In vitro biofilm forming capacity on abiotic contact surfaces by outbreak−associated *Vibrio harveyi* strains

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PEER REVIEW

ABSTRACT

Objective: To evaluate the *in vitro* biofilm forming capacity on abiotic food contact surfaces by *Vibrio harveyi* (*V. harveyi*) strains.
Methods: Thirty six Gram−negative *V. harveyi* strains were isolated from various street vended seafood outlets in a food processing line and evaluated for their ability to produce mucoid biofilms on food contact surfaces using a microplate assay. Phenotypic characterization of mucoid biofilm producing *V. harveyi* strains were screened on Congo red agar, thiosulfate−citrate−bile salts−sucrose agar and tryptic soy agar, respectively.
Results: Only five *V. harveyi* strains (14%) were mucoid biofilm producers characterized by formation of black colonies, whereas the remaining 31 strains (86%) were not capable of producing biofilm characterized by formation of red colonies or pinkish−red colonies with darkening at the centre. The morphological, physiological and biochemical characteristics of these isolates were studied using standard protocols. Strain identification was confirmed by polymerase chain reaction targeted to species−specific polymerase chain reaction primers VH−1 and VH−2 corresponding to variable regions of *V. harveyi* 16S rRNA sequence. All the biofilm−forming strains showed resistance to at least three antimicrobial compounds tested. *V. harveyi* strains isolated from various seafood were able to form biofilms of different capacity, and the strains VB267, VB238 and VB166 isolated from cat fish, shrimp and eel fish exhibited significantly greater biofilm forming ability compared to other isolates.
Conclusions: It can be concluded from the present study that the strain VB166 was able to better attach and form subsequent biofilms on glass and stainless steel compared to high density polyethylene. These properties allow these bacteria to survive, proliferate and persist in street vended seafood outlets.

1. Introduction

Vibrio infections remain a serious threat to public health and are frequently associated with disease outbreaks due to consumption of the seafoods obtained from contaminated water. These pathogens are generally associated with the skin lesions and abrasions, gut and skin of marine animals, phytoplankton, sediments and suspended detritus[1]. *Vibrio harveyi* (*V. harveyi*) is the major causal organism of vibriosis, which causes impending devastation to diverse ranges of marine invertebrates over a wide geographical area. However, over the past few decades, bacterial strains of this

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species have been documented as significant pathogenic agents and a cause for high rates of shrimp mortality in the aquaculture industry worldwide[2]. Once considered as an opportunistic pathogen, *V. harveyi* is now known to cause mass mortalities in penaeid prawn farms across the world. Other commercially important marine organisms are also affected, including pearl oysters (*Pinctada maxima*)[3], fish (*Solea senegalensis*)[4], *Hippocampus* sp.[5] and lobsters (*Panulirus homarus*)[6]. Additionally, *V. harveyi* has also been reported as a major pathogen of an escalating list of aquatic animals, including finfish, bivalves, and *Artemia franciscana* nauplii[7]. These diseases have been identified as major setback in Philippines, Japan, Southeast Asia and European countries, causing stern loss of juvenile prawns in several hatcheries[8].

Bacterial biofilms are complex communities of microorganisms embedded in a self–produced matrix and adhere to inert or living surfaces[9]. They have been observed on an array of biotic and abiotic surfaces and considered to be the prevailing microbial lifestyle in most aquatic environments[10]. The formation of biofilms on food, food–processing surfaces, and water distribution systems constitutes an augmented risk for product contamination with pathogenic bacteria[11]. The complexity of biofilms increases resistance to a number of environmental stresses and also enhances antimicrobial resistance due to delayed penetration of the antimicrobial drugs[12]. The potential for *Vibrio* biofilms to act as a reservoir of pathogenic bacteria has been well reported in clinical infections[13].

Polyvinyl–chloride, polyethylene, glass and polystyrene materials are the main abiotic surfaces used in the aquaculture installations. These inert surfaces can be colonized by several bacteria such as *V. harveyi* in shrimp farm[2]. Microbial biofilms formed by *Vibrio* sp. occur on a variety of food contact surfaces that act as a possible source of contamination, including conveyors, collators, hand tools, gloves, gaskets and tools made with stainless steel, utensils, polymeric materials and glass[14,15]. In aquaculture installations, *Vibrio* sp. is frequently isolated from seawater before and after filtration and UV sterilization[16]. The extensive production of biofilm by microorganisms may seriously affect the quality and safety of the processed food and could also pose an impending risk to human health[17]. An improved understanding of bacterial adhesion process is needed for production of microbiologically safe and good quality products in the food industry. Unfortunately, there is no information available regarding the biofilm formation of *V. harveyi* isolates obtained from street vended seafood and thus forms the basis of this study.

2. Materials and methods

2.1. Bacteriological Sampling

Raw seafood samples were collected randomly from June to October 2010 in sterilized containers from street vended seafood outlets, spread over 10 regions within Krishna District of Andhra Pradesh in Southern India and were stored at 4 °C until further analysis. Various parts (skin lesions, abrasions and gut) of seafood animals were considered as a potential source of inoculum.

2.2. Bacterial isolation and culture media

Bacterial strains were isolated according to the method previously described by Ben Kahla–Nakbi *et al*[16]. Briefly, swabs were prepared from different parts as mentioned in sampling and were inoculated into sterile tryptic soy broth (TSB), and incubated at 37 °C for 24 h at 120 r/min on an orbital shaker. The 24 h grown culture broth was serially diluted and spread on TSB agar for isolation. Obtained bacterial strains were stored in TSB: glycerol mixture (1:1 v/v) at −80°C until further use. Biofilm formation of selected isolates were tested with seafood broth prepared by blending different seafood samples (100 g each) separately in 250 mL of water and centrifuged at 8 675 r/min, 4 °C for 10 min to obtain clear supernatants, that were autoclaved before use. The 3% NaCl was added to all the test media used.

2.3. Qualitative detection of mucoid biofilm–producing bacteria

Qualitative detection of biofilm formation was studied by culturing the strains on Congo red agar (CRA) plates as described previously[18]. The media contained TSB broth (37 g/L), sucrose (0.8 g/L), agar (10 g/L) and Congo red stain (0.8 g/L). The Congo red stain was prepared as a concentrated aqueous solution, autoclaved separately and added to the media after the agar had cooled to 55 °C. The CRA plates were inoculated with all the isolates listed in (Table 1) and incubated aerobically for 24 h at 37 °C. After incubation, pigmented colonies (generally black colour) were considered as mucoid phenotype positive.

Table 1

β–lactamase, biofilm production and antimicrobial resistance profiles of *V. harveyi* isolates.

Strain ID	Growth in NaCl 3 %	6%	β–lactamase production	Biofilm Production	Phenotype of strains on CRA	Resistant Antimicrobials
VB103	+	+	+	+	Pink	AMP, CFP, TET, AK,
VB211	+	−	+	+	Pink	AMP, CFP, CF
VB140	+	−	−	+	Pink	CFP, CF, AK
VB267	+	+	+	+++	Black	AMP, CFP, CF, OTET, AK, NA
VB45	+	−	+	+	Pink	AMP, CF, AK, TMP
VB178	+	+	+	+	Pink	AMP, CFP, CF, OTET, TMP
VB234	+	+	+	+	Pink	AMP, CF, AK, TMP
VB168	+	+	+	+	Pink	AMP, CFP, CF
VB171	+	+	+	++	Black	AMP, CFP, CF
VB298	+	−	−	+	Pink	CFP, CF, OTET, TMP
VB160	+	−	−	+	Pink	CF, AK, TMP
VB238	+	+	+	+++	Black	AMP, CFP, CF, OTET, AK, NA
VB166	+	+	+	+++	Black	AMP, CFP, CF, OTET, AK, NA
VB197	+	+	+	+	Pink	AMP, CF, AK, TMP
VB258	+	−	+	+	Pink	AMP, CF, AK, TMP
VB108	+	+	+	++	Black	AMP, CFP, CF, OTET, TMP

Data are presented as: positive (+), negative (−) for growth in NaCl, black colonies (phenotype of strains on CRA plates); +: Pink colour colonies; ++ and +++: black colonies. AMP: Ampicillin, CFP: Cephalosporins, CF: Cephalothin, CLP: Chloramphenicol, TET: Tetracycline, OTET: Oxytetracycline, AK: Amikacin, KAN: Kanamycin, CIP: Ciprofloxacin, NA: Nalidixic acid, TMP: Trimethoprim.

2.4. Bacterial Identification

The bacterial isolates tested positive for biofilm production on CRA plate were screened further on selective *Vibrio* specific media thiosulfate–citrate–bile salt–sucrose agar. A total of 16 isolates, obtained after consecutive subculturing of separated colonies based on mucoid colony phenotypes, were further confirmed to species level by following the set of biochemical keys for identification of environmental *Vibrio* isolates[19]. Genomic DNA was extracted from the selected isolates by phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) method and the obtained DNA was suspended in 1xTris–EDTA buffer and stored at −20 °C until required. The DNA samples were subjected to polymerase chain reaction (PCR) using species–specific primers VH1F′ (5′ACC GAG TTA TCT GAA CCT TC 3′) and VH2R′ (5′GCA GCT ATT AAC TAT ACT ACT 3′), which specifically amplifies a 413 bp fragment of the 16S rRNA gene sequence[20].

2.5. β–lactamase bioassay and antimicrobial susceptibility

β–Lactamase activity was assayed by the acidometric method[21]. Briefly, 125 μL of aqueous solution containing 0.2% of soluble starch (w/v) and 1% benzyl penicillin (w/v) was added to the wells of 96 well microtitre plates. A total of 50 μL of 12 h grown bacterial cultures were mixed with the above solution and incubated for (30±2) °C for 3 min. The release of penicillinoic acid was determined by adding 125 μL of aqueous solution containing 0.5% of iodine (w/v) and 1% of potassium iodide (w/v). Decolourization of the sample (<3 min) was considered as a positive reaction. Antibiotic resistance profile of the isolates was examined by Kirby–Bauer′s disk diffusion method according to the standard procedures outlined in the Clinical and Laboratory Standards Institute Guidelines[22]. Briefly, overnight cultures were spread evenly over Mueller–Hinton agar with a sterile cotton swabs. The tested antimicrobial disks included ampicillin (10 μg), cephalosporin (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), tetracycline (30 μg), oxytetracycline (30 μg), amikacin (30 μg), kanamycin (30 μg), ciprofloxacin (5 μg), nalidixicacid (10 μg) and trimethoprim (10 μg). After 24 h of incubation at 37°C, the zone of inhibition around the individual disc was measured and interpreted as per manufacturer′s guidelines.

2.6. Microtiter plate biofilm production assay

Sixteen selected isolates were cultured overnight at 37 °C in TSB broth and diluted 1:100 in 0.1% sterile peptone water. 10 μL of diluted culture was dispensed into 96–well microplate containing 90 μL of sterile TSB. The plates were incubated for 24 h at 37 °C without shaking. Bacterial cultures were removed, and wells were rinsed twice with sterile deionized distilled water to remove loosely attached bacteria. A bout 150 μL of 0.25% crystal violet was added to each well and the plate was incubated for 30 min at (30±2) °C. The plate was further rinsed twice with sterile deionized

distilled water and air dried for 10 min. Crystal violet dye bound to the biofilm was solubilized with 150 μL of 70% ethanol for 30 min at (30±2) °C. The absorbance was measured at 595 nm using a microplate reader. Further, 3 discerning isolates which showed greater biofilm formation, were checked for their ability to form biofilm in different seafood broths *viz*. Indian white shrimp (*Penaeus merquiens*), milkfish (*Chanos chanos*), Indian mottled eel (*Anguilla bengalensis bengalensis*), gaint tiger prawn (*Penaeus monodon*), sardine (*Sardinella gibbosa*) catla (*Catla catla*), tuna (*Euthynnus affinis*), sea crab (*Portunus sanguinolentus*), flathead mullet (*Mugil cephalus*), sea bass (*Lates calcarifer*), pungas catfish (*Pangasius pangasius)*, catfish (*Chana marulias*), tilapia (*Tilapia mossambica*), and channa (*Channa striata*) as mentioned above.

2.7. Biofilm formation on contact surfaces

Selected isolate designated VB166 was chosen for biofilm formation studies on various food contact surfaces and media (TSB and seafood broths). Strain VB166 was grown overnight in TSB at 37 °C at 120 r/min. The culture was diluted 1:100 in 0.1% peptone water and inoculated into 200 mL of prepared seafood broth and TSB to a final bacterial concentration 10^5 cells/mL. Aseptically, 15 mL of broth was dispensed into Petri dishes containing stainless steel (2×2 inches) coupon, high density polyethylene (HDPE) (2×2 inches) coupon, and glass (1×2 inches). The Petri dishes were incubated at 37 °C for 24 h without shaking. The experiments were performed in triplicate. After incubation, glass, stainless steel and HDPE coupons were transferred aseptically into a sterile Petri dish and rinsed twice in 15 mL of deionized distilled water. The contact surfaces were then stained with 15 mL of 0.25% crystal violet for 30 min at room temperature. The crystal violet bound to the biofilm was solubilized with 5 mL of 70% ethanol for 30 min, and the absorbance was determined at 595 nm.

2.8. Alcian blue staining of mucoid biofilm of strain VB166

The mucoid biofilm of the bacterial cells was stained with 0.1% (w/v) alcian blue solution in 0.5 mol/L acetic acid, pH 2.5, which binds specifically to acidic polysaccharides[23]. *V. harveyi* strain VB166 was pre–cultured for about 24 h. Aliquots of 200 μL of pre–culture was inoculated into glass bottom dishes containing 4 mL of TSB. After 24 h of incubation, the culture medium was removed, and the microcolonies on the glass surface were washed with phosphate buffered saline (pH 7.2). Then they were stained with alcian blue solution for 30 min at room temperature. After rinsing with distilled water, the specimens were observed under an optical microscope.

2.9. Scanning electron microscopy observation of strain VB166

Aliquots of 50 μL of precultured *V. harveyi* strain VB166

was inoculated into microtiter well plates. Each well contained 1 mL TSB and a 12 mm diameter circular glass cover slip. After 24 h of incubation under aerobic conditions at 37 °C, the biofilms formed on the cover slips were fixed using 2% glutaraldehyde in 0.175 mol/L phosphate buffer at room temperature for 1 h. After washing with phosphate buffer solution, the cells were dehydrated through graded series of acetone: 50% acetone (1×15 min); 70% acetone (1× 15 min); 90% acetone (1×15 min) and 100% acetone (3×15 min). The coupons were thereafter air dried in a critical point dryer, mounted on copper studs and coated with gold plasma and examined using the JOEL 5800 LV scanning electron microscope.

2.10. Statistical analysis

All data from biofilm quantitative assays were expressed as mean±SD with each assay conducted in triplicate. The Mann Whitney *U*–test was used for quantification of the biofilms using the SPSS 13.0 statistics package for Windows.

3. Results

3.1. Identification of mucoid biofilm–producing V. harveyi strains

Phenotypic mucoid biofilm production was assessed by culturing the isolated strains on CRA plates (Table 1). All *V. harveyi* strains grown on CRA medium resulted in three colony morphotypes with different colours (red, pinkish–red and black). Among 36 *V. harveyi* strains tested in this study, only five strains (14%) were mucoid biofilm producers (VB267, VB238, VB166, VB171 and VB108) characterized by black colonies as depicted in Figure 1 for strain VB166, whereas the remaining 31 strains (86%) were considered as non–biofilm producers that are characterized by red colonies or pinkish–red colonies with darkening at the centre. These five isolates were further identified by standard morphological, physiological and biochemical tests following the schemes of Alsina and Blanch[19]. All strains were Gram–negative facultative anaerobes, motile and curved rods, and they exhibited salt requirement in a range (3%–6% NaCl) (Table 1). These strains were tested oxidase, catalase and gelatinase, amylase, chitinase positive, and produced green mucoid phenotypes on thiosulfate–citrate– bile salt–sucrose agar plates. In addition, these isolates failed to utilize sucrose, indole, melibiose, mannitol, sorbitol, ethanol, histidine and proline, and were positive to arginine dehydrolase, o–nitrophenyl–β–d–galactoside test, nitrate reductase and sensitive to the vibriostatic agent 0/129 at 120 μg. Furthermore bacterial identification was confirmed by means of PCR targeted to species–specific primers VH–1 and VH–2 for the identification of five selective *V. harveyi* isolates, which corresponded to variable regions of *E. coli* 16S rRNA gene sequence targeted between the bases 59–87

and 453–473 and comparable with the amplification product obtained by the type strain *V. harveyi* MTCC 3438 (Figure 2).
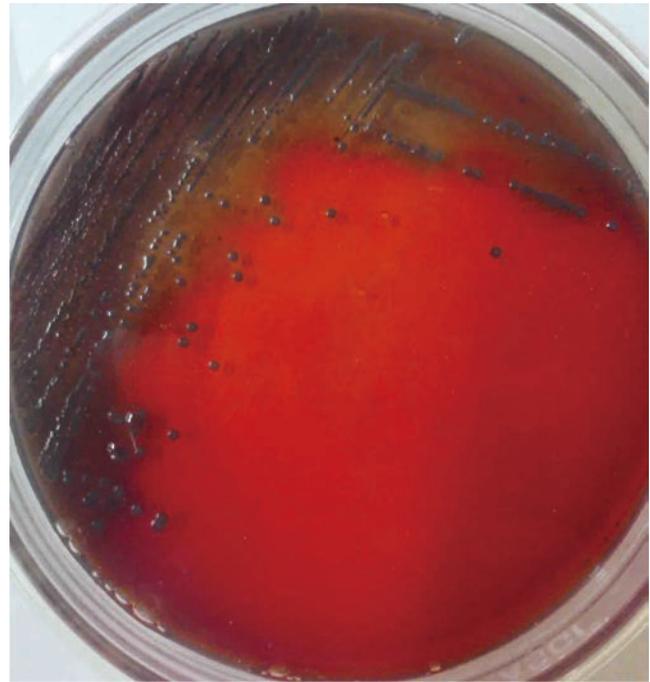
Figure 1. Mucoid biofilm production by *V. harveyi* strain VB166, which denotes defined black colonies on CRA plates.
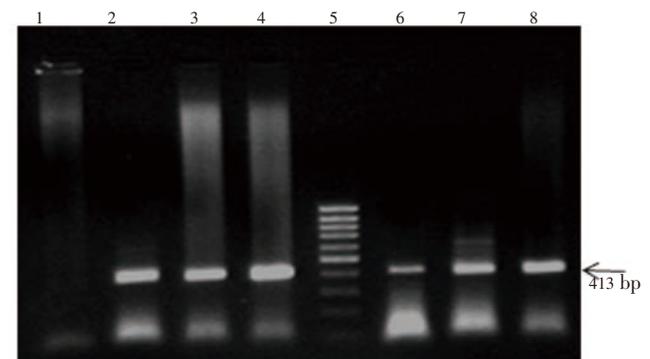
Figure 2. Rapid PCR to identify *V. harveyi* bacterial isolates from seafood samples using species specific 16s rRNA primers, Lane 1: negative control *E. coli* DNA; Lane 2–4: VB267, VB238, VB166; Lanes 5: 100 bp DNA ladder; Lanes 6–7: VB171, VB108; Lane 8: positive control *V. harveyi* MTCC 3438.

3.2. β–lactamase bioassay and antimicrobial susceptibility

Most of the isolates were able to cleave the β–lactam ring and produce penicillinoic acid. The results of β–lactamase assay showed that 75% of the strains produced β–lactamase, but overall, for 87% of the tested isolates, the production of β–lactamase correlated with the resistance to ampicillin and cephalosporin. Resistance to β–lactams is often the result of β–lactamases that inactivate the antibiotics[24]. However, previous studies reported the production of β–lactamase by bacteria with high resistance to β–lactam antibiotics[25]. The results of the antibiotic sensitivity tested by the disk diffusion method are summarized in Table 1. Ampicillin resistance was observed in 87% of the strains tested, whereas 75% of resistance was detected in cephalosporin

and cephalothin. However TET and OTET resistance was observed in 25% and 31% of the strains respectively. No resistance was detected for chloramphenicol, kanamycin and ciprofloxacin, however intermediate resistance was observed among 50% of the strains in case of amikacin, nalidixic acid and trimethoprim. Multiple resistances to ampicillin and cephalosporin coupled to cephalothin and/or TET/OTET was observed (Table 1).

3.3. In vitro biofilm formation

Biofilm formation and bacterial growth of the sixteen *V. harveyi* isolates were studied in TSB. A significant variation in the bacterial growth profiles were noted between the strains. These strains produced greater biofilm in TSB with 3% NaCl compared to TSB without salt (data not shown). Therefore, TSB with salt was used to compare biofilm formation by all isolates. Further, there was large variation in biofilm–forming capacity among the *V. harveyi* isolates from the seafood processing line, with values ranging from 0.1 (isolate VB178) to 1.6 (isolate VB166) (Figure 3).
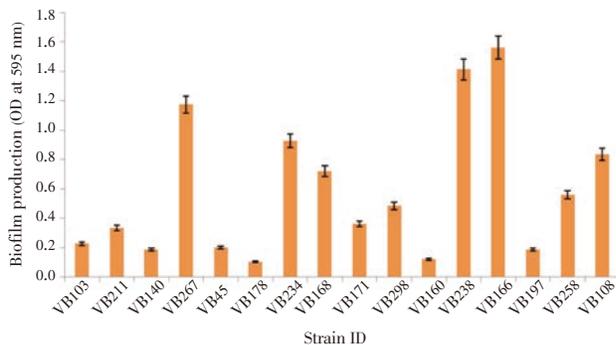
Figure 3. Microtiter plate assay for biofilm formation capability of *V. harveyi* isolates from seafood sources.

However, biofilm formation by *V. harveyi* strains VB267, VB238 and VB166 isolated from cat fish, shrimp and eel fish were significantly greater compared to other isolates. These abilities differed with the strains irrespective of their site of isolation. In the microplate assay, statistical analysis of the mean crystal violet staining (optical density values) allowed us to distinguish three significantly different groups ($P<0.05$) with higher (VB166, VB238 and VB267), intermediate (VB234, VB108 and VB168) and lower (VB178 and VB160) levels of crystal violet staining. Significant variation was noticed in the ability of each strain to grow in different test broths after 24 h incubation (Figure 4). Highest optical density value obtained for strain VB166 (1.55) was considered as 100%. In comparison with strain VB166, VB238 evidenced 90% whereas, VB267 showed only 75% respectively. Consequently, these isolates *i.e.*, VB166, VB238 and VB267, were further selected to check for the biofilm formation capacity on natural nutrient sources (prepared as broths). The catfish and shrimp broths were able to support greater amounts of growth in *V. harveyi* strains, when compared to other seafood broths. As shown in Figure 4, there is a statistically significant difference ($P<0.005$) in the biofilm formation as displayed by the strains, with VB166, VB238

showed the greatest biofilm production and VB267 showing the lowest biofilm production in seafood broths.
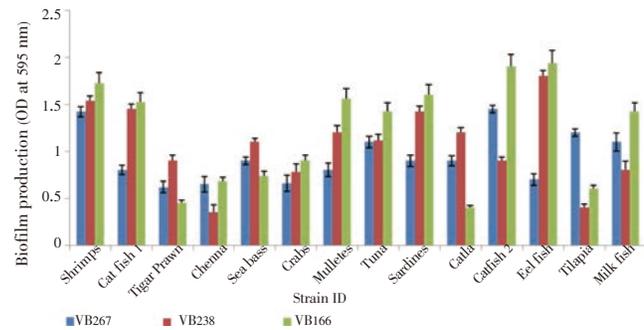
Figure 4. Biofilm formation in seafood broths. Biofilm formation capability of *V. harveyi* strain VB267 (dark bars), strain VB238 (hatched bars) and strain VB166 (light bars) in test broths.

3.4. Biofilm formation on contact surfaces

The ability of the *V. harveyi* strain VB166 isolated from catfish to form a mucoid biofilm was evaluated on various food contact surfaces in shrimp, crab, mullet, tuna, catfish, eel fish and sardine broth respectively (Figure 5).
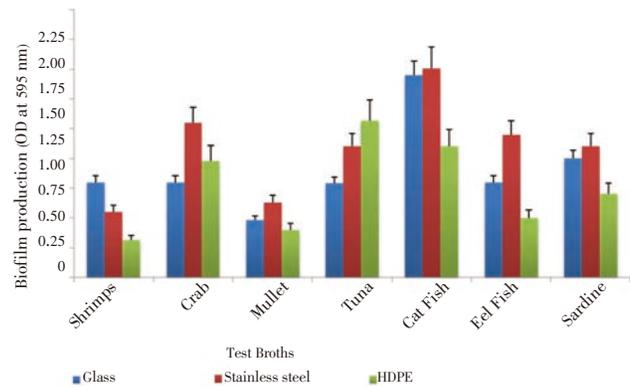
Figure 5. Biofilm formation of strain VB166 on different contact surfaces. Biofilm formation by *V. harveyi* strain VB166 on Glass, stainless steel and HDPE surfaces in various test broths.

This strain was tested for its strong ability to produce biofilm in the selected seafood broths, ability to produce β–lactamase and multidrug resistance. *V. harveyi* strain VB166 formed significantly greater biofilm on the surface of glass and stainless steel in selected test broths compared to HDPE (Figure 4). Crystal violet staining of strain VB166 biofilm on glass and stainless steel coupons submerged in selected test broth is depicted in Figure 6. However, there was no significant correlation between mucous production, adhesion and biofilm formation on various food contact surfaces used in this study.
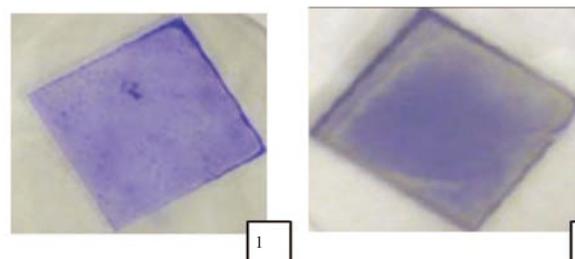
Figure 6. Crystal violet staining of strain VB166 biofilm on 1) glass and 2) stainless steel coupons.

3.5. Alcian blue staining and scanning electron microscopy

Biofilm formed on stainless steel surface was tested for alcian blue staining. Microscopic observations revealed alcian blue stained biofilm as green color matrix, which clearly indicates the biofilm formation of strain VB166 on the stainless steel surface (Figure 7a). Similarly, glass and HDPE surfaces were also tested for bound biofilm formation by strain VB166 and evidenced the similar pattern under optical microscope. The fact that SEM micrograph showed a thinly dispersed biofilm supports the interpretation of how the biofilm associates between cells in nature (Figure 7b). In fact, in their sample that produced the greatest amount of mucoid biofilm; the bacterial cells could not be seen at all and were presumed to be covered only by biofilm but not cells.
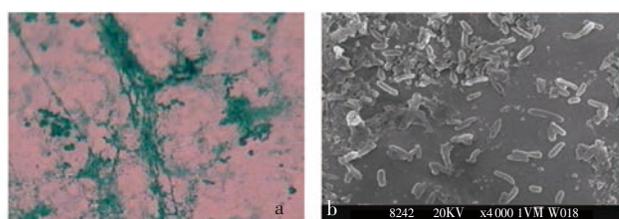
Figure 7. a: Alcian blue staining of mucoid biofilm of strain VB166 which specifically binds to acidic polysaccharides; b: SEM micrograph showing the mucoid biofilm of *V. harveyi* strain VB166 associated with the cells.

4. Discussion

In the present work, we report mucoid biofilm formation abilities on various food contact surfaces and the effect of several antimicrobials on selected biofilm producing *V. harveyi* strains. Microbial adhesion to surfaces is mediated by the interaction between both microorganism and substratum in a complex process, which is influenced by several physico–chemical properties including hydrophobicity and surface charge[11]. Seafood can be either contaminated in aquatic environment (primary contamination) or subsequently during the preparation (secondary contamination). Street vended seafood poses a high risk of being secondarily contaminated, due to street–trading conditions. Food items that are exposed at the street environment for about 8–12 h without refrigeration or protection provide highly favorable conditions for microbial growth and subsequent biofilm formation on their surfaces. *Vibrio* species are naturally occurring bacteria of aquatic habitats, including marine and estuarine environments and aquaculture settings worldwide. Infections by *Vibrio* species may occur after consumption of insufficiently cooked seafood[26]. Besides their potential pathogenicity to humans, many *Vibrio* species have also been described as important fish and shellfish pathogens and implicated as a pathogen causing mortalities in a variety of aquaculture

target species[24,27]. The present study evaluated the biofilm capacity of *Vibrio* sp. from street vended seafood outlets that operate in unsafe and unsanitized environment. The results obtained in this study, showed that *V. harveyi* isolates are able to produce mucoid biofilm on abiotic surfaces. Interestingly, *V. harveyi* strain VB166 was categorized as strong biofilm–producer, developing black colonies on CRA plate, despite the fact that all the strains isolated were from different seafood sources, and evidenced a considerable variability in biofilm production as observed during microtiter plate assay and alcian blue staining of contact surfaces. Apparently, the biochemical tests and PCR based amplification results confirmed that these are *V. harveyi* isolates. In our earlier study, we reported a rapid PCR–based and species–specific detection technique to facilitate early detection and identification of bioluminescent and non–bioluminescent *V. harveyi* isolates from the seawater[28].

Antibiotic resistance in marine bacteria is a serious concern for commercial establishments. Interestingly, several marine bacteria acquired resistance towards different antibacterial agents by changing the permeability of outer membrane porin channels, plasmids, transposons and integrons[29]. Occurrence of these antibiotic–resistant *V. harveyi* isolates is mainly due to the continuous usage of several broad spectrum antibiotics in the aquaculture sites. Interestingly, all the biofilm–forming strains showed resistance to at least one antimicrobial. From the above results, it can be anecdotal that the biofilm production of *V. harveyi* strains increased slightly due to increased resistance to various antibacterial agents. Few studies on antibiotic resistance in shrimps showed that strains of *V. harveyi* isolated from shrimp larvae are resistant to erythromycin, kanamycin, penicillin G, and streptomycin[16]. This is in consonance with the findings of Abraham *et al*[30]. Chromosomally borne class A β–lactamase genes may contribute to resistance, including changing the permeability of outer membrane porin channels (leading to reduced drug influx) and penicillin binding proteins[21,31]. However, the present study indicated that strains isolated from seafood sources formed strong biofilms and were able to produce β–lactamase, a potential contributing factor for antimicrobial resistance.

Bacteria have the ability to attach, colonize, and form biofilms on a variety of surfaces[32]. Contamination with *Vibrio* sp. can cause pricey product recalls by producers and severe health problems. It is interesting to note that *V. harveyi* strain VB166 isolated from catfish was able to form significant biofilms in all tested broths at room temperature. The strain VB166 was able to attach and form subsequent biofilms on glass and stainless steel compared to HDPE. Continuous usage of salt water in stainless steel containers leads to the corrosion of surfaces,

which subsequently enhances the bacterial attachment to abiotic surface, by means of pili or flagella, followed by the production of mucoid biofilm[33]. Recent studies revealed that the ability of vibrios to form biofilms (*i.e.* matrix–enclosed, surface associated communities) depends upon specific structural genes and regulatory processes (two–component regulators, quorum sensing and c–di–GMP signaling)[34]. The carboxylated and sulphated polysaccharides in biofilm clearly indicates its presence after alcian blue staining on abiotic surface. The localization of the soluble and bound carbohydrates was visualized microscopically using alcian blue to stain the anionic sugars. These results evidently support the biofilm formation on stainless steel by the strain VB166. The ability of *Vibrio alginolyticus* strains adhering to epithelial cell lines (Hep–2 and Caco–2), fish mucus and their ability to form a biofilm on different surfaces (glass, polyethylene and polyvinyl–chloride) was previously reported by Snoussi *et al*[35]. Abdallah *et al*. similarly reported the slime production from two seafood–borne pathogens *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains on CRA plates and ability to adhere to abiotic and biotic surfaces[36]. Biofilm formation of marine *Vibrios* on various surfaces has been reported elsewhere for *V. harveyi* and *Aeromonas* sp. respectively[2,37]. However other researchers have observed similar results which showed that few pathogenic strains are also able to produce and adhere on various abiotic contact surfaces including glass, stainless steel, rubber and polypropylene *viz.* *Escherichia coli* O157:H7[38], *Listeria monocytogenes*[39–40], *Acinetobacter*[41], *Salmonella* sp.[42–47]. These extracellular polymers shield the bacteria against harsh environment and serve as a source of microbial contamination during seafood processing. In addition to increasing resistance to sanitation, biofilm formation in food processing facilities increases opportunities of contamination of the processed product[12]. Due to this increased resistance to stress, biofilms pose a serious threat in seafood industry.

Understanding the ability of bacteria, specifically *Vibrio* sp., to form biofilms in seafood processing industry enables the food processors to take better control over prevention of contamination from *Vibrio* biofilms. An ideal cleaning and sanitation procedure should prevent bacterial accumulation and subsequent biofilm formation rather than focus on biofilm removal. Therefore, equipment design, cleaning and sanitizing procedures in food industry should always consider the prevention and removal of bacterial biofilms in order to prevent the attached bacteria. Understanding the factors contributing to biofilm formation is of utmost importance. Furthermore, this exemplifies the importance of considering food safety when selecting materials for food processing equipment and surfaces. The discovery of new biofilm control strategies, following

the specifications desired to be used in food industry, and based on the use of biological–based solutions with high antimicrobial activity and specificity seem to be a step ahead in overcoming the biofilm resistance issue. Further studies are warranted to elucidate and determine the mucoid biofilm structure–function relationship and biofilm inhibition assay.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Comments

Background

Vibrio species are naturally occurring bacteria of aquatic habitats and aquaculture settings worldwide. Infections by *Vibrio* species may arise after consumption of insufficiently cooked seafood. However these biofilms shield the bacteria against harsh environment and serve as a source of microbial contamination during seafood processing. In addition to increasing resistance to sanitation, biofilm formation in food processing facilities increases opportunities of contamination of the processed product. Owing to increased resistance to stress, biofilms pose a serious threat in seafood industry.

Research frontiers

This study is performed in order to evaluate the bacterial potential of biofilm formation isolated from seafood sources, to assess their ability to produce β–lactamase, a potential contributing factor for antimicrobial resistance, and to determine the *in vitro* biofilm forming capacities on abiotic contact surfaces.

Related reports

These results evidently support the similar reports on the ability of *Vibrio alginolyticus* adhering to epithelial

cell lines (Hep–2 and Caco–2), fish mucus and their ability to form a biofilm on different abiotic contact surfaces by Snoussi *et al*. Abdallah *et al*. similarly reported the slime production from two seafood–borne pathogens *Vibrio alginolyticus* and *Vibrio parahaemolyticus* and their ability to adhere to abiotic and biotic surfaces.

Innovations and breakthroughs

The data regarding biofilms formation potential of *V. harveyi* on seafood are scarce. In the present study, authors have demonstrated that 14% of VH strains isolated from various seafood were potential biofilm producers and exhibited resistance to antimicrobial compounds. Strains isolated from catfish, shrimp and eel fish exhibited greater biofilm forming ability compared to isolates from other seafood. The VH strain VB166 showed better attachment and formation of biofilm on glass and stainless steel surfaces compared to HDPE.

Applications

The results of the present study suggest that performing a microplate quantitative bioassay for the detection of biofilm formation on various contact surfaces is very essential in disease endemic areas. From the literature survey it has been found that the ability of bacteria, specifically *Vibrio* sp., to form biofilms in seafood processing industry enables the food processors to take better control over prevention of contamination from *Vibrio* biofilms. This approach will make the seafood industry an environmentally feasible technology.

Peer review

This is a valuable research work in which authors have evaluated the *in vitro* biofilm forming capacity on abiotic food contact surfaces by *V. harveyi* strains. The activity was assessed based on 14% of the VH strains which were potential biofilm producers and showed resistance to antimicrobial compounds. This study also demonstrated that the VH strain VB166 better attaches and forms biofilm on glass and stainless steel surfaces compared to HDPE. This study has promising applications in seafood industry with special reference to human health perspective.

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