Antibiofilm Activity of Coelomic Fluid from *Holothuria Scabra* (Sandfish) Against Extended Spectrum Beta-Lactamase (ESBL) Producing *Escherichia Coli*

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Abstract: Urinary tract infection (UTI) is one of the most common bacterial infections; its recurrence and infectivity can be attributed to formation of biofilms. Recent studies about antimicrobial peptides from echinoderms imply that these proteins can inhibit biofilms formed by Gram positive and Gram negative bacteria. This study aims to evaluate the antibiofilm activity of coelomic fluid from *Holothuria scabra* against ESBL producing *E. coli*. The ability of the test organism to form biofilms was evaluated using the tube method wherein weak to strong biofilm formation was observed. The coelomic fluid was assessed for its ability to inhibit the biofilm formed by the ESBL-producing *E. coli* by using the biofilm inhibition assay. The coelomic fluid inhibited the biofilms and it was observed that the most effective concentration was 25% concentration. Biofilm inhibition may be contributed to the presence of low molecular weight antimicrobial peptides in the coelomic fluid ranging from 2-4 kDa as detected by SDS PAGE.

Keywords: Antibiofilm, E. coli, Sea cucumber, Holothuria scabra

INTRODUCTION

Urinary tract infection (UTI) is one of the most common bacterial infections which affects millions of people (Delcaru et al., 2016; Mireles, Walker, Caparon, & Hultgren, 2015). About 80% of UTI is due to Uropathogenic *Escherichia coli* (UPEC) (Eberly et al., 2017; Delcaru et al., 2016); uropathogens cause infections by forming biofilms on the surface of catheter materials, bladder walls and within epithelial cells of the bladder (Eberly et al., 2017; Kostakioti, Hadjifrangiskou, & Hultgren, 2013). Biofilms are bacterial communities enclosed in extracellular polymeric substances (EPS) made up of proteins, amyloid fibers,

exopolysaccharides and extracellular DNA (Serra, Mika, Richter, & Hengge, 2016; Miquel, Lagrafeuille, Souweine, & Forestier, 2016; Zago et al., 2015). After some time, the bacteria within the extracellular matrix will respond to the signals from the environment causing the dispersion of bacteria which may revert to planktonic form or continue producing biofilms elsewhere. The uropathogens can form dormant reservoirs in the urothelium which is thought to be responsible for the recurrent infections (Eberly et al., 2017).

In addition, biofilms are frequently associated with chronic infections and non-healing infections (Fleming & Rumbaugh, 2017; Sønderholm et al., 2017). These biofilms greatly slows down treatment by protecting the enclosed bacteria from both the host immune response and antimicrobial therapy (Eberly et al., 2017). Furthermore, biofilms are accountable for up to 1000-fold increase in antibiotic tolerance by limiting antimicrobial penetration, and enzymatic inactivation of drugs connected with the formation of antibiotic resistance subpopulations in the biofilm itself (Fleming & Rumbaugh, 2017; Coulter, Mclean, Rohde, & Aron, 2014).

Antibiotic resistance of biofilms has been reported to be notably higher than those of planktonic bacteria (Qi et al., 2016). The effectiveness of antibiotics has been threatened since the emergence of antibiotic-resistant bacteria; it has been a worldwide dilemma and a growing concern in the field of medicine (Golkar, Bagazra, & Pace, 2014; Wright, 2014; Gould & Bal, 2013). *E. coli, Klebsiella* species and other Gram negative bacteria are able to resist antibiotics by producing plasmid-mediated enzymes called Extended Spectrum Beta- Lactamase (ESBL). These organisms can also produce cross resistance to aminoglycosides, quinolones, cotrimoxazole and cloramphenicol (Kim, Yang & Kim, 2017; Kumar, Singh, Ali, & Chander, 2014). They are also known to show false sensitive zone of inhibition in the Kirby-Bauer disk diffusion method (Kumar et al., 2014).

ESBLs were first isolated in 1983 from Germany but are now prevalent worldwide (Shakya, Shrestha, Maharjan, Sharma, & Paudyal, 2017). In fact, out of 50,895 bacterial isolates from 22 hospital laboratories in 14 regions of the Philippines, 27% of *E. coli* were producers of ESBL (RITM & DOH, 2015). The number of ESBL producing *E. coli* may still be falsely low since most of them cannot be detected by routine susceptibility tests (Kumar, et al., 2014).

Marine invertebrates have been suddenly given increased attention in the field of medicine and microbiology due to their bioactive compounds. Marine invertebrates include echinoderms that inhabit the sea floor; phylum echinodermata includes sea stars (asteroids), sea urchin (echinoids), sea cucumbers (holothurians), brittle stars (ophiuroids) and sea lilies (crinoids). Since most of them are exposed to various infectious organisms, they have developed defense strategies to survive in contaminated environments. They provide their own protection and homeostasis through natural immune response wherein among the humoral components, the primary defense molecules are the antimicrobial peptides (AMPs) (Schillaci et al., 2013). These antimicrobial peptides are amphipathic and small cationic proteins composed of less than 100 amino acids (Chiaramonte & Russo, 2015) that can be found in the coelomic fluid together with the coelomocytes, and some minerals (Jiang et al., 2017). AMPs are able to interact with the hydrophobic lipid bilayer of microbial membranes due to their hydrophobicity. AMPs also have the ability to attach to negatively-charged bacterial membranes since they are positively charged particles. However, the mechanism on how AMPs attach to Gram-positive and Gram-negative cell walls is still not yet understood. The most widely accepted mechanism of AMPs is their permeability and formation of pores within the cytoplasmic membranes (Chiaramonte & Russo, 2015).

Different studies showed that sea cucumbers contain metabolites that act as chemical defense to avoid potential predators (Datta, Nath, Talapatra, & Swarnakar, 2015). Sea cucumbers have many reported bioactive compounds with antibacterial, antiviral, antioxidant, cytotoxicity, antitumor, and anticancer activities (Khademvatan, Eskandari, Saki, & Foroutan-Rad, 2016). They are echinoderms from the class holothuroidea with soft and worm-like bodies. In Oriental countries, like the Philippines, Malaysia, Japan, Korea, and China with extensive fisheries, sea cucumbers are used as food and in folk remedies for treating disorders. One of the most commonly cultured tropical sea cucumber species is *Holothuria scabra* or sandfish which is classified under the family Holothuriidae (Kodama, Sumbing, Lebata-Ramos, & Watanabe, 2015).

H. scabra lives in the shallow water on soft sediments in the Pacific region. It has a grayish-black upper side with dark wrinkles; and the underside is paler in comparison. It is covered in calcareous spicules in the form of buttons and tablets (Hamel et al., 2013).

Figure 1. *Holothuria scabra*

To date, there have been no previous researches conducted about the potential of using the coelomic fluid from *H. scabra* in inhibiting biofilms formed by ESBL-producing *E. coli.* This study aims to investigate the antibiofilm potential of coelomic fluid from *H. scabra*, determine the concentration that inhibited the growth of the ESBL producing *E. coli*, and detect the presence of low-molecular weight antimicrobial peptides in the coelomic fluid that may be responsible for the antibiofilm effect of the coelomic fluid against the test organism. This may provide a natural and low-cost source of antibiofilm agent against ESBL producing *E. coli* that has a lesser risk for antimicrobial resistance.

MATERIALS AND METHODS

Collection and Identification

Ten live and fresh sea cucumbers, *H. scabra* were collected in the gulf of Brgy. San Miguel, Bauan, Batangas and was authenticated at the University of the Philippines-Los Banos Museum of Natural History.

Preparation of Coelomic Fluid

The coelomic fluid (CF) was collected by cutting the anterior-dorsal part of the animals with a scalpel and draining the fluid into a plastic cup containing an isosmotic anticoagulant solution (0.5 M NaCl, 20 mM Tris–HCl, 30 mM EDTA; pH 7.4) (ISO-EDTA) (Schillaci et al., 2013). The coelomic fluid was centrifuged at 4000 × g at 4°C for 20 minutes. The liquid extract from the coelomic fluid was then frozen until analysis (Sellem, Brahmi, Mnasser, Rafrafi, & Bouhaouala-Zahar, 2017).

Test Organism

The bacterial culture in this study includes ESBL producing *Escherichia coli* which was obtained from Batangas Medical Center and recovered onto nutrient agar.

Biofilm Formation using Tube method

A loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 72 hours. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water and the tubes were dried in an inverted position. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times (Hassan et al., 2011).

Biofilm inhibition assay

Biofilm inhibition was carried out in 96 wall plates adopting modified method of biofilm inhibition spectrophotometric assay. First, 100µl of cell suspension of ESBL producing *E. coli* was added into 96 well titer plate and different concentrations of coelomic fluid as 25%, 50%, 75% and 100% were added and incubated at 37º C for 3 days. The negative control used in this method was 100 uL of distilled water. After the incubation, the liquid suspension was removed and 100 µL of 1% w/v aqueous solution of crystal violet was added. Following staining at room temperature for 30 minutes, the dye was removed and the wells were washed thoroughly. Then, 95% ethanol was added and incubated for 15 minutes. The ethanol solution was read spectrophotometrically at 570nm using HumaReader HS (Human Diagnostics™). Inhibition mediated reduction of biofilm formation was calculated by the following formula: (Namasivayam & Roy, 2014).

$$\% \text{ inhibition} = \frac{\text{OD control} - \text{OD treatment} \times 100}{\text{ODcontrol}}$$

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a gel electrophoresis chamber using a 8.6 x 6.7 cm Any kD ™ Mini-PROTEAN ® TGX™ Precast Protein Gels. The buffer consisted of 25 mM Tris, 192 mM glycine, and 0.1% SDS. After migration, separating gels were

stained with Coomassie brilliant blue G-250, and were washed using deionized water (Kwabena, Greg, Moorhead, & Raymond, 2003).

Statistical analysis

Percent of biofilm inhibition was statistically analyzed using Analysis of Variance (ANOVA) with post hoc test using Tukey and Dunnett method to determine which concentration of the coelomic fluid is ideal for biofilm inhibition and to compare the negative control with the concentrations of the coelomic fluid. Differences were considered significant at $p<0.05$. Each experiment was performed thrice and in triplicate (Schillaci et al., 2013).

RESULTS AND DISCUSSION

I. Coelomic Fluid

The ten *H. scabra* collected from Brgy. San Miguel, Bauan, Batangas yielded 300 mL of coelomic fluid as shown in Figure 2. The coelomic fluid extracted looked like seawater, was colorless, has a slightly thick consistency and has a fishy odor. This is similar to the description of Chia and Xing (1996), wherein they stated that there is a virtual similarity between the coelomic fluid and seawater but is less alkaline and has slightly more saline. The coelomic fluid also comprised majority of the *H. scabra's* whole body weight.

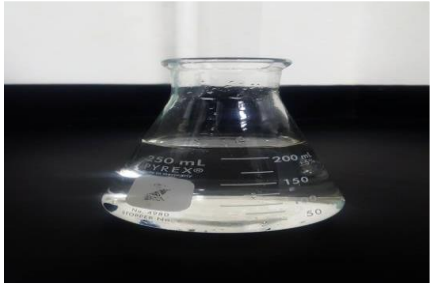

Figure 2. Coelomic fluid extracted from *H. Scabra*

II. Biofilm Formation using Tube method

Table 1.Detection of Biofilm Formation using Tube method

	Negative Control	Tube 1	Tube 2	Tube 3
Trial 1	0	3 +	2+	1+
Trial 2	0	3+	1+	2+
Trial 3	0	1+	3+	1+

Legend: 0 = None; 1+ = weak; 2+ = Moderate; 3+ = Strong

Table 1 and Figure 3 show the results of the biofilm formation test using tube method. Positive biofilm formation gave a visible film line seen at the wall and bottom of the tube and the equivalent of the biofilm was scored as 1-weak, 2-moderate, and 3-high/strong (Hassan, et al., 2011). Strong biofilm formation was observed in tube 1 of both trials 1 and 2 and tube 2 of trial 3 since a thick visible film lined the wall of the tube. On the other hand, tube 2 of trial 1 and tube 3 of trial 2 showed moderate biofilm formation. Weak biofilm formation was observed in tube 3 of both trial 1 and 3 and in tube 1 of trial 3. Therefore, the tube method showed biofilm formation at all trials. It basically shows that the test organism has the capability to form biofilms on the tubes. The results obtained can be correlated with the results of the study of Yilmaz and Guvensen (2016) wherein the ESBL producing *E. coli* isolated from the cage birds developed a biofilm at different levels as showed by the tube method.

II. Biofilm inhibition assay

Table 2.Biofilm Inhibition Assay Results

Concentration of Coelomic Fluid	% Biofilm Inhibition
Negative Control	0
25%	93.88
50%	28.75
75%	21.19
100%	14.53

Table 2 shows the results of the biofilm inhibition assay. Based on the results, the negative control had 0% biofilm inhibition which indicates that it did not inhibit the biofilm. Meanwhile, the 25% concentration of coelomic fluid exhibited the greatest biofilm inhibition after 3 trials at 93.88%. The 50%, 75%, and 100% concentrations had lesser biofilm inhibition at 28.75%, 21.19%, and 14.53%, respectively. This means that increasing the concentration of coelomic fluid led to decreased effectiveness as the percentage of biofilm inhibition declined. This conforms to the study of Yu et al. (2011), stating that the antimicrobial activity of some AMPs decline under high-salt conditions since coelomic fluid is found to be more saline than seawater.

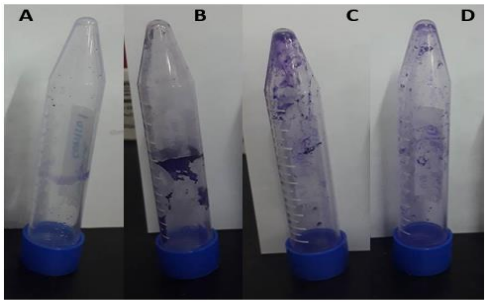

Figure 3. Results of Biofilm Formation Test Using Tube method (A. Negative control B. 3+ biofilm formation C. 2+ biofilm formation D. 1+ biofilm formation)

Table 3. Comparison on the Concentrations and the Negative Control

Concentration of Coelomic Fluid	p-value	Interpretation
25%	0.003	Significant
50%	0.646	Not Significant
75%	0.840	Not Significant
100%	0.950	Not Significant

Legend: F-value = 5.383; p-value = 0000; Significant at p-value < 0.05; Dunnet test

Table 3 compares the effectiveness of the coelomic fluid concentrations with the negative control. The table reveals that there is a significant difference between the 25% concentration of coelomic fluid and the negative control as it reached the p-value of 0.003 which is < 0.05. This implies that there was a major difference seen when using the 25% concentration when compared to the negative control. On the other hand, using higher concentrations of coelomic fluid at 50%, 75%, and 100% generated results that were similar to the negative control in effectiveness as evidenced by the p-values of 0.646, 0.840, and 0.950, respectively. In relation to this, Tam, Lu, and Yang (2002), pointed out that increasing the ionic strength in high-salt conditions weakens the activity of AMPs and its interaction with electrostatic charges. This is based on the mechanism of action of AMPs via interaction with microbial membranes which are influenced by the AMPs negative-charged phospholipids; the phospholipids are said to be electrostatic. Also, according to Yu et al. (2011), AMPs decline in effectiveness under physiological and high-salt conditions.

Table 4.Multiple Comparison of Different Concentration Used

Concentration of Coelomic Fluid		p-value	Interpretation
Negative	25%	0.007	Significant
	50%	0.817	Not Significant
	75%	0.934	Not Significant
	100%	0.983	Not Significant
25%	Negative	0.007	Significant
	50%	0.115	Not Significant
	75%	0.0000	Highly Significant
	100%	0.028	Significant
50%	Negative	0.817	Not Significant
	25%	0.115	Not Significant
	75%	0.310	Not Significant
	100%	0.981	Not Significant
75%	Negative	0.934	Not Significant
	25%	0.000	Highly Significant
	50%	0.310	Highly Significant
	75%	0.647	Not Significant
100%	Negative	0.983	Not Significant
	25%	0.028	Significant
	50%	0.981	Not Significant
	75%	0.647	Not Significant

Legend: F-value = 5.383; p-value = 0000; Significant at p-value < 0.05

Table 4 shows the multiple comparison of different concentrations used in biofilm inhibition assay. The data presents the different concentrations of coelomic fluid and their corresponding p-values. When the 25% concentration was compared with the negative control, the resulting p-value was 0.007 which shows a significant difference. This means that the 25% concentration can inhibit the biofilm. On the other hand, when the negative control was compared with 50%, 75% and 100% concentrations, the p-values of each concentration were greater than 0.005 which means that there was no significant difference. This implies that the negative control, 50%, 75%, and 100% concentration have no antibiofilm effect. This supports the finding that increasing the concentration of coelomic fluid is actually detrimental in biofilm inhibition instead of being helpful. It also implies that 25% is much better than the other concentrations overall. This contradicts the belief that increasing concentration yields better results. The loss of antibiofilm activity may be

attributed to the increasing salt levels of the higher concentrations; at higher salt levels, antimicrobial activity is diminished (Kandasamy & Larson, 2006). In addition, antimicrobial peptides are highly sensitive to salts since higher levels of salts correlate well with decreased antimicrobial activity (Chu et al., 2013).

In association to this, the coelomic fluid of the *H. scabra* has been proven to contain bioactive compounds which suppressed the growth of the biofilms. Since echinoderms have been living in an environment challenged by several microorganisms, they have developed an efficient defense mechanism (Schillaci et al., 2013). The echinoderm humoral immune response produces proteins stored within the cells with hemolytic and hemagglutinating properties. These include antimicrobial peptides released from the coelomocytes (Schillaci, Cusimano, Russo, & Arizza, 2014).

Beauregard et al. (2001) first discovered antimicrobial peptides (AMPs) in echinoderms when a ~ 6 kDa peptide in the coelomic fluid of the sea cucumber, *Cucumaria frondosa* was noted to be active against Gram-positive and Gram-negative bacteria (Schillaci et al., 2014). In general, AMPs are <10 kDa as they are only composed of up to 100 amino acids. They vary in the amino acid sequence and structural arrangement and generally are cationic with a net charge of +2 to +9 due to excess acid with the hydrophobic part opposite to the hydrophilic part. These properties permit the peptides to be soluble in water while being able to concurrently react with the hydrophobic layer of the bacterial membrane (Schillaci et al., 2014).

IV. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

In this study, SDS-PAGE revealed the presence of 2 kDa, 2.155 kDa, 2.467 kDa, 4.292 kDa and 4.517 kDa protein in the coelomic fluid extracted from *H. scabra*. The presence of these low molecular weight proteins in the coelomic fluid of *H. scabra* may have been the reason behind the biofilm inhibition. Although only few researches were conducted to study the mechanisms of action of the AMPs against biofilms, it shows that AMPs have properties that aid them to act on different stages of biofilm formation. Some AMPs prevent the adhesion of bacterial cells to the surface or to other cells; other AMPs destroy the cells before becoming a part of the biofilm structure. There are also other AMPs that cause detachment of cells, and impede regulatory signals that are responsible for motility, modulation of the immune

system, and interfere with matrix synthesis (Batoni, Maisetta, & Esin, 2016).

Furthermore, characteristics of a novel antibiotic class are possessed by AMPs which includes broad spectrum activity, low incidence of bacterial resistance and the ability to form pores in the bacterial cytoplasmic membrane. They demonstrate steadiness at different pH and temperature ranges and low virulence against eukaryotic cells which may provide a wide therapeutic window. There has been a report that AMPs can inhibit and disrupt even well-established biofilms at low concentrations. They show synergy with antibiotics by disturbing the biofilm matrix and promoting the diffusion of bacterial cells, and neutralize endotoxins. Additionally, the ability of bacteria to resist AMPs are rare and may be attributed to their affinity to negatively charged lipid bilayer of bacterial membranes (Chung & Khanum, 2017).

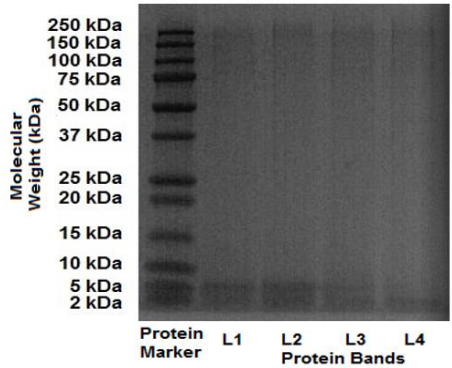

Figure 4. SDS PAGE result of the coelomic fluid from *H. scabra* (L1. 4.517 kDa & 2kDa L2. 4.517 kDa and 2.467 kDa L3. 4.292 kDa and 2kDa L4. 2.155 kDa)

CONCLUSION

The coelomic fluid obtained from *H. scabra* displayed antibiofilm activity against the ESBL- producing *E. coli* as shown by the results of the biofilm inhibition assay. This activity may be attributed to the presence of antimicrobial peptides ranging from 2-4 kDa in the coelomic fluid. The most effective concentration that inhibited the biofilm was 25% concentration of coelomic fluid while increasing the concentration of the fluid did not significantly enhance the inhibition. The researchers believe that the coelomic fluid of *H. scabra* can provide an interesting source of potential antibiofilm agents against ESBL *E. coli*.

RECOMMENDATION

Further studies are recommended in relation with the antimicrobial properties of coelomic fluid from *H. scabra.* Extraction and isolation of the antimicrobial peptides from the coelomic fluid of the sea cucumber can also be performed for the identification of the antimicrobial peptide and to utilize it directly against biofilms.

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