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Biofilm Formation of Halophilic *Vibrio harveyi* Strain Vh265 on Various Food Contact Surfaces

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Abstract: Twenty six gram-negative *Vibrio harveyi* strains were isolated from various 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 (CRA), Thiosulfate-citrate-bile salts-sucrose agar and Tryptic soy agar, respectively. The battery of tests viz. morphological, physiological and biochemical characteristics of the selected isolate were studied using standard protocols. *V. harveyi* strains isolated from various seafood's were able to form biofilms of different capacity, given that the strain Vh265 isolated from sardine fish exhibited significantly greater biofilm forming ability compared to other isolates. The strain Vh265 was able to better attach and form subsequent biofilms on stainless steel compared to glass and HDPE. These properties allow these bacteria to survive, proliferate and persist in street vended seafood outlets.

Key words: Mucoid biofilm % Congo red agar % *Vibrio harveyi* and High Density polyethylene

INTRODUCTION

Vibrio species are naturally occurring bacteria of aquatic habitats, including marine and estuarine environments and aquaculture settings worldwide [1-3]. *Vibrio* infections remain a serious threat to public health commonly associated with outbreaks of *Vibrio* infections due to the consumption of foods and water contaminated with human feces or sewage, raw fish and seafood, or associated with the exposure of skin lesion, such as cuts, open wound and abrasions to aquatic environments and marine animals [4-7]. *Vibrio harveyi* is the major causal organism of vibriosis, which causes impending devastation to diverse ranges of marine invertebrates over a wide geographical area. *Vibrio harveyi* is a serious pathogen of marine fish and invertebrates, particularly penaeid shrimp [8]. Several studies have suggested that biofilms may be important for survival, virulence and stress resistance of *Vibrio* spp. [9- 12].

Microbial biofilms are attracting attention of scientists in different areas such as the medical field, aquatic environment, food processing industries etc. Microbial biofilms may be detrimental and undesirable in food processing premises. Biofilms by pathogenic bacteria such as *Salmonella* [13-16], *Klebsiella* [15-18], *Pseudomonas* [19], *Campylobacter* and enterohaemorrhagic *E. coli* O157:H7 [16] and *Listeria* [19-20] have been reported. Such biofilms could be an incessant source of contamination to foods coming in contact with them when formed on contact surfaces. The extensive production of biofilm by microorganisms may seriously affect the quality and safety of the processed food and could also pose a potential risk to humans [21]. A better understanding of bacterial adhesion process is needed for production of microbiologically safe and good quality products in the food industry. This study was undertaken to understand the ability of *Vibrio harveyi* strain to form biofilms on potential food contact glass surfaces.

MATERIALS AND METHODS

Bacteriological Sampling: Raw seafood samples were collected randomly from Aug to October 2010 in sterilized containers from street vended seafood outlets spread over 10 regions within Krishna District, Southern India and were kept at 4°C until further analysis. Different parts of seafood sources were considered as source of inoculums for the study.

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Table 1: Biofilm production of *V. harveyi* isolates. Data are presented as, positive (+), negative (-) for growth in NaCl, black colonies (phenotype of strains on CRA plates), + and ++, Pinkish red color colonies; and +++, black colonies

	Growth in NaCl					
Strain ID	3 %	6%	Source/Origin	OD_{595} ± SD	Biofilm Production	Phenotype of strainson (CRA)
Vh100	+	+	Shrimp	1.08	+	Pinkish red
Vh210	+	-	Tiger Prawn	1.22	+	Pinkish red
Vh130	+	-	Seabass	1.30	+	Pinkish red
Vh265	+	+	Sardine	1.72	+++	Black
Vh451	+	-	Mullete	1.06	+	Pinkish red
Vh170	+	+	Tilapia	0.98	+	Pinkish red
Vh236	+	+	Catla	0.78	+	Pinkish red
Vh160	+	+	Cat fish	0.98	+	Pinkish red
Vh179	+	+	Eel fish	1.46	++	Pinkish red
Vh292	+	-	Crab	1.65	+	Pinkish red
Vh161	+	-	Channa	1.40	+	Pinkish red
Vh230	+	+	Clam	1.20	++	Pinkish red
Vh163	+	+	Oyester	1.67	++	Pinkish red

Bacterial Strains and Culture Broth: Eighty six *Vibrio* strains used in this study were isolated from internal organs of aquacultured street vended seafood according to method previously described by Ben Kahla *et al*. [22]. Bacterial strains were stored in a tryptic soy broth (TSB) glycerol mixture (1:1v/v) at -80ºC until ready for use. They were grown overnight in TSB. Each seafood sample (100 g) was blended separately with 250 ml of water, centrifuged (8,000×*g*) at 4°C for 10 min and filter sterilized using 0.2µm membrane filters.

Phenotypic Characterization of Biofilm-producing Bacteria: Qualitative detection of biofilm formation was studied by culturing the strains on Congo red agar (CRA) plates as described previously by Jain and Agarwal [23]. The constituents of the media were brain heart infusion 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 when the agar had cooled to 55°C. CRA plates were inoculated with all the isolates listed in (Table 1) and incubated aerobically for 24 h at 37°C. After incubation, the pigmented colonies (generally black colour) were considered as mucoid phenotype positive, whereas unpigmented bacteria (formed pinkish red, smooth colonies with a darkening at the centre) were interpreted as mucoid phenotype negative strains. Plates were checked and the color was noted after 24 h.

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 (TCBS) agar. The 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 [24].

Microtiter Plate Biofilm Production Assay: The twenty six selected mucoid *Vibrio* isolates were cultured overnight at 37°C in TSB. Turbidity to measure bacterial growth was observed by determining absorbance at 595 nm. Mucoid biofilm-forming capability of each isolate was determined by a crystal violet binding assay [25]. Overnight cultures were diluted (1:100) in 0.1% peptone water. Ten µl of diluted culture was dispensed into wells of 96-well microplates containing 90µl of TSB with and without salt (10g tryptone and 5 g yeast extract per liter of deionized water). The plates were incubated for 24 h at 25°C. Bacterial cultures were removed and wells of the 96-well microplates were rinsed twice with deionized water to remove loosely attached bacteria. Each well was stained with 150µl of 0.25% crystal violet for 30 min at room temperature. The wells were rinsed twice with deionized water. After drying, the crystal violet bound to the biofilm was solubilized with 150µl of 70% ethanol for 30 min. The absorbance was measured at 595 nm using a microplate reader.

Biofilm Formation on Contact Surfaces: The selected *Vibrio harveyi* strain Vh265 isolated from seafood was

overnight cultured in TSB at 37°C. The culture was diluted (1:100) in 0.1% peptone water and inoculated into 200 ml of prepared TSB with and without NaCl and carbon source at the final concentration of 10^5 cells/ml. Aseptically, 15 ml of broth was dispensed into Petri dishes containing stainless steel (2X2 inches), coupon, high density polyethylene (HDPE) coupon, (2X2 inches) and glass (1X2 inches). The Petri dishes were incubated at 25°C for 24 h. After incubation glass, stainless steel and HDPE were transferred into a sterile Petri dish and rinsed twice in 15 ml of deionized water. The contact surfaces were stained with 15 ml of 0.25% crystal violet for 30 min at room temperature. The wells were rinsed twice with 15 ml of deionized water. 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.

Statistical Analysis: All data from biofilm quantitative assays were expressed as Mean±Standard Deviation (S.D) 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.

RESULTS

Identification of Biofilm-producing *Vibrio Harveyi* Strain: Phenotypic mucoid biofilm production was assessed by culturing the investigated strains on CRA plates (Table 1). All *V. harveyi* strains grew on CRA medium giving three morphotypes with different colour (red, pinkish-red, black). Among 26 *Vibrio* strains tested in this study, one strain Vh265 was determined as potent mucoid biofilm producers characterized by black colonies, whereas the remaining strains were non-biofilm producing characterized by pinkish-red colonies with darkening at the centre. The bacterial isolate was identified by standard morphological, physiological and biochemical tests following the schemes of Alsina and Blanch (1994) set of keys for identification of *Vibrio* sp. All the strains were Gram-negative; facultative anaerobes, motile, curved rods, salt requirement (3-6% NaCl), oxidase, catalase and gelatinase, amylase, chitinase positive; and produced green mucoid phenotypes on TCBS agar plates. In addition, these isolates failed to utilize sucrose, indole, melibiose, mannitol, sorbitol, ethanol, histidine and proline and were positive to arginine dehydrolase, ONPG test, nitrate reductase and sensitive to the vibriostatic agent 0/129 at 120µg (Table 2).

Table 2: Biochemical set of keys for species level identification of mucoid biofilm producing bacteria *Vibrio harveyi* strain Vh265.

Form	Curved rods	6	+
Motility	Motile	8	+
Gram Stain	Negative	10	-
Spore	Nonforming	**Production of**	
O-F test	-	Amylase	+
Catalase	+	Gelatinase	+
Oxidase	+	Alginase	-
Gas from D-glucose	-	β-lactamase	+
Esculin Hydrolysis	+	Chitinase	+
Voges-Proskauer reaction (24h)	+	**Utilization of**	
Reduction of NO_3 to NO_2	+	D-Xylose	+
ONPG Hydrolysis (24h)	+	D-Glucose	+
Growth in TCBS	Yellow	L-Arabinose	+
Arginine hydrolysis	+	D-Galactose	+
Inhibition by 0/129		Sucrose	-
10µg	Resistant	Cellobiose	+
120µg	Sensitive	Indole	-
Growth at		Melibiose	-
20°C	+	D-Mannitol	-
30°C	+	D-Sorbitol	-
37°C	+	Lactose	+
40°C	+	L-Arginine	+
43°C	-	L-Ornithine	+
Growth at % NaCl		Ethanol	-
0	+	L-Histidine	-
3	+	L-Proline	-

Bacterial Growth and Biofilm Formation: Biofilm formation and bacterial growth by the sixteen *Vibrio harveyi* isolates were observed in TSB. There was significant variation in bacterial growth between strains. Strains produced greater biofilm in TSB with 3% NaCl and 0.5% carbon source compared to TSB without salt and carbon source (data not shown). Therefore, TSB with salt and glucose was used to compare biofilm formation by all isolates. Further, there was large variation in biofilm-forming capacity among the *Vibrio harveyi* isolates from the seafood processing line, with values ranging from 0.78 (isolate Vh236 from Catla fish) to 1.72 (isolate Vh265 from Sardine fish). However, biofilm formation by *Vibrio harveyi* strain isolated from Sardine fish was significantly greater compared to other isolates. These abilities differed with the strains irrespective of their site of isolation. In the microplate test, statistical analysis of the mean crystal violet staining OD values of the isolates classified as strong biofilm producers allowed us to distinguish three significantly different groups (p-value<0.02) with higher (Vh265), intermediate (Vh179,Vh230,Vh163) and lower (Vh100, Vh210 and Vh130, Vh451,Vh170,Vh161,Vh292,Vh160 and Vh236) levels of crystal violet staining. There was a significant variation in the ability of each strain to grow in different test broths at 24 h incubation.

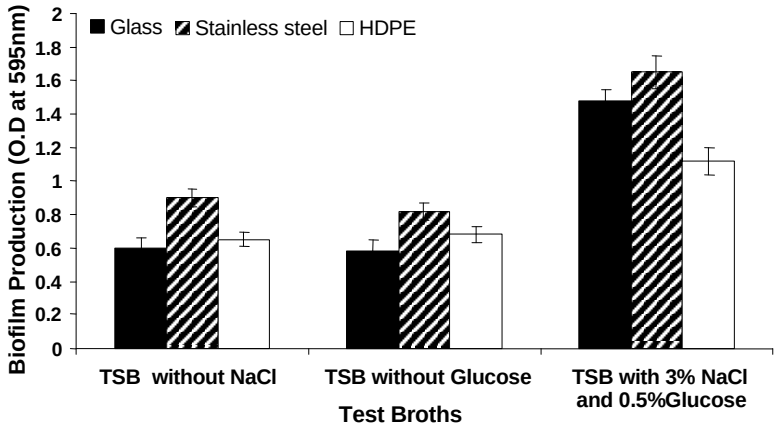

Fig. 1: Biofilm formation by *Vibrio harveyi* strain Vh265 on glass, stainless steel and HDPE surfaces in various test broths

Biofilm Formation on Contact Surfaces: The ability of the *Vibrio harveyi* strain Vh265 isolated from Sardine fish to form a mucoid biofilm was evaluated on various food contact surfaces in TSB broth. This strain was tested because of its strong ability to produce biofilm in the selected broths, *Vibrio harveyi* strain formed significantly greater biofilm on the surface of stainless steel in TSB compared to glass and HDPE (Fig. 1). However, there is no correlation between mucous production and adhesion and biofilm formation on various food contact surfaces used in this study.

DISCUSSION

Seafood poses a high risk of being contaminated, due to open-trading conditions. Food items that are exposed at the open environment for about 8-12 h without refrigeration or protection, provides highly favorable conditions for microbial growth and subsequent biofilm formation. Formation of biofilm, extracellular polysaccharides that shield the bacteria against harsh environment, can serve as a source of microbial contamination during processing. This study evaluated ability of *Vibrio harveyi* strain Vh265 isolated from seafood to form biofilm on food on various contact surfaces. The ability of *Vibrio harveyi* strain to form biofilm on food contact surfaces would eventually decrease the effectiveness of sanitation processes and therefore increases the risk of contamination.

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) [26]. Abdallah *et al*. [27] 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. Biofilm formation of marine *Vibrios* on various surfaces has been reported elsewhere for *V. alginolyticus* [28], *V. cholera* [29] and *V. harveyi* [30, 31] respectively.

Microbial contamination is a liability for food processors. The ability to effectively control contamination will greatly benefit consumer food safety and the economic welfare of the manufacturer. Understanding the ability of *Vibrio harveyi* strain to form biofilms on various surfaces that come into contact with food in different sectors of the food industry should provide some insights on how to prioritize or strategize processing conditions that enhance safeguarding of our food supplies. Based on the above results, it can be concluded that *Vibrio harveyi* strain can survive on food contact surfaces forming biofilm. A better 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 *Vibrio harveyi* isolates obtained from seafood processing lines and thus forms the basis of this study. An ideal cleaning and sanitation procedure should prevent bacterial accumulation and subsequent biofilm formation rather than focus on biofilm removal [32]. 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 transfer of attached bacteria. Understanding factors

contributing to biofilm formation are 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 and biofilm inhibition assay.

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