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Biofilm formation of aquatic pathogen, *Vibrio campbellii* on different contact surfaces

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Abstract
The present study was aimed at empirically ascertaining the ability of a pathogenic *V. campbellii* isolate to form biofilms on different surfaces, commonly used in shrimp aquaculture facilities. Biofilm formation ability on three different surfaces (plastic, galvanized metal and tile) was studied and, biofilm cells were enumerated at 48 h, 96 h and 192 h by spread plate technique in replicates and reproducibility was checked by independent experiments. In this study *V. campbellii* exhibited the highest potential to form biofilms at 48 h ($6.27 \times 10^6 \pm 4.81 \times 10^6$ CFU/mL) and 96 h ($5.82 \times 10^6 \pm 2.50 \times 10^6$ CFU/mL) on tile surface. At 192 h, however, *V. campbellii* showed high potential to form biofilm on plastic surface ($3.49 \times 10^6 \pm 0.02 \times 10^6$ CFU/mL). Albeit, *V. campbellii* showed lower affinity for galvanized metal surface than tile and plastic surfaces at all three time points. Study concludes that biofilm forming ability of *V. campbellii* on tile and plastic is higher than that of on galvanized metal, and the use of tile and plastic in shrimp aquaculture facilities should be reconsidered.

Keywords: biofilm, luminous vibriosis, abiotic surfaces, shrimp aquaculture

1. Introduction
Luminous vibriosis, caused by the members of Harveyi clade Vibrios (e.g. *Vibrio harveyi* and *V. campbellii*) [1] is one of the multitude of infections of vibriosis [2] and a major constrains in shrimp farming industry. *V. campbellii*, pathogenic, gram negative, oxidase positive, rod or curved shaped and facultative anaerobic autochthonous microflora is one of the causative agents of the luminous vibriosis. Although, *V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus* have been frequently reported as pathogens, less attention has been given to *V. campbellii*. Opportunistic pathogens such as some *Vibrio* species become pathogenic when they are in the form of biofilms [3], an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance matrix [4]. These pathogens form biofilms as a contrivance for their better survival in aquatic ecosystems and, show higher virulence inside the host due to high degree of resilience of biofilms to external disturbances, such as antibiotics & internal host defense mechanisms [5] like phagocytosis [6]. Non- cellular materials including mineral crystals, corrosion particles, clay or silt particles or blood components, depending on the environment in which the biofilm develop may also be found in the biofilm matrix [4]. Biofilms are ubiquitous due to the phenotypic elasticity of bacteria [5]. Pathogenic strains of microbial cells within biofilms are remarkably differing genetically and phenotypically from their planktonic counterparts, enhancing their virulence and susceptibility to antibacterial agents or drugs [5]. Biofilm formation is a multistage, complex process comprising five main stages; (1) Development of a surface conditioning film, (2) Movement of micro-organisms into close proximity with the surface, (3) Adhesion (reversible and irreversible), (4) Growth and colonization of micro-organisms, and (5) Biofilm cell detachment/ Dispersal [5]. Biofilm formation is governed by different environmental signals, such as pH, temperature (system and ambient), availability of certain nutrients and presence of oxygen [7, 5] along with characteristics of substratum, hydrodynamics and characteristics of aqueous medium and various properties of the cell surface [4]. Tile, plastic and galvanized metal are commonly used surface materials, having diverse array of applications in shrimp culture set up. Plastic mixed with fiber glass are used to construct aerators, while tile and galvanized metal are used for the purpose of constructing shrimp ponds. Present study ascertained the potential of *V. campbellii*, one of the causative agents of luminous vibriosis in cultured shrimp to form pathogenic biofilms on different surfaces, commonly used in shrimp culture facilities.

2. Materials and Methods

The present study was conducted in October to December 2016 at the laboratory of Livestock production, Faculty of Agricultural Sceinces, Sabaragamuwa University of Sri Lanka. Ability of biofilm formation of *V. campbellii* (LMG 21363) on three different contact surfaces (tile, plastic, and galvanized metal) at different time points (46 h, 96 h, and 192 h) was empirically studied. All the surfaces used in the study were coupons, small in size, 4 cm^2 (2 x 2 cm), and sterilized prior to the experiment. Tile and galvanized metal coupons were sterilized by autoclaving at 121 ^{0}C for 15 min. Plastic surfaces were cleaned with detergent and washed with distilled water thoroughly and sterilized by washing with absolute alcohol and then dried properly under the laminar flow. Subsequently, they were sterilized under UV light for 30 min. Following the sterilization, six coupons of each surface were transferred to sterilized petri plate under aseptic conditions. Thus, three plates were prepared for each surface. Surfaces were conditioned for 24 h in Tryptic Soy Broth (TSB) before inoculating the *V. campbellii* isolate.

Culture of *V. campbellii* (LMG 21363), kindly donated by Laboratory of Aquaculture and Artemia Reference Center, Faculty of Bio Science Engineering, Ghent University, Belgium was recovered on TCBS (Thiosulphate Citrate Bile Sucrose sugar) (Oxoid Limited, USA) agar plates. Single *V. campbellii* were grown in TSB (containing 1% NaCl) by re-culturing and incubating overnight at 28 ^{0}C. Cell density was determined spectrophotometrically at 600 nm. Afterwards, 10^5 CFU/ mL of *V. campbelli* cells were inoculated to each conditioning petri plate except the controls. Plates were then incubated at ambient temperature (27±2 ^{0}C) until the sampling was done at three time points;48 h, 96 h and 192 h post- inoculation. At each time point, area of 4 cm^2 on each surface in triplicates were scraped using sterilized scalpels. The scrapings were then washed with 1.5 mL of sterilized distilled water and collected into sterilized micro centrifuge tubes. At each sampling point TSB in each petri dish was replaced by freshly prepared sterilized TSB. Collected samples were subjected to serial dilution in sterilized distilled water. Biofilm cells were enumerated using spread plate method. Following the spreading of 1 mL of each sample, plates were incubated at 28 ^{0}C for 24 h. Finally, cells were counted and expressed as CFU/mL.

Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS Version 16). Multiple comparisons were done with Duncan multiple range tests. Microsoft office Excel 2013 was used for the graphical illustrations. Results were presented as mean ± SE.

3. Results and Discussion

The present study was conducted to determine the ability of a pathogenic *V. campbellii* to form biofilms on three different surfaces, commonly used in shrimp farming facilities at three different time points, 48 h, 96 h and 192 h post inoculation. Un-inoculated control that contained only the surfaces on TSB medium did not show any biofilm cells, attached to any of the surfaces and it exclude any contamination throughout the experimentation.

Number of colonies on three studied surfaces showed an increasing trend from 48 h (1.3059 $x10^8$ CFU/mL) to 96 h (1.3498 $x10^8$ CFU/mL) and the trend declined from 96 h to 192 h post incubation (5.536 $x10^7$ CFU/mL). The highest biofilm formation by *V. campbellii* isolate was observed at 48 h and 96 h on tile surface, and at 192 h, plastic exhibited the highest biofilm formation. In contrast, galvanized metal surface showed the lowest biofilm formation ability at 48h, 96 h and 192 h post incubation. Results indicated that biofilm forming ability of pathogenic *V. campbellii* on different surfaces were significantly different ($p<0.05$) at 48 h post incubation. The highest number of colonies were formed on tile surface ($6.27x10^6 \pm 4.81x10^4$ CFU/mL) followed by plastic ($4.76 x10^6 \pm 4.92 x10^4$ CFU/mL) and galvanized metal surface ($2.72 x10^6 \pm 2.49 x10^4$ CFU/mL), respectively (Table 1). Moreover, there was a significant difference ($p<0.05$) in biofilm formation by *V. campbelli* isolate on three different surfaces at 96 h post incubation. The highest number of colonies were observed on tile ($5.82x10^6 \pm 2.5x10^4$ CFU/mL) whilst the lowest number of colonies were observed on galvanized metal ($2.21x10^6 \pm 1.14x10^4$ CFU/mL). Next to the tile, plastic surface showed the higher number of colonies ($4.34 x10^6 \pm 3.26 x10^4$ CFU/mL). At 192 h post incubation, biofilm formation by *V. campbellii* on three surfaces was significantly different ($p<0.05$) from each other and plastic showed the highest number of colonies ($3.49 x10^6 \pm 2.66 x10^4$ CFU/mL) followed by tile ($0.51 x10^6 \pm 1.14 x10^4$ CFU/mL) and galvanized metal ($0.4x10^4 \pm 0.0051x 10^4$ CFU/mL).

Table 1: Number of colonies formed by *V. campbellii* (LMG 21363) on tile, plastic and galvanized metal surfaces at 48 h, 96 h and 192 h post incubation.

Contact surfaces	Mean number of colonies (CFU/mL)		
	48 h	96 h	192 h
Tile	$6.27x10^6 \pm 4.81 x10^4$ c	$5.82 x10^6 \pm 2.50 x10^4$ c	$5.1 x10^5 \pm 1.14 x10^4$ b
Plastic	$4.76 x10^6 \pm 4.92 x10^4$ b	$4.34 x10^6 \pm 3.26 x10^4$ b	$3.49 x10^6 \pm 2.66 x10^4$ d
Galvanized metal	$2.72 x10^6 \pm 2.49 x10^4$ a	$2.21 x10^6 \pm 1.14 x10^4$ a	$4.0 x10^3 \pm 0.005 x10^4$ a

* Different superscripts in each column indicate the statistically significant difference in values at 95% confidence intervals.

The present findings were significant as *V. campbellii* is one of the pathogenic bacteria, responsible for the luminous vibriosis in shrimps, cultured fish and molluscs, decreasing survival rates and increasing mortalities in hatcheries and grow-outs of shrimp farms, resulting massive economic losses to the industry. Biofilms are difficult to eliminate once formed. Thus, pathogenic biofilms are of a highly concerned field nowadays, mainly in aquaculture, food processing, medical field, dentary and other industries like production of papers [8].

Biofilm formation is characterized by several steps where each step is regulated by number of factors. But the factors regulating biofilm formation and adhesion of microbial cells to different surface are still not well understood. Basically, substratum effect, hydrodynamics, characteristics of the aqueous medium and properties of the cells govern the biofilm formation on different surfaces [6]. But in this study the only dynamic factor was the surface, so that all the variation in biofilm formation that observed within the study was mainly due to the different physical and chemical

properties of the different surfaces tested.

At 192 h post incubation, plastic exhibited the highest number of colonies revealing the higher potential of plastic to host *V. campbelli* (LMG 21363) than other surfaces. The present findings are in accordance with Karunasagar *et al.* . . . . . [9] in which authors have found the highest *V. harveyi* cell density on plastic surfaces. Also, a study by Kolari [10] on biofilm formation on different surfaces found that plastic provided significant benefit over stainless steel, i.e., biofilm formation was higher in plastic and less prone to contamination rather than in stainless steel. The present findings are well supported by the study carried out by Zacheus *et al.* . . . . . .[11] in which authors have found that volume of heterotrophic bacterial cells were slightly higher on Polyvinyl Chloride surfaces over other tested surfaces.

Hydrophilic and hydrophobic characters of studied surfaces may affect the affinity of microbial cells. Hydrophobic and non-polar surfaces are more preferred than hydrophilic and polar surfaces by microbes. Teflon and other polystyrene/ plastic are hydrophobic and non- polar surfaces, whilst metals are hydrophilic surface [4, 12]. This may result the highest mean number of colonies on plastic at 192 h post incubation and the lowest mean number of colonies on galvanized metal at 48 h, 96h and 192 h post incubation. This argument is well supported by findings of Fletcher and Loeb [13] in which they had showed a preference of hydrophobic surfaces by a marine *Pseudomonas* sp. Also Karunasagar *et al.* . . . . . [9] have shown the lowest *V. harveyi* cell density on stainless steel. Present findings are in accordance with Marques *et al.* . . . . . [14] in which authors have found less viable number of *Staphylococcus aureus* on stainless steel. In contrast, Kefford and Marshall [15] have revealed that adhesion of *Leptospira biflexa Serovar patoc* 1 (*L. patoc*) was consistently higher on hydrophobic surfaces over hydrophilic surfaces (glass and plastic). Lack of comprehensive studies limit the discussion of present findings on how tile hosted the highest number of *V. campbellii* at 48 h and 96 h post incubation.

Detachment occurred in almost all the three surfaces, reducing mean number of microbial colonies regardless the physiochemical properties of the surfaces. Souza *et al.* . . . . . [16] has revealed that there is no significance influence of surface type or incubation period for the detachment rate of *Staphylococcus aureus.* Kolari [10] has found that repositioning of biofilm cells was not substratum dependent by observing patterns of repositioning of *Deinococcus geothermalis* biofilm cells on glass and stainless steel surfaces.

The attachment of bacteria to submerged solid surfaces has become a major concern in aquatic environments due to the negative consequences caused by high degree of resilience of biofilms. Although the study revealed a reduction in mean number of colonies of *V. campbellii* isolate from 48 h to 192 h, most of the studies have found that the number of adhered cells on the surface increase as a function of time. However, findings of the Souza *et al.* . . . . . [16] is in agreement with present study that adherent cells always did not increase over time. Present findings warrant further studies on substratum effect of biofilm formation over the time in shrimp aquaculture facilities.

4. Conclusion

The present findings indicate that usage of plastic and tile should be minimized in shrimp aquaculture facilities and it is a necessity to investigate alternatives for tile and plastic. However galvanized metal showed comparatively low number of *V. campbellii* (LMG 21363) colonies. Thus, it is recommended to use galvanized metal in shrimp farming facilities rather than plastic and tile.

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