* as a resourceful model. This pilot study was therefore undertaken to evaluate antibiotic susceptibility and virulence potential of biofilm forming NCV's isolated from edible shrimp (*Penaeus monodon*).

Materials and Methods

Sampling

10 shrimp sampling, from four retail fish markets in Cochin, South India over a three month period from January to March 2017 were done. The head and tail were removed and the gut homogenized with 0.85% saline; 25g of homogenate added to 225mL of Alkaline Peptone Water (APW) pH 8.6, and incubated at 37^0 C for 24 hours. Two loopful of culture from pellicle of each flask with APW were plated on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates (HiMedia, Mumbai, India) and incubated at 37^0C for 24 hours. Green and yellow colonies (3-5mm diameter) were randomly selected and inoculated onto *Vibrio*-specific agars such as *Vibrio* Alginolyticus agar (VAL) [9] and *Vibrio* Parahaemolyticus Sucrose Agar (VPSA) (HiMedia, Mumbai, India) and incubated overnight for confirmation.

Biochemical characterization of bacterial strains

Phenotypic characterization of the isolates was as outlined in

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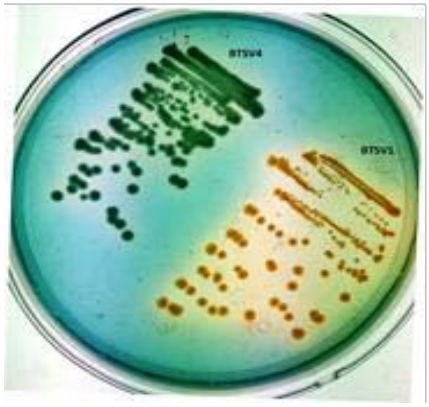

Figure 1: Bluish green colony of *V. parahaemolyticus* (BTSV4) and yellow colony of *V.alginolyticus* (BTSV1) in VPSA.

Bergey's Manual of Systematic Bacteriology, by using KB007 Hi-*Vibrio*™ Identification Kit.

Molecular confirmation of *Vibrio* species

Genomic DNA was isolated and purified and a portion of the 16S rDNA was amplified using a universal primer pair for 16S rDNA**.** The sequences for the primer pair is as follows:-Forward primer -5' AGAGTTTGATCCTGGCTCAG 3'. Reverse primer -5'ACGGCTACCTTGTTACGACTT 3'.The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm [10]. A phylogenetic tree was constructed by comparing the present isolates with *Vibrio* phylogeny retrieved from GenBank database by the Neighbor-Joining method [11] using the MEGA 4 software [12]. The obtained sequences were aligned and submitted in GenBank.

Screening of biofilm formers among *Vibrio* isolates

Qualitative assessment of biofilms: Congo Red Agar method [13] and Tube adherence method [14] were used with modifications for qualitative assessment of biofilms methods. The bacterial strains were incubated overnight on CRA plates and observed for the presence of rough black crystalline colonies which are indicative of biofilm production. For tube adherence test, strains were grown in nutrient broth under shaking conditions to allow biofilm formation and then transferred to glass test tubes, incubated without shaking for 18 hours at 28°C; the culture broth was discarded and the biofilm at the interface between the air and medium was visualized using 1% Crystal Violet (CV). BTSD2 (*Bacillus licheniformis,* accession no: KF573745) [15] was used as positive control, while un-inoculated Trypticase soy broth medium was used as a negative control for the biofilm assays.

Quantitative assessment of biofilms: The quantitative estimation of biofilm was performed by microtiter plate method [16,17]. Briefly, the overnight cultures of *Vibrios* were diluted (1:10 dilution) and 20 µl of the diluted broth was added to 300 µl of TSB in 96-well flat-bottomed microtiter plate. After overnight incubation, the contents of each well were aspirated with PBS buffer and vigorously shaken in order to remove any non-adherent bacteria. The remaining bacteria were fixed with methanol for 15 minutes and later stained for 5 minutes with 1% CV solution. Excess stain was rinsed off and the biofilms, upon drying were extracted with 33% (v/v) glacial acetic acid per well. All tests were repeated thrice independently, and statistically analyzed.

Investigation of *Vibrio* biofilms under different stress conditions

Influence of incubation temperature on biofilm formation was investigated at 4°C, 28^{0}C and 37^{0}C for 24, 48 and 72 hours under static conditions using micro-titre plate method.

Influence of static and dynamic (shaking) conditions on biofilm formation - Dynamic conditions was achieved by incubating on a horizontal shaker at 150 rpm. The microtitre plates were incubated for 24, 48 and 72 hours at 28^{0}C and 37^{0}C under static and dynamic conditions.

Influence of NaCl on biofilm formation used 1%, 3%, 5% 8% and 10% concentrations of NaCl in TSB for 24 hours under static conditions.

Antibiotic susceptibility testing

Antibiotic sensitivity of *Vibrio* isolates were tested on Mueller–Hinton (MH) agar in accordance with the Kirby- Bauer method [18], with 14 antibiotics (HiMedia, Mumbai, India) belonging to different classes, namely ampicillin (10µg/disc), azithromycin (15µg/disc), carbenicillin (100µg/disc), cefixime (5µg/disc), cefuroxime (30µg/disc), chloramphenicol (30µg/disc), co-trimoxazole (25µg/disc), gentamicin (10µg/disc), nalidixic acid (30µg/disc), rifampicin (5µg/disc), streptomycin (10µg/disc), tetracycline (30µg/disc), trimethoprim (5µg/disc) and vancomycin (30µg/disc). Fresh cultures were inoculated into Luria -Bertani broth and incubated until Optical density equaled MacFarland 0.5, plated on Mueller–Hinton agar and antibiotic discs were placed. Upon incubation at 37°C for 18–20 hours, growth inhibition around discs was measured and the results were interpreted as per the manufacturers' instructions.

In vitro pathogenicity assays for *Vibrios*

(a) Exoenzyme profiling was done by incorporating aesculin**,** starch and tributyrin into the basal medium and the plates were observed to determine the exo-enzyme production [19].

(b) Hemolytic assay-The test organisms were spot inoculated on blood agar plates, incubated at 37^{0}C for 24 hours and alpha, beta or gamma hemolysis were categorized based on the lytic zones produced [20].

(c) Auto aggregation assay and Suicide Phenomenon - Auto aggregation assay was performed according to Kos *et al.* [21] with modifications. Bacteria were grown for 18 hours at 37^{0}C in nutrient broth with 1% NaCl, harvested by centrifugation at 5000g for 15min, washed twice and resuspended in Phosphate Buffered Saline (PBS) to get approximately 10^8 CFU $ml^{-1.}$ Cell suspension (4mL) was vortexed for 10 sec and auto aggregation was determined during 5 hours of incubation at room temperature. Every hour 0.1mL from the upper suspension was transferred to another tube with 3.9mL of PBS and the absorbance (A) was measured at 600nm. Auto aggregation percentage is expressed as $1-(A_t/A_0) \times 100$, where A_t represents the absorbance at time t =1, 2, 3, 4 or 5 hours and A_0 the absorbance at t = 0.

Table 1: Biochemical Identification of Isolates.

Biochemical Tests	SV1	SV2	SV4	MV5
Gram staining	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods
Oxidase Test	positive	positive	positive	positive
MOF Test	A/A	A/A	A/A	A/A
Voges Proskauer's	_	_	_	_
Arginine Utilization	_	_	_	_
Salt tolerance (1%)	+	+	+	+
ONPG	_	+	_	_
Citrate Utilization	_	_	_	_
Ornithine Utilization	+	_	+	_
Mannitol	+	+	+	+
Arabinose	_	+	+	+
Sucrose	+	+	_	_
Glucose	+	+	+	+
Salicin	_	_	_	_
Cellobiose	_	_	_	_

+ = Positive; - = Negative; A/A= Acidic Slant/Acidic Butt (yellow)

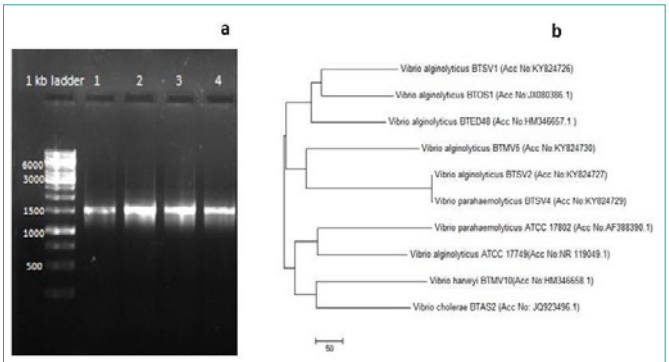

Figure 2: (a) Agarose gel showing 16S rDNA gene amplification: lane 1: BTSV1, lane 2: BTSV2, lane 3: BTSV4, lane 4 BTMV5 **2(b)** Showing phylogenetic analysis of the *Vibrio* isolates.

Figure 3: (a) Congo red agar plate with biofilm producers**. 3(b)** Comparative view of stained tubes showing biofilm attachment- A & B-highly adherent strains., C-weakly adherent strain.

Nutrient Broth with 0.5% glucose was inoculated and incubated at 37°C for 24 hours to determine suicide phenomenon. Strains showing the suicide phenomenon spontaneously pelleted, while those lacking this characteristic showed uniform broth turbidity [22].

(d) Autoagglutination & Precipitation after boiling -.*Vibrio* strains were inoculated into Brain Heart Infusion Broth (BHIB) and incubated at 28°C for 18 hours (static conditions) and observed for Self-Pelleting (SP). Absence of growth in the broth phase and appearance of large aggregates as a button in the bottom of the tube is the clear indication of self-pelleting. Such strains were designated as SP^{+}. Later, the tubes were vortexed for 30 seconds and split into two equal fractions. One aliquot was held at room temperature for 1 hour, and the other one was placed in a boiling-water bath for the same period of time. Upon the end of incubation, the tubes were cooled and compared with unheated controls. Strains which exhibited a reduction in the turbidity after heat treatment (compared with unheated controls) were considered positive for Precipitation After Boiling ($PAB^{+.}$). The Relative Degree of Precipitation (RDP) was calculated by measuring the absorbance at 540 nm according to the following formula: RDP = A_{540} (untreated) - A_{540} (heated) [23].

(e) Surface hydrophobicity - Cell surface hydrophobicity of bacteria was analyzed by determining microbial/bacterial adhesion to hydrocarbons (MATH or BATH) as per Rosenberg *et al.* [24]. Overnight bacterial cultures were centrifuged and the pellets were washed twice with PBS and resuspended in PBS (pH 7.4) to get OD_{600}=0.1. 0.5 ml p-xylene was added to 1.2 ml aliquots. The tubes were incubated at 30°C for 10 mins, vortexed, allowed to stand at RT and the lower aqueous phase was removed and measured at $OD_{600.}$ The results were expressed as the percentage decrease in absorbance (OD_{600}) of the lower aqueous phase compared with OD_{600} of the initial cell suspension.

In-vivo pathogenicity assays using *C.elegans*

(a)Nematode, general maintenance and reagents- The nematode *C. elegans* Wild Type N2 was propagated on Nematode Growth Medium (NGM agar) in 6 cm diameter plates and fed with *Escherichia coli* OP50 grown in Luria Bertani broth as per Brenner [25].

(b) Solid killing assay with *C. elegans*- Evaluation of *C. elegans* life span feeding on the experimental strains was carried out as per

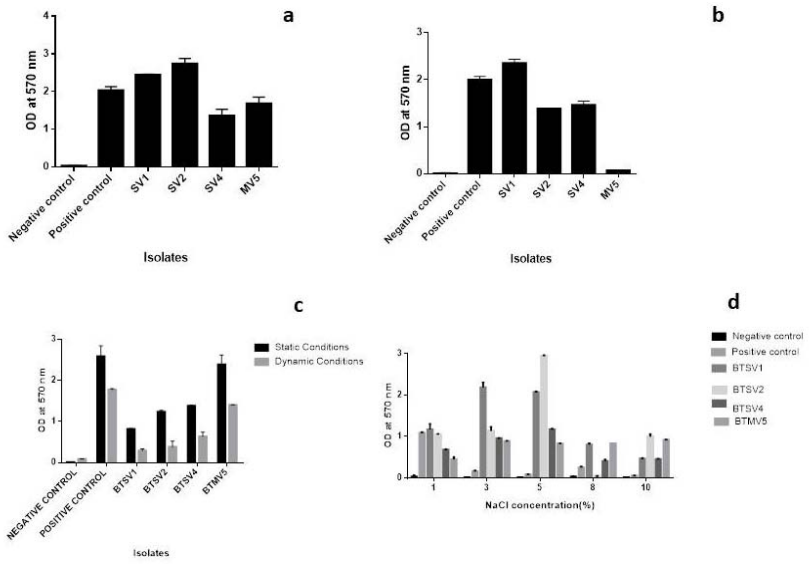

Figure 4: Influence of temperature on biofilm activity upon 72 hours of incubation **(a)** at 28°c **(b)** 37°c **(c)** Biofilm activity under static and dynamic conditions. **(d)** Influence of NaCl on biofilm formation. (Negative control-TSB and Positive control-BTSD2).

Aballay *et al.* [26]. Individual bacteria were inoculated into 5 mL of LB and grown at 28°C until it reached 0.5 OD_{600}.10 µL was spread on NGM plates and incubated overnight. Approximately 20-25 age-synchronized (adult bleaching) L4 stage hermaphrodites were transferred from a lawn of *E. coli* OP50 to a lawn of the test organism, and incubated at 20°C for 24 hours. The worms were then seeded onto plates containing OP50 and scored for dead nematodes at 24 h intervals for a period of 10 days with a dissecting stereomicroscope (Labomed, CZM6) for viability.

(c)Pharyngeal pumping assay- The control and infected worms were placed on NGM plates seeded with OP50 and *Vibrios* respectively, and pharyngeal pumping was observed using a stereomicroscope for 30 consecutive seconds [27].

Statistical analysis

All experiments were repeated thrice and the experimental data points were plotted using GraphPad Prism (Version 6.0, CA, USA). Data was expressed as Mean values ± Standard Errors of the Mean (SEM). The time taken for 50% of the nematodes to die (time to death 50, TD50) was calculated using the equation: Y = Bottom + (Top – Bottom)/ (1 + 10^ ((LogEC50 –X)*Hill Slope)), where X is the logarithm of days and Y is the average of dead worms.

Statistical evaluations of survival analysis between Control OP50 and pathogen groups were performed by one-way ANOVA followed by Student–Newman–Keul's test. A p-value of less than 0.05 was considered to be significant.

Results

Isolation and biochemical identification of bacterial isolates

The shrimp gut homogenate plated on TCBS agar gave green/ yellow colonies, which were picked, and streaked on *Vibrio*-specific agars; three isolates (BTSV1,BTSV2 and BTMV5) appeared greenish yellow on VAL agar and one isolate (BTSV4) appeared bluish-green on VPSA were considered presumptive for *V. alginolyticus* and *V. parahaemolyticus* respectively (Figure 1). The 4 isolates were biochemically characterized and identified using KB007 Hi-*Vibrio*™ Identification Kit (Table 1).

Table 2: MAR Index of *Vibrios*.

Sl No	Isolates	A	B	MAR Index (A/B)
1	BTSV1	9	14	0.62
2	BTSV2	6	14	0.42
3	BTSV4	5	14	0.35
4	BTMV5	5	14	0.35

A= No of Resistant Antibiotics; B = Total No of Antibiotics Tested

Molecular confirmation of *Vibrio* isolates

PCR based 16S rDNA amplification (Figure 2a) and sequence analysis confirmed the molecular identity of *Vibrios* - three strains of *V. alginolyticus* (BTSV1, BTSV2, BTMV5) and one of *V. parahaemolyticus* (BTSV4). The sequences were submitted in GenBank and the accession numbers KY824726 (BTSV1), KY824727 (BTSV2), KY824729 (BTSV4), and KY824730 (BTMV5) obtained.

Phylogenetic analysis using MEGA 6 to understand their interrelatedness based on the Neighbour-Joining analysis of 1000 resampled data sets is depicted in the Figure 2b.

V. alginolyticus strain BTSV2 and *V. parahaemolyticus* strain BTSV4 claded together which indicated of their genetic relatedness. The other two strains of *V.alginolyticus* (BTSV1 and BTMV5 existed as a separate clade. However, the isolates showed a closer similarity to two *V.alginolyticus* strains (BTOS1 and BTED48) retrieved from GenBank database using BLAST analysis.

Qualitative biofilm formation assays

Congo red agar method helped to differentiate strong, moderate and weak biofilm producers. Strain BTSV1 & BTSV4 produced black crystalline colonies whereas BTSV2 & BTMV5 showed smooth red

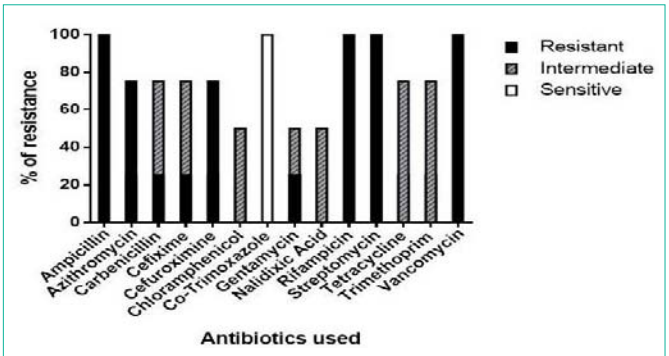

Figure 5: Antibiotic profiling of *Vibrio* isolates.

coloured ones. According to the intensity of color, the isolates were categorized as strong (BTSV1 & BTSV4), moderate (BTSV2, and BTMV5) (Figure 3a).

Tube adherence method: A thick visible film lined the wall and bottom of the tube with strains BTSV1 & BTSV4, indicative of strong adherence while those with strains BTSV2 & BTMV5 had less visible film formation, suggestive of their weakly adherence to glass materials (Figure 3b).

Quantitative investigation of *Vibrio* biofilms under different stress conditions

Incubation temperature influenced biofilm formation: At 4 °C due to reduced growth, the biofilm formation was less evident for all strains tested. In contrast to the lower temperature, biofilm was much prominent at 28^{0}C and 37^{0}C, and the biofilm architecture was sustained up to 72 hours for all cases. BTSV2 was the strongest biofilm producer at 28^{0}C and BTSV1 at 37^{0}C (Figure 4a and 4b). The biofilm formation increased with increased incubation period except for BTSV2 and BTMV5.

Influence of static and dynamic (shaking) conditions on biofilm formation: All isolates produced biofilm in a dynamic environment. That said, the rate of biofilm formation was less than that in the static environment. It was also observed that differences in temperature (28^{0}C and 37^{0}C) and an increase in incubation time did not impact biofilm formation under dynamic conditions. Hence, biofilm assay at 37^{0}C for 24 hours under both conditions is depicted in Figure 4c.

Influence of NaCl on biofilm formation: Biofilm development at 5% NaCl was highest in the microtitre plate (Figure 4d).

Antibiotic susceptibility testing

Antibiogram showed that all four *Vibrios* were multiple antibiotic resistant bacteria with MAR index > 0.2 (Table 2).

The isolates were characterized by resistance to 13 among 14 antibiotics tested including third generation Cephalosporins such as Cefixime. However, none were resistance to Co-trimoxazole (Figure 5).

In- vitro assays for pathogenicity

Exoenzyme production: More than one hydrolytic enzyme was produced by the isolates which was indicative of their ability to digest nutrients present in food material. The isolates also exhibited a distinctive alpha hemolysis (partial hemolysis) pattern on blood agar plates. The results were summarized in the table below (Table 3) and (Figure 6).

Auto aggregation assay: Aggregation increased with the increase in incubation time. Auto aggregating phenotype was noted in all isolates, with highest of 90% by BTSV1 (Figure 7a).

Suicide Phenomenon, auto agglutination & precipitation after boiling and Surface hydrophobicity

Thick pellets were observed for the isolates SV1 and SV2 at the bottom of the tubes upon 18 hours of incubation under static conditions, indicating that the isolates were positively suicidal (SP+) where as others were non-suicidal (SP-) strains (SV4 and MV5). The isolates were positive for auto agglutination test and precipitation after boiling (PAB+).

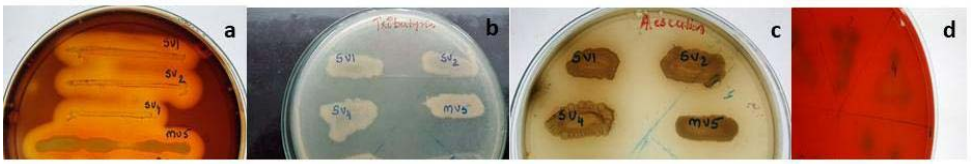

Figure 6: Exoenzymes produced by *Vibrios* **(a)** Starch hydrolysis **(b)** Lipid hydrolysis **(c)** Aesculin hydrolysis **(d)** Hemolytic assay.

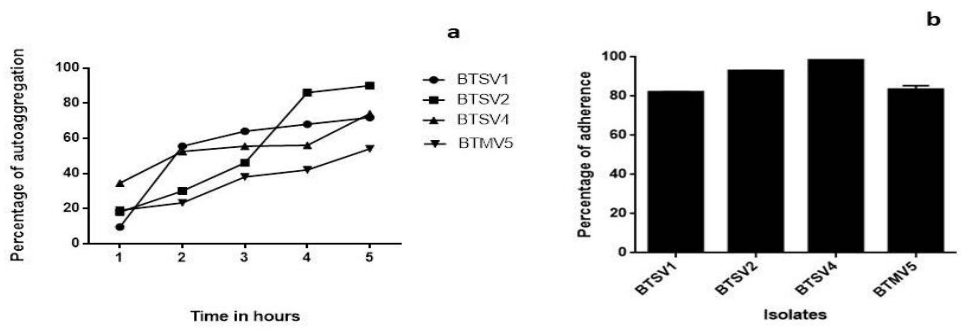

Figure 7: (a) Auto aggregation pattern of isolates **(b)** Cell surface hydrophobicity of isolates.

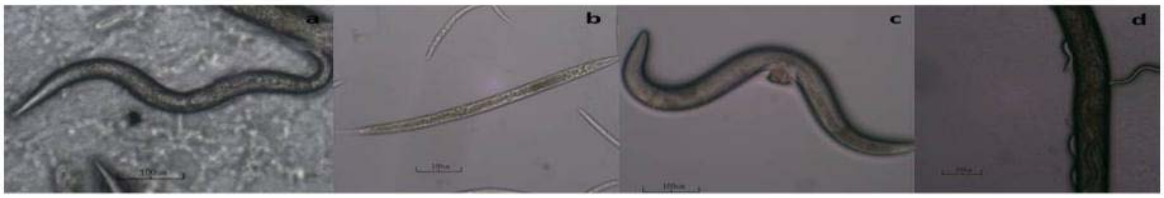

Figure 8: (a) Healthy live *C.elegans* in OP50 **(b)** Dead nematode in OP50 (long and immotile) **(c)** Protruding Vulva formation **(d)** Internal hatching. Scale bar represents 100 µm.

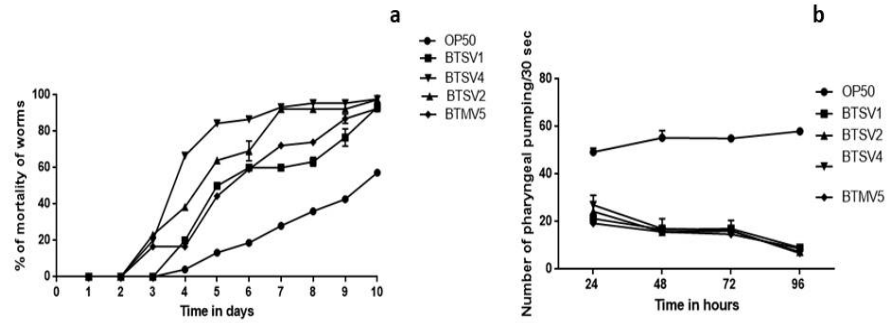

Figure 9: (a) Survival score analysis of *C.elegans* **(b)** Pharyngeal pumping assay.

Percentage of hydrophobicity values less than 20 are considered as weakly hydrophobic. The isolates tested were all strongly hydrophobic on showing adherence to xylene, with BTSV4 maxing at 98.28% (Figure 7b).

Survival score analysis by solid killing assay of *C. elegans*

Since the *Vibrios* exhibited strong biofilm formation under different stress conditions, a *C. elegans* nematode infection model was used to evaluate their *in-vivo* pathogenicity. Wild-type *C. elegans* (N2) remained viable and healthy on the plates fed with its standard laboratory food i.e. nonpathogenic *Escherichia coli* strain OP50. Dead nematodes appeared as long, immobile rods, which failed to respond to external stimuli like a gentle tap with a worm loop. The TD50 value for OP50 was calculated as 12.95 ± 0.473 days. By contrast, nematodes exposed to test *Vibrios* exhibited significantly ($P<0.05$) reduced lifespan with abnormal phenotypic behavior like reduction in pharyngeal pumping, protruding vulva and internal hatching (Figure 8).

The TD50 values of the isolates BTSV1, BTSV2, BTSV4 and BTMV5 were 5.16 ±0.809 days, 4.43 ± 0.410 days, 3.57 ± 0.460 days and 5.60 ± 0.443 days respectively. The strain BTSV4 exhibited lowest of the TD50 values. *Vibrios* required approximately 10 days for complete killing of the nematode (Figure 9a).

Pharyngeal pumping assay

The pharyngeal pumping rate was monitored for 96 hours (four days) to assess nematode food preference. The pumping rate of the worms fed with *E. coli* OP50 was found to be significantly ($p<0.05$) higher than pathogen fed animals and remained stable up to 4 days. The normal grazing behavior of the nematodes was affected by their exposure to *Vibrios* and their pumping rates notably reduced after 96 hours (Figure 9b). Worms exposed to *V. parahaemolyticus* (BTSV4) recorded the lowest pumping when compared to *V.alginolyticus*

Table 3: Showing the enzyme profiling of *Vibrios*.

Isolates	Starch hydrolysis	Lipid hydrolysis	Aesculin hydrolysis	Hemolytic assay
BTSV1	+	+	+	Alpha hemolysis
BTSV2	+	+	+	Alpha hemolysis
BTSV4	+	+	+	Alpha hemolysis
BTMV5	+	+	+	Alpha hemolysis

+ = Positive; - = Negative

strains mentioned in the study.

Discussion

In biofilms, bacterial aggregates are enclosed in a self-produced polysaccharide matrix attached to each other and to a biological/non-biological surface [28,29]. Foodborne illnesses due to biofilm formers are difficult to abolish as they may be 100 times more resistant to antimicrobials compared to planktonic cells. Although virulence has been directly related to multidrug resistance, the mechanism of virulence, antibiotic resistance, and biofilm formation still remains elusive. The limited documentation in the international literature on the emergence of drug-resistant biofilm formers in market fresh shrimp was the trigger to survey these aspects. This study will therefore provide information on the existence of strong biofilm forming non-cholera *Vibrios* isolated from fresh and healthy shrimps available at local fish markets in Cochin, South India. In addition, the antibiotic resistance profiling of these biofilm producers indicates copious use of chemicals/drugs in shrimp farming, threatening human health and safety.

Halophilic 'Non-Cholera *Vibrios*' (NCVs) are natural inhabitants of the aquatic ecosystems, chiefly associated with bacterial infections affecting all species of cultured shrimps. *V. alginolyticus* and *V.parahaemolyticus* have been etiologically associated with mass mortality of cultured shrimps and seafood-associated bacterial

gastroenteritis throughout the world [30-32]. The key factor enabling *Vibrios* to adapt to environmental stress and transmission is the ability to form matrix-enclosed communities called biofilms [33]. With this ability to adhere to abiotic and biotic surfaces allowing their persistence and survival under aquaculture settings, the resultant *Vibrios* is may cause huge economic losses in the seafood industry [34]. Glass and polystyrene materials in aquaculture installations can be colonized by *Vibrios* as an initial step towards host colonization and thereafter to mariculture animals and seafood consumers. The isolates in this study could attach to glass and polystyrene materials at varying degree.

Studies investigating biofilm detection are performed under static conditions, though there are few reports of biofilms on surfaces under dynamic conditions. All strains in this study remained biofilm formers under both these conditions. An interpretation of this phenomenon remains unclear at this moment. The fact that shaking improves oxygenation and favorable conditions for growth of many bacteria can be attributed to this finding. It is our understanding from this study that biofilm forming bacteria can emerge as potent contaminants in water recirculation systems of various aquaculture settings, posing serious health problems in reared animals.

Nutrient availability and salinity influence biofilms by *Vibrios* [35]. A recent study by Han *et al.* [36] reported decreased biofilm formation at 4-10^{0}C, while, temperature increase enhanced biofilm formation, virulence, and quorum sensing of *V. parahaemolyticus* on seafood (crab, shrimp) and contact surfaces. This study too corroborates the aforesaid facts that *Vibrio* biofilms tend to increase with the increase in temperature and NaCl concentration. That said, the species diversity and their gene regulatory mechanisms may also play a major share in bacterial adherence.

The compact nature of biofilms contributed by matrix polymers, makes bacteria inaccessible to natural and artificial agents, and hence the ability to penetrate and destroy. Increased antibiotic resistance is a trait associated with biofilm bacteria and well elucidated in *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*), and in *Vibrio cholera*e. However, a correlation of antibiotic resistance and biofilm formation is still unclear in the case of non-cholera *Vibrios* [37].

Until recently, *Vibrios* were considered susceptible to many antibiotics commonly used in aquaculture. However, the past decade saw the emergence of multiple antibiotic resistant *Vibrios.* Resistance of *V. harveyi* strains to 20 different antibiotics was reported from India.13 among 14 of the antibiotics tested in our study were commonly used as prophylactic agents in shrimp farming. Among the antibiotics, the resistance to Co-trimoxazole was absent. This is in agreement with the antimicrobial pattern of luminous bacteria from shrimp farms of West Bengal studied by Sengupta *et al.* [38] who reported *Vibrios* resistant to β-lactam antibiotics and tetracycline. Only one previous study reported prevalence of antibiotic-resistant *Vibrios* from fish markets of Cochin [39] with *V. parahaemolyticus* strains resistant to ampicillin, streptomycin, and carbenicillin, but 100% susceptibility to nalidixic acid and tetracycline. They also reported 70% of isolates susceptible to trimethoprim, chloramphenicol, and gentamicin. Our study differs by indicating an alarming increase of resistance among *Vibrios* to a broader class of antibiotics within a short span of five years. The resistance exhibited by our isolates towards third generation cephalosporins will definetly raise the question of understanding antibiotic resistant profiles and risk factors for each pathogen.

Among the 11 species placed under 'Harveyi Clade' (Vibrionaceae), *V. alginolyticus* and *V. parahaemolyticus*, besides being major pathogens of marine shrimp, fish, and molluscs, are used as model organisms in biofilm studies [40], quorum sensing and multi-chromosomal genome organization [41]. The infectious life cycle of any pathogen involves different stages such as gaining entry, establishment and multiplication, avoidance of host defenses, causing damage or mortality and finally exit from the host [42]. All these mechanisms are aided by the expression of virulence factors at the different stages of infection. Several biochemical characteristics of the *Vibrio* isolates along with the analysis of their virulence factors and surface characteristics were used to determine the relationship between these factors and the pathogenic potential of the species. An essential step in the successful invasion of host requires adherence to host surfaces by flagella or pili [43,44] followed by production of extracellular polysaccharides eventually leading to biofilm formation [45]. Lytic enzymes produced by *Vibrios* enable them to procure nutrients present in the host and disseminate to different parts of host tissue. Among them, hemolysins, lipases, and gelatinases are well documented [46,47]. The ability of *V.alginolyticus and V.parahaemolyticus* strains mentioned in this study to produce multiple exo enzymes, as well as hemolysins make them prime contaminants in seafood. Experimental studies reveal the genes involved in chemotaxis also influence auto-aggregation and hydrophobicity of pathogenic bacteria. An interrelationship between these factors and biofilm is critical to expanding the knowledge regarding the development of biofilms in static and flowing aquatic environments [48].

Caenorhabditis elegans, a genetically traceable multicellular organism is an attractive host to address fundamental questions regarding pathogenicity of various Gram negative human pathogens. Our study looked into the pathogenic potential of isolated *Vibrios* using wild type *C.elegans* N2 strain. *Vibrio alginolyticus* infection often results in colonization of bacteria in worm gut within a short span of 8 hours [49]. The exposure of the nematodes for 24 hrs to three *V. alginolyticus* strains specified in our study also documented abnormalities and mortality due to the infection over a 10-day period. Virulent *V.parahaemolyticus* was reportedly associated with pharyngeal damage and distention leading to death of the nematode in 48 hours [50]. The worms fed on *V.parahaemolyticus* strain BTSV4 exhibited the lowest number of flings probably due to the same impact of the infection around the pharyngeal region.

Conclusion

The findings of the study support the conclusion that the presence of antibiotic resistant biofilm formers will compromise the safety and acceptability of seafood thereby co-equally endangering aquatic and human life. Furthermore, good aquaculture practices with special attention to food safety, environmental protection, and the well-being of farmed organisms must be implemented to safeguard food security and sanitation in future. An extensive elucidation of the molecular mechanisms involved in the infection or pathogenesis of *Vibrios* is

mandatory to illustrate the notion of widespread contamination of antibiotics in shrimp aquaculture and remold new ways to combat this calamity.

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