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Identifying biofilm forming bacteria in cow milk in Mekong Delta, Viet Nam

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ABSTRACT

Bacterial biofilms are agglomeration of bacterial cells, stuck to the material surfaces of material in wet environments and formed by a self-produced matrix. The formation of bacterial biofilms is a great risk for the milk processing industry, as the survival of many bacterial species in cow milk may lead to many problems such as microbial spoilage, deterioration in quality, and consumer health risks. This study aimed to identify biofilm formation bacteria from cow milk. The experiment included isolation; biofilm forming assay in 96-well microtiter plates and the identification of microbial isolates using classical and molecular biological methods. A total of 14 bacterial isolates from 10 cow milk samples were evaluated for their biofilm formatting ability. Among them, four isolates were identified as moderate and strong biofilm producers. These four isolates belong to the genera Serratia and Aeromonas. Out of the 4 isolates, Serratia marcescens VL41 was classed as a strong biofilm producer while Aeromonas veronii ST15, Aeromonas sp. ST17, Serratia marcescens VL13 were classed as moderate biofilm producers respectively. The findings of this study suggest that it is necessary to discover the contamination causes and prevention of genera Serratia, and Aeromonas into cow milk.

1. INTRODUCTION

Bacterial biofilms are formed by communities of microorganisms engulfed in a self-produced polymeric matrix that adheres to biotic and abiotic surfaces (Šuláková et al., 2019). The presence of biofilms is considered as an evident health hazard because besides bacteria insignificant to health they may also contain pathogenic microorganisms (Vlková et al., 2008). Biofilms contain many microbial diseases in both animals and humans and are associated with many animal diseases. Animal health and disease control are extremely dependent on the ability to control bacterial biofilm formation (Tasneem et al., 2018).

In the dairy industry, microbial biofilms form many surfaces of technological systems in factories and dairy farms present a large risk because of the presence of many species of microorganisms. So, the quality and safety of raw cow milk and final products may be affected due to microbial spoilage, and deterioration in quality. Dairy biofilms included bacterial extracellular polymeric substances, milk residues, protein, and calcium phosphate. heat-resistant proteases and lipases secreted from biofilm

formatting bacteria into cow milk can reduce milk quality (Weber et al., 2019). Bacteria living in biofilm are strong against antibacterial agents than planktonic cells of the same species. Therefore, conventional sanitation and disinfectant agents find it difficult to kill the bacteria. Furthermore, the DNA transmission from the cell to the cell occurred in a microorganism community and the biofilm development could be controlled by their conjugation mechanisms. Specific microorganism was discovered on internal surfaces of equipment in dairy processing lines, genetically closely related a lot of strains persisted in communities of biofilm forming microorganisms (Schlegelová et al., 2010). Some genera *Pseudomonas, Aeromonas, Serratia, Acinetobacter, etc.* with a predominance of the genus *Pseudomonas* were reported.

There are a large number of methods for detecting the bacterial community composition of cow milk such as the molecular method (PCR) has been applied to detect bacteria in raw milk (Weber et al. 2019).

To increase the bacterial quality of cow milk produced by the cow-farmers and control the better quality of hygienic conditions from the aspect of sanitation and disinfection of the contact surfaces, it is necessary to identify bacterial strains responsible for biofilm formation in cow milk. The article focused on the biofilm forming assay of bacterial isolates from cow milk in the 96-well microtiter plate and identified them by molecular method.

2. METHODS

2.1. Sources of samples and isolation of bacterial isolates

A total of 10 cow milk samples were taken randomly from cow milk farms in Vinh Long (5 samples) and SocTrang (5 samples) provinces in the Mekong Delta, Vietnam. The samples were contained in sterile plastic tubes, labeled before being carried to the lab, and stored in a refrigerator (410°C, 24 h) for isolation in Cetrimide - Agar (CA) media (45.3 g L^{-1} Cetrimide agar, 10 mL L^{-1} Glycerol, 3.4 g L^{-1} agar) (Merck, Germany) (Flint and Harley, 1996) in the College of Natural Sciences, Can Tho University. Firstly, the cow milk sample was plated on CA medium and incubated at 37°C for 24–48 h; and then the culture was streaked on the medium to receive single colonies (Nguyen et al., 2006). Steps of the bacterial isolation included dilutions and spreading on sterilized Petri plates containing the CA media. Serial dilutions (10^{-2}, 10^{-3}, and 10^{-4}) of composite samples were prepared. 10 µL of the sample of 10^{-4} dilution was plated on Petri plate containing the CA agar media in sterile conditions. The plates were incubated at 37°C for 48 h. Colonies were subcultured on the CA agar plates by striking technique and re-incubated at 37°C for 48 h. This isolation process is carried out in the same medium until a single isolate is obtained. The pure isolate was cultured on the CA culture medium in a sterilized Eppendorf tube (1.5 mL) and put in a refrigerator at 10°C for 3 months.

2.2. Biofilm formatting assay of bacterial isolates

The biofilm formatting evaluation of bacterial isolates was carried out according to the method of Stepanovic et al. (2007): The bacterial isolates were suspended in 4 mL of sterile TSB-Y (Tryptone Soy Broth) medium (30 g L^{-1} of TBS (Merck, Germany); Y: 5 g L^{-1} Yeast extract (Himedia, India)), incubated in shaker (200 rpm: round per minute) during 12 h. After incubation, 1 mL of the culture was transferred into a tube containing 9 mL of the sterile TSB-Y medium. The culture tube was mixed well with the vortex machine and then inoculated at 37ºC for 12 h. Then, 200 µL of each bacterial suspension was dropped into 96-well flat-bottomed sterile polystyrene microtiter plates, repeated 3 times. Non-inoculated TSB-Y medium wells were used as negative controls, repeated 3 times. The plates were incubated at 37°C for 48 h. The bacterial suspension was aspirated, and each well was washed three times with 200 µL of sterile PBS 1x (Phosphate-buffered saline solution pH 6.8; 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4). Thereafter, the biofilm was fixed with 200 µL of methanol for 20 minutes (min), and later removed. The plates were stained with 200 µL of crystal violet solution at 1% for 15 min at ambient temperature; removed and washed with the 200 µL sterile PBS 1x in triplicate to clean the crystal violet solution completely. Then, each well of the plate was added 200 µL of 95% ethanol solution to each well for at least 20 min at room temperature. The absorbance (OD) measured at 570 nm of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (ODc). The ODc value was determined: $OD_C = OD_{Control} + 3*SD$ ($OD_{Control}$: the mean OD of the negative control; SD: standard deviation). The classification of the biofilm-forming ability of the bacteria is as follows (Stepanovic et al., 2007): no biofilm-forming ($OD_{Sample} \leq ODc$), weak biofilm-forming (ODc <

$OD_{Sample} \leq 2ODc$), moderate biofilm-forming ($2ODc < OD_{Sample} \leq 4ODc$), and strong biofilm-forming ($4ODc < OD_{Sample}$).

The average OD values were calculated and evaluated as an index of biofilm formation levels.

2.3. Examination of morphological characteristics

The isolates were selected based on their moderate and strong formation capacities and identified by their colony morphology, Gram-staining, and cell morphology according to the protocols of Nguyen et al. (2006). Before testing, the pure isolates were subcultured onto CA agar plates and incubated at 37°C for 48 h. Morphological characters of the colonies were carried out based on their shape, size, color, margin, and elevation on the media. Cell morphologies of the isolates were observed using optical microscopes (Olympus BX51 Microscope 1000x).

2.4. Identification of biofilm-forming bacteria

Moderate and strong biofilm-producing isolates were sequenced 16S rRNA genes. The gene was amplified by the polymerase chain reaction (PCR) method.

The extraction of bacterial DNA from bacterial suspension (1 mL from a TSB medium cultured at 30°C for 24h, and 120 rpm) was performed following the protocols of Neumann et al. (1992). The bacterial gene was amplified primers: 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3') primers (Frank et al., 2008) in a DNA thermocycler (C1000 Thermal Cycler - BioRAD). The PCR was performed in a total volume of 50 µl in 0.2 mL Eppendorf tubes containing 25 µL master mix, 0.5 µL primer 27F (0.25 µM); 0.5 µL primer 1492R (0.5 µmol), 10 µL of DNA, and 14 µL BiH2O. The amplification was performed in the conditions, including 95°C for 6 min; then 40 cycles at 95 °C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final step at 72°C for 5 min. The PCR products (10 µL) were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Sanger sequencing of the partial 16S rRNA gene of selected isolates was carried out. Finally, the 16S rRNA sequence of the isolate was searched in BLAST (Basic Local Alignment Search Tool) to determine the similarity, cut-off of 99%.

The selected sequences of bacterial strains from the BLAST and the isolates were used for phylogenetic tree building from evolutionary distances in MEGAX software.

2.5. Statistical analysis

All data from biofilm forming ability was analyzed and evaluated, and graphs were drawn using Microsoft Excel 2010 software. The LSD (least significance difference) was used to check the significant differences in collected data.

3. RESULTS AND DISCUSSION

3.1. Biofilm formation assay of bacterial isolates

A total of 14 isolates were collected from 10 cow milk samples, including 6 isolates from Soc Trang (ST11, ST14, ST15, ST17, ST31, ST47); 8 isolates from Vinh Long (VL11, VL13, VL15, VL17, VL22, VL31, VL40, VL41). Their biofilm-forming ability was determined. The OD measurement results of bacterial isolates are presented in Figure 1.

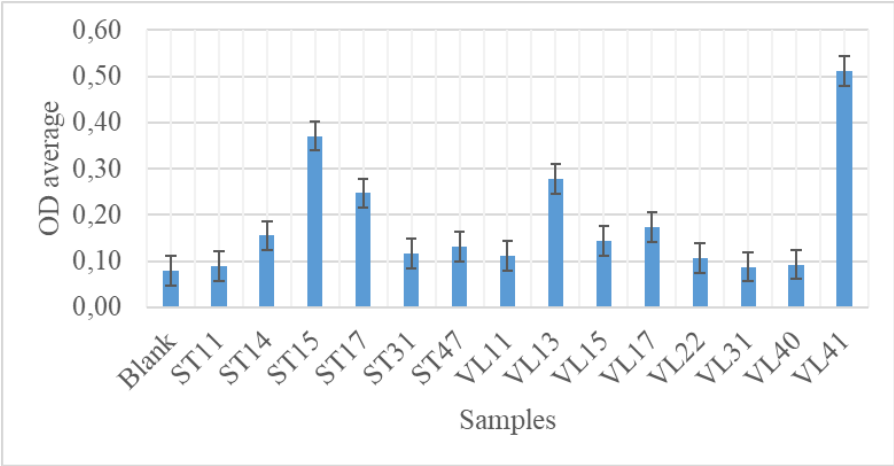


Figure 1. Biofilm formation ability ($OD_{average}$) of bacterial isolates

The results show that 8/14 (57.1%) of the isolates were biofilm producers, among them 1, 3 and 4 isolates were strong, moderate, and weak biofilm producers, respectively. Out of eight isolates, two (the ST15, the ST17) moderate biofilm producers were obtained from the cow-milk farms in Soc Trang and two moderate (the VL13) and strong (the VL41) biofilm producers were obtained from Vinh Long. The calculated value of ODc was 0.117. The VL41 showed the highest OD value (0.5106), statistically significant differences from others ($p < 0.05$) which suggests that this isolate was the strongest biofilm producer.

Biofilm formation can be affected by various environmental factors, phenotypic and genetic factors. Normally, biofilm-formatting is increased by cell motility (supported by flagella). Al-kafaween et al. (2019) highlighted that moderate biofilm formation on the 96-well microtiter plates at 37°C by both *Pseudomonas aeruginosa* and *Streptococcus pyogenes* was seen after two days. On day two, the OD values for *Pseudomonas aeruginosa* and *Streptococcus pyogenes* were 0.12 and 0.13 respectively, and the OD values were lower than the OD values of 4 (the ST15, the ST17, the VL13, and the VL41) as moderate and strong producers (OD values > 0.2) in the present study.

The 4 isolates as moderate and strong biofilm producers were selected for further studies, including the determination of their morphological characteristics and identification.

3.2. Morphological colony and cell characteristics of four bacterial strains classed moderate and strong biofilm producers

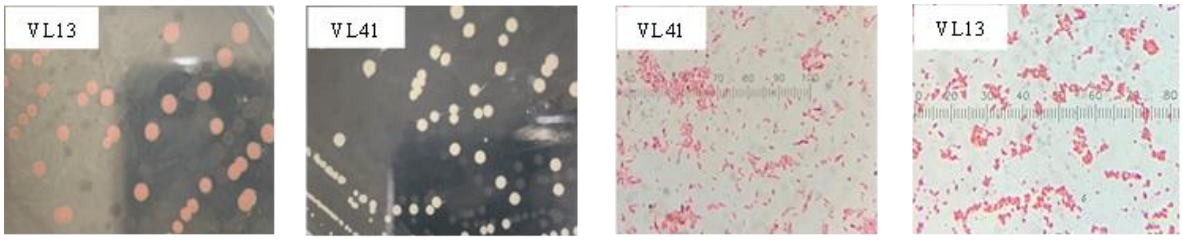
The growth performance of the bacterial isolates on the CA agar is shown in Table 1. From table 1, it can be seen that all of the colonies (ST15, ST17, VL13, VL41) exhibited a circular shape, entire margin, raised elevation, with sizes ranging from 0.5 to 2.2 mm. The color of the ST15, ST17, VL1, and VL41 isolates was opaque white, clear white, pink, and white respectively.

The bacterial cells were short rod-shaped (S-R) under the microscope. Gram staining determination of the bacterial isolates showed that all of them were Gram-negative bacteria (Table 1).

Table 1. Characteristics of 4 bacterial isolates as moderate, strong biofilm producers

Name	Colony characteristics					Gram	Cell morphology
	Shape	Color	Edge	Elevation	Diameter (mm)		
ST15	Circular	Opaque white	Entire	Raised	1.0 - 2.0	-	S-R
ST17	Circular	Clear white	Entire	Raised	0.5 - 1.3	-	S-R
VL13	Circular	Pink	Entire	Raised	1.8 - 2.0	-	S-R
VL41	Circular	White	Entire	Raised	1.2 - 2.2	-	S-R

Note: '- ': Negative; S-R: Short rod-shaped



a b

Figure 1. The colony image of VL13 and VL41 isolates (a); Gram staining of VL13 and VL41 isolates in Olympus BX51 Microscope (1000x) (b)

3.3. Identification of the moderate and strong biofilm bacterial trains

The four isolates as moderate and strong biofilm producers were designated as the ST15, the ST17, the VL13, and the VL41 respectively. After the genetic sequences of the isolates were sequenced, the sequences were loaded to the genetic sequence database of the National Center for Biotechnology Information. The alignment results are shown in Table 2:

Table 2. Identification of homology sequence in GenBank

Strains	Nucleotides number	Results of searching in BLAST	Percentage Identity (%)	Accession number
ST15	1,350	*Aeromonas veronii* strain JX16102	100	KY767538.1
ST17	1,346	*Aeromonas* sp. Sh2	100	KT186103.1
VL13	1,384	*Serratia marcescens* strain SeEW01	100	MK961214.1
		Serratia marcescens strain S308	100	KP718760.1
VL41	1,364	*Serratia marcescens* strain TV	100	GU213910.1
		Serratia marcescens strain L1	100	MT434357.1
		Serratia marcescens strain NPK2 2 18	100	MN691673.1
		Serratia marcescens strain NPK2 2 14	100	MN691669.1
		Serratia marcescens strain NPK2 2 13	100	MN691668.1
		Serratia marcescens strain NPK2 2 9	100	MN691664.1
		Serratia marcescens strain NPK2 1 44	100	MN691653.1
		Serratia marcescens strain NPK2 1 43	100	MN691652.1
		Serratia marcescens strain NPK2 1 42	100	MN691651.1
		Serratia marcescens strain NPK2 1 38	100	MN691647.1
		Serratia marcescens strain AOE2019	100	MK813904.1

The isolated strain ST15 was closely related to *Aeromonas veronii* strain JX16102 (KY765538.1), which with an identity of 100%; the isolated strain ST17 was closely related to *Aeromonas* sp. Sh2 (KT186103.1) with a homology of 100%.

The isolated strain VL13 was 100% identity closely related to *Serratia marcescens* train SeEW01 (MK961214.1), and S308 (KP718760.1) respectively. Therefore, the strain VL13 identified in this study belongs to the *Serratia marcescens* and is named *Serratia marcescens* strain VL13.

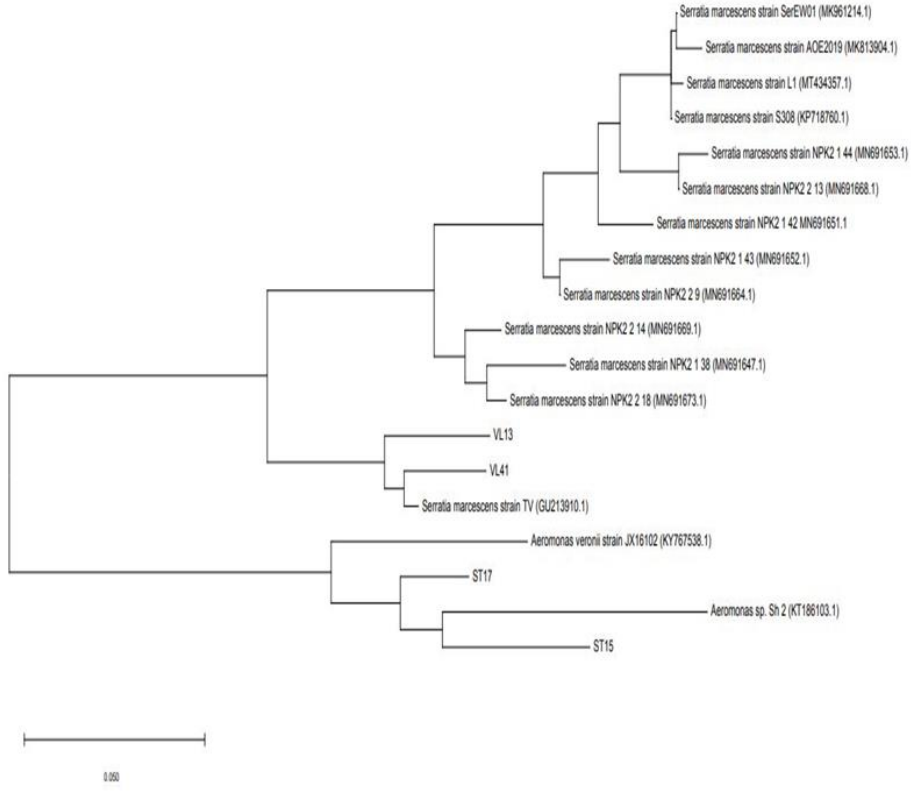


Figure 3. Phylogenetic tree showing the relative position of ST15, ST17, VL13, VL41 isolates, and other strains by the maximum-likelihood method of complete 16S rRNA sequences.

The sequence of VL41 showed 100% similarity to *Serratia marcescens* strain TV (GU213910.1), L1 (MT434357.1), NPK2 2 18 (MN691673.1), NPK2 2 14 (MN691699.1), NPK2 2 13 (MN691668.1), NPK2 2 9 (MN691664.1), NPK2 1 44 (MN691653.1), NPK2 1 43 (MN691652.1), NPK2 1 42 (MN691651.1), NPK2 1 38 (MN691647.1), AOE2019 (MK813904.1) respectively. So, the VL41 isolate was identified as Serratia *marcescens* strain VL41.

The phylogenetic position of the four isolated bacterial strains (ST15, ST17, VL13, VL41) and 15 different *Aeromonas* sp., *Serratia* sp. from GenBank (Table 2) were examined for their relevant phylogenetic relationships via the neighbor-joining method. A phylogenetic tree was constructed using MEGAX software and the genetic distance of each strain was analyzed. The results are illustrated in Figure 3.

The isolated strains VL13 and VL41 were clustered with the strain, *Serratia marcescens* (Figure 3). It can be concluded that the strains VL13, VL41 bbelongto *Serratia marcescens.* Moreover, the isolated strains ST15, and ST17 were clustered to *Aeromonas* sp. (Figure 3). In addition, from the results seen in Table 2, it can be concluded that the strain ST15 belongs to *Aeromonas veronii* and the strain ST17 belongs to *Aeromonas* sp.

Bacterial groups in cow milk have great impacts on expiry date, spoilage, and yields of cow milk. Psychrotrophic bacteria can form extracellular enzymes (proteases, lipases) which may engender putrefaction of milk. Many species belonging to psychrotrophic bacteria are potentially toxic, antibiotic resistant, and potentially harmful to humans. Psychrotrophic bacteria in raw milk such as *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Serratia*, *etc.* can produce heat-stable enzymes. These enzymes may cause putrefaction of the quality of milk products during storage. The diversity of bacterial species in cow milk are closed relations to milking-conditions, environmental conditions of cow farms. Furthermore, most psychrotrophic bacteria can form biofilms on various milk storage and processing equipment (Yuan et al., 2019).

Aeromonas could produce biofilms on food products and food contact surfaces that was a source of cross contamination in food process industries (Nagar et al., 2016). In the study of Ahmed et al. (2014), *Aeromonas* spp. was isolated from 25 random samples of raw cow's milk from the cities of Diarb Negm and Zagazig in Egypt. Identification of the bacteria showed that some species including *Aeromonas trota*, *Aeromonas hydrophila*, *Aeromonas janda* and *Aeromonas caviae* were prevailed with percentages of 40%, 25%, 25%, and 10% respectively. Among them, *Aeromonas hydrophila* produced extracellular proteolytic and lipolytic enzymes. These enzymes are widely and demonstrated to be the primary sources for destroying milk and milk products.

Some species belonging to *Aeromonas* are responsible for human infections such as *Aeromonas caviae*, *A. hydrophila* (Hoel et al., 2019). Among them, *A. veronii* is associated with gastroenteritis, respiratory inflammation, etc. (Tekedar et al. 2020); the *Aeromonas veronii* isolates carried at least four or more virulence genes (Li et al., 2020).

Serratia spp. has been associated with bovine mastitis in taking care of livestock. Serratia spp. could produce biofilms on the inner surface and cause spoilage at different points along milk processing lines (Friman et al., 2019).

Serratia marcescens often attach to internal stainless-steel surfaces of pipelines, and sections of pasteurization equipment pipelines of milk-processing plants and can cause destroy milk and milk products (Cherif-Antar et al., 2016). S. marcescens associated mastitis outbreaks in two dairy cattle farms in Finland. So, herd health control is necessary to be able to remove the contaminant sources from S. marcescens in cow milk (Friman et al., 2019).

From the above results, it is necessary to control and monitor the microbial quality in raw milk, including Aeromonas sp., Aeromonas veronii, Serratia marcescens by the improved management of herd health, management of milking, milk collection, and sources of water for cleaning of milk cows.

4. CONCLUSION

Among 14 bacterial identified isolates, four bacterial strains as classed moderate and strong biofilm producers were found. The strongest biofilm producer was *Serratia marcescens* VL41. Three isolates classed as moderate biofilm producers included *Aeromonas veronii* ST15, *Aeromonas* sp. ST17, and *Serratia marcescens* VL13. Further investigations are needed to identify the exact contamination causes and extracellular proteolytic and lipolytic enzymes of these strains into cow milk and improve the control of the received quality of cow milk quality and livestock health.

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