Original Research Article

Role of *Pseudomonas aeruginosa* Biofilm in the Development of Antibiotic Resistance in *Escherichia coli*

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Submission Date: 25-04-2018; Revision Date: 25-07-2018; Accepted Date: 30-08-2018

ABSTRACT

The study aimed to assess the role of *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilm in the development of antimicrobial resistance of *Escherichia coli* (*E. coli*). *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 25922) were cultured on cetrimide agar and eosin methylene blue (EMB) agar. The two microorganisms were mixed to prepare three bacterial suspensions using tryptic soy broth (TSB) in increasing concentrations, such as: 1:1, 2:3 and 1.5:3.5. Bacterial suspensions were transferred into MBEC™ Assay kits which was subjected to agitation and incubated at 37°C for 24 hrs and 48 hrs. Formed biofilms on peg lids were obtained for Gram staining and cultured using EMB agar to evaluate the biofilm matrix and presence of bacteria. Polymerase chain reaction (PCR) was conducted to determine significant changes in *E. coli* genes that matched the *Tn1696 aacC1*, *Tn10 tetRA* and *Tn903 aph* resistance genes of *E. coli* which codes for gentamicin, tetracycline and kanamycin, respectively. Results showed strong bands to *Tn1696 aacC1* gene for gentamicin resistance at the highest concentration. No bands were visualized for tetracycline and kanamycin resistance. The results therefore establish that coexistence of both organisms in a biofilm leads to the development of the antimicrobial resistance of *E. coli* to gentamicin.

Key words: Antimicrobial resistance, Biofilms, *E. coli*, *P. aeruginosa.*

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INTRODUCTION

Antibiotic resistant bacteria (ARB) impose a big threat to global health and challenge the effectiveness of antimicrobial agents to treat common infectious diseases.[1,2] Infections from ARB are encountered constantly in the healthcare setting resulting to patients' longer recovery time and higher hospital bills.[3] In response to this, health care professionals opt to prescribe medications that are more expensive and can be toxic to the patient.[4] Among the mechanisms that cause ARBs is through biofilms.[5,6] Biofilm is a slimy film formed due to bacterial adherence to surfaces in aqueous environment. It becomes more complex and teemed as it aids the influx of other bacteria to thrive hence, creating a dense community.[6] Burmolle *et al.*[6] further explains that interactions of multi-bacterial strains trigger co-aggregation of cells, conjugation and protection from eradication when the biofilm is exposed to antimicrobial compounds.

Nosocomial infection nowadays is one of the worldwide public health problems and a major serious effect of biofilm formation associated to high morbidity and mortality rate in the developing countries.[7] A recent study conducted reveals that both bacteria and fungi are responsible for acquiring hospital-associated diseases but, the former is accountable for the 90% of infection.[8] Microbial species that have a major role in this are *P. aeruginosa, S. aureus* and *E. coli*.[8] Apart from their ability to colonize the body of immunocompromised patients, their capability to attach to medical devices like catheter, incubators and sinks is the leading factor to

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DOI : 10.5530/ajbls.2018.7.2

produce biofilm.[5,8] This further creates a ground for developing urinary tract infections (UTI), pneumonia, septicemia and several related diseases.

Peleg and Hooper[9] elaborated that gram-negative bacteria have been indicated to be responsible for more than 30% of nosocomial infections where the highest percentage of 47% is implicated to ventilator-associated pneumonia and urinary tract infections (UTIs) with 45% falls on the second. The said study expounded that in line with UTIs, most of the cases are associated with urethral catheterization wherein the risk of bacteriuria increases by 5-10% per day considering *E. coli* as the most etiologic gram-negative organism followed by *P. aeruginosa*, *Klebsiella* spp., *Enterobacter* spp. and *Acinetobacter baumannii*.[7,10,11] Another threat imposed by biofilm to healthcare setting is its competence to grow to medical devices thus, increasing the cases of nosocomial infection.[5,10,12] Hospitals are places where disinfection and sterilizations are the crucial means of killing bacteria on several medical equipment.[12] However, such protocols are now considered ineffective because biofilm can withstand or tolerate the effects of disinfectants hence, promoting the transmission of antibiotic- resistance genes (ARG) among the biofilm members leading to the production of multidrug resistance bacteria.[4,12]

The increased number of nosocomial infection cases has been implicated to biofilms found in hospital premises.[11,13] In fact, more than 60% of hospital-acquired infections worldwide are attributed to biofilm-forming bacteria on medical devices. Catheters are the most common utilized medical device.[10] The study emphasized that the said device becomes a suitable environment to support biofilm growth after exposure to body fluids following subsequent infections.[10,13] Furthermore, *Staphylococcus aureus, S. epidermidis, Escherichia coli* and *Pseudomonas aeruginosa* are the microorganisms most often associated in catheter-related infections.[11]

P. aeruginosa and *E. coli* can form biofilm in hospital settings, however, many strains of the latter are found to be weak biofilm formers.[6,7] It has shown that *P. aeruginosa*, on the other hand is known to be an excellent biofilm-forming microbe and can greatly affect the growth of other organisms existing in the biofilm.[10,14-17] Therefore to observe a clinical set up of biofilm formation, *P. aeruginosa* ATCC 27853 strain was used to form biofilm leading to the development of the antibacterial resistance of *E. coli* ATCC 25922 strain.

This study provides a clear understanding on the emergence of ARB through discovering the mechanism occurring between the coexisting microorganisms in biofilm.[4] Since *P. aeruginosa* and *E. coli* are both pathogenic bacteria, any genetic modification intensifying their antimicrobial resistance caused by biofilm interaction calls for immediate attention and subsequent response not only from the healthcare community but also from the public.[4,13] Moreover, this study will broaden and enhance the extent of bacterial identification and drug susceptibility testing in laboratory settings. Latest studies about the antimicrobial resistance of *E. coli* will aid the healthcare providers in prescribing appropriate medications to the patients affected by its associated infections.[18,19]

MATERIALS AND METHODS

Study Design

The study used pre-test and posttest experimental quantitative approach in meeting its goals. Laboratory setups were used to determine the effect of *P. aeruginosa* biofilms on the antimicrobial resistance of *E. coli*. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used in the study to ensure that no resistance genes were present, as *E. coli* ATCC 25922 is used as a control strain in antimicrobial susceptibility testing.[20,21] Cetrimide agar (CA) (HiMedia Laboratories, Mumbai, India) and Eosin Methylene Blue agar (EMBA) (HiMedia Laboratories, Mumbai, India) were used to subculture *P. aeruginosa* and *E. coli*, respectively.[22,23] For the biofilm formation, MBEC™ Assay (Innovotech Inc., Alberta, Canada) was utilized.[24] The primers *Tn1696 aacC1, Tn903 aph* and *Tn 10 tetRA* which encode for gentamicin, kanamycin and tetracycline resistance genes of *E. coli* were used for the polymerase chain reaction (PCR).[25-28]

Preparation of Bacterial Inoculum

Four to five colonies each of *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were obtained using an inoculating loop and the growth were transferred into 5 mL of tryptic soy broth and were mixed to ensure homogenous turbidity.[24] In an adequate light, both the bacterial suspension tube and the 0.5 McFarland turbidity standard were visually compared against a paper with a background and contrasting black lines.[29] The bacterial suspension tubes were then incubated at 37°C for 15 min before streaking into their selective agar plates.

Using an inoculating loop, *P. aeruginosa* ATCC 27853 *and E. coli* ATCC 25922 were streaked on cetrimide and eosin methylene blue (EMB) agar.[22-23] The plates were then incubated at 37°C for 18-24 hrs. *E. coli* ATCC 25922 plated on EMB agar was the control strain used for the polymerase chain reaction and gene changes was determined.[21]

Biofilm Formation

The Calgary procedure[24,30-32] of biofilm formation with some modifications was followed for the growth of the biofilm. Mainly, four to five colonies of *P. aeruginosa* ATCC 27853 strain from cetrimide agar were obtained and were placed on 5mL of tryptic soy broth. Another four to five colonies of *E. coli* ATCC 25922 strain were then placed on a separate 5 mL Tryptic Soy Broth (TSB) (HiMedia Laboratories, Mumbai, India) tube. Both bacterial suspensions were compared to 0.5 McFarland standard and were adjusted by either adding tryptic soy broth and or bacterial colony until the turbidity matched with that of the McFarland standards.[31] The two bacterial suspensions were incubated at 37°C for 15 min afterwards. Mixtures of *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 suspensions were made using three concentrations. First mixture contained 50% of *P. aeruginosa* and 50% of *E. coli*, the second mixture has 40% *P. aeruginosa* and 60% *E. coli* and the third mixture contained 30% *P. aeruginosa* and 70% *E. coli* making concentrations of 1:1, 2:3 and 1.5:3.5, respectively. The ratio of the concentrations corresponds to the milliliters of bacterial suspensions that were pipetted on a sterile empty tube to make a 5mL of mixed bacterial suspension. After the mixtures were prepared, the tubes were incubated at 37°C for 15 min.

Under a biosafety cabinet, 150µL of bacterial suspension containing 50% *P. aeruginosa* and 50% *E. coli* concentration (1:1 ratio) were pipetted on each of the 32 wells (columns 1-4, rows A-H) of the MBEC™ Assay. The next 32 wells (columns 5-8, rows A-H) were dispensed with 150µL each of bacterial suspension containing the 40% *P. aeruginosa* and 60% *E. coli* concentration (2:3 ratio). The last 32 wells (columns 9-12, rows A-H) were dispensed with the same volume of bacterial suspension each having the 30% *P. aeruginosa* and 70% *E. coli* concentration (1.5:3.5 ratio).

The MBEC™ Assay cover was then placed fitting the hydroxyapatite coated peg lids into the wells containing the bacterial suspension.[24] The assay was then agitated at 225rpm before incubating at 37°C for 24 hrs.

Isolation of Microorganisms from Bacterial Biofilms and Verification of Biofilm Formation through Microscopy

Using different sterile swabs, *E. coli* ATCC 25922 isolates were scraped and inoculated on 5mL of tryptic soy broth each. The bacterial suspensions were compared with 0.5 McFarland standard until equal turbidity were attained and were then incubated at 37°C for 15 min. The suspensions were then plated at separate EMB agars afterwards and were then incubated at 37°C for 24 hrs.

Slide spreads of each concentration from the two incubation periods were also prepared and gram stained and viewed under the microscope to check for the presence of a biofilm matrix that would verify that biofilm formation occurred after incubation.

Molecular Analysis of *E. coli* Resistance

To find out if antibiotic genes emerged after the coexistence of *E. coli* ATCC 25922 with *P. aeruginosa* ATCC 27853, *Tn1696 aac1*, *Tn10 tetRA* and *Tn903 aph* genes were detected through polymerase chain reaction.[26,28]

RESULTS

Under the light microscope using the oil immersion objective with a total magnification of 1000x, all concentrations in the two-time intervals exhibited the presence of matrix. The presence of the polysaccharide matrix confirmed that biofilm formation occurred between the two microorganisms (Figures 1 and 2).

Within 24 hrs, all quadrants in 1:1 concentration exhibited many growth. In 2:3 concentration, it was observed that quadrants I, II and III showed many growth while quadrant IV displayed only a few growth. Like in the first concentration, 1.5:3.5 concentrations showed many growth on the entire plate. Within 48 hrs, the plate with a concentration of 1:1 exhibited many growth in quadrants I and II while quadrants III and IV exhibited

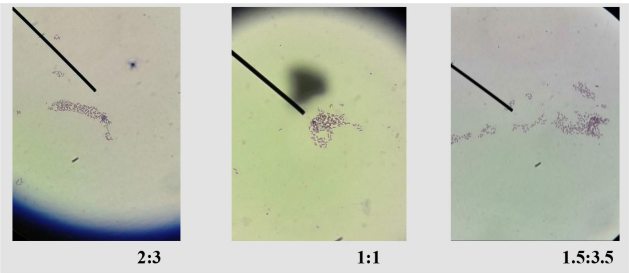

Figure 1: Biofilm matrix under light microscopy after 24-hr incubation (1000x magnification).

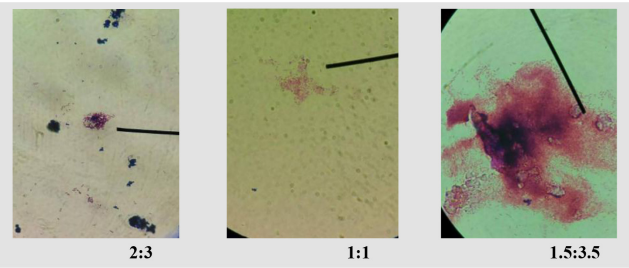

Figure 2: Biofilm matrix under light microscopy after 48-hr incubation (1000x magnification).

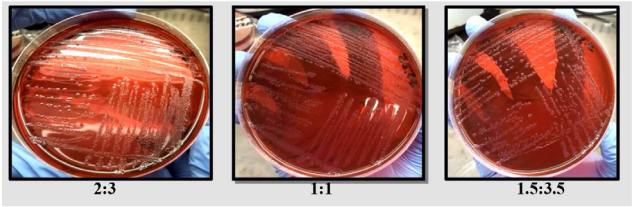

Figure 3: Growth *P. aeruginosa* and *E. coli* biofilm in EMB agar after 24 hrs of incubation.

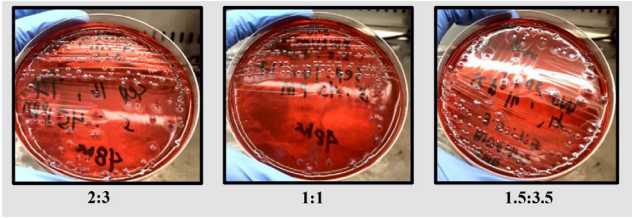

Figure 4: Growth *P. aeruginosa* and *E. coli* biofilm in EMB agar after 48 hrs of incubation.

no growth. In concentrations 2:3 and 1.5:3.5, all quadrants displayed only few growths.

Concentrations of 1:1 and 2:3 demonstrated a pinpoint colony while 1.5:3.5 appeared larger compared to other concentrations after 24 hrs. After extending the incubation for 48 hrs, 1.5:3.5 concentrations displayed a large colony at quadrant III compared to smaller colonies formed at concentrations 1.5:2.5 and 2:3. This proves that after the extended incubation, the biofilm formed on the peg lids have more time to recruit more bacteria in the biofilm establishing denser and larger colony on EMB agar.[32]

Within 24 hrs, concentrations 1:1 and 2:3 both exhibited pinpoint colonies in quadrants I, II and III while showed small colonies in quadrant IV. Concentration 1.5:3.5 produced small colonies in quadrants I, II and III whereas medium colonies in quadrant IV. Within 48 hrs, concentration 1:1 had small colonies in quadrants I and II while showed no growth in quadrants III and IV. In 2:3 concentration, small colonies where seen. Lastly, concentration 1.5:3.5 displayed small colonies in quadrants I and II, large colonies in quadrant III and medium colonies in the fourth quadrant (Figures 3 and 4).

Samples were obtained from the peg lids after verifying the formation of biofilm and were streaked on EMB agar to check for their growth. Many colonies formed from quadrants I, II and III after incubation of 24 hrs with slight differences on quadrant IV at concentration 1:1. After 48 hrs of incubation, many colonies still formed at concentration 1.5:2.5. However at higher concentration, few colonies have been established. This proves that as the incubation prolongs, it gives more

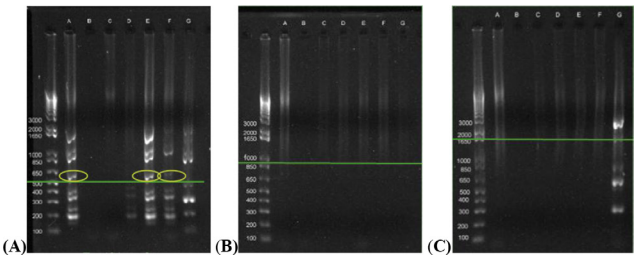

Figure 5: Agarose gel electrophoresis profiles of PCR products amplified using primers (A) (Gentamicin, 616 bp) Tn1696 aacC1 F and Tn1696 aacC1 R., (B) (Kanamycin, 944 bp) Tn903 aph F and Tn903 aph R. and (C) (Tetracycline, 1996 bp) Tn10 tetRA F and Tn10 tetRA R.

time for the bacteria to aggregate and combine with one another constructing one large colony.[32] Colonies also gave slightly pink appearance owing to its capability to ferment glucose due to the presence of *E. coli* and these appeared to be mucoid due to the extracellular polysaccharide matrix produced during the biofilm formation.

The encircled bands on Figure 5A represent the developed resistance in *E. coli* to Gentamicin at 616 bp, which is represented by the straight line.

DISCUSSION

Tn1696 aacC1 gene denotes for *E. coli* gentamicin resistance in 616 bp. Samples A, E and F had positive bands in the said size however, no visible bands appeared of Samples B, C and D. Given that Sample A and E had the same concentration, the latter showed more intense bands after a 48 hr incubation time. Samples B and F had equal concentration of *E. coli* and *P. aeruginosa* however, visible bands were displayed on the latter after incubating for 48 hrs.

On the other hand, samples C and D did not have visible bands despite have dissimilar incubation time. These samples do not confer negative result. Their concentrations have affected the visibility of the bands on the gel. In running a PCR, the concentration of the gel, the primer and the sample will affect the PCR product. The agarose gel concentration is too strong compared to that of the sample's that result to the dilution of the bands. In addition to the strength of the gel, the presence of the dimers on the gel added further to the weakening of the intensity of the bands.

Furthermore, bands being displayed were not as consistent and firm as they should be because the primers where designed to use in genetic recombination procedure. In this procedure a vector is being inserted in the *E. coli* genes in order to generate an exact DNA sequence signifying a resistance to gentamicin. Nevertheless, in this study, the change in the DNA sequence took place

after the co-existence of *E. coli* and *P. aeruginosa* in a biofilm but did not guarantee an absolute DNA sequence alteration just like in the primer.

Tn903 aph gene codes for kanamycin resistance in 944 bp.[28] Samples A to G showed no bands in the gel, thus conferred negative result. However, some factors can affect the visibility of bands. Compared to the antibiotic concentration, the primer used corresponds to 20 ug/mL concentration, which is higher to gentamicin with 10 ug/mL. In running a PCR, the concentration of the gel, the primer and the sample can affect the PCR product. In Figure 5B, the agarose gel concentration showed little compatibility to the primer's concentration that resulted to the dilution of the bands. In addition to the strength of the gel, the presence of the dimers on the gel added further to the weakening of the intensity of the bands.

Tn10 tetRA gene denotes for *E. coli* tetracycline resistance in 1996 bp.[28] The primer used paralleled the antibiotic concentration of 15ug/mL, which is higher than gentamicin but lower than kanamycin. Sample A displayed a weak intensity of the band as compared to the ladder. Sample G on the other hand had the most visible bands. Since no bands were shown at 1996 bp, it is a negative result (Figure 3C). However, hazy bands imply dilution of the bands due to the variation in the concentration of the primer and the gel. The incompatibility of the concentrations resulted to the dilution of the bands as well the presence of the dimers on the gel. The study conducted by Culotti and Packman[14] and Taylor, Cerqueira, Oliveira, Nicolau and Azevedo[33] both confirmed that *E. coli* and *P. aeruginosa* can be coexisted in a biofilm. According to Culotti,[14] *P. aeruginosa* can greatly increase the ability of *E. coli* to persist and grow in aquatic environments. In the study of Taylor *et al*.[33] although the two organisms can be grown together, there is a significant decrease in the growth of *E. coli* due to its less adaptability in a dual species biofilm and have only enhanced ability to form a mono-species biofilm when presented as a single colonizer. This phenomenon explains what may have happened in this study.

It is considerable that it was the biofilm matrix which affected the ability of *E. coli* to ferment the EMB agar and not produce the characteristic green metallic sheen. Despite not having the characteristic growth, the distinct mucoid colonies in the agar in addition with the microscopic results and observations support the formulated hypothesis of a biofilm formation between the two organisms. Among the three primers, *E. coli* showed bands with the greatest intensity to gentamicin resistance gene particularly in 616 bp which is the intended target size of the primer. On a study of *E. coli* isolates by Paraoan, Rivera and Vital,[34] it was found out that some of their isolated *E. coli* have gentamicin resistance though not as high as the other antibiotics used such as cephalothin. This establishes that *E. coli* can really develop resistance against the antimicrobial agent, gentamicin. The primer encoding for tetracycline resistance gene displayed slightly vivid bands, not specifically on 1996 bp and lastly, no bands were seen on 944 bp which is the target size for kanamycin resistance gene. PCR results revealed evident bands in the sample bearing the highest concentration of *E. coli* with the ratio of 1.5:3.5. In addition, the incubation time affected the growth of *E. coli* in the biofilm as seen in the samples B and F which consist equal concentration of *E. coli* and *P. aeruginosa*, 1:1. After the 24 hr incubation, hazy bands were seen on Sample B however, after a 48 hr incubation time, Sample F produced vivid bands on its column. Therefore, the coexistence of *E. coli* and *P. aeruginosa* in a biofilm leads to the development of the antimicrobial resistance of *E. coli*.

CONCLUSION

In conclusion, the coexistence of *P. aeruginosa and E. coli* in a biofilm lead to the development of the antimicrobial resistance of *E. coli* to gentamicin which served as proof of the emergence of antibiotic resistant bacteria (ARB).[4] Infections from ARB are encountered constantly in the healthcare setting and patients take longer time to recover due to the inefficiency of first generation drugs which can be as a result of biofilms.[4,7] In addition, longer incubation time and higher concentration of *E. coli* in a biofilm contributes to higher probability of the bacteria to develop resistance to gentamicin. Development of resistance to gentamicin is quite alarming as antibiotic resistant *E. coli* to the drug has been seen in hospital setting.[35] Therefore, proper management of possible medical instruments (most especially in catheters) and areas where both *P. aeruginosa* and *E. coli* may coexist is needed as to avoid a possible infection with an antibiotic resistant *E. coli* which may result to a nosocomial infection more difficult to treat.

ACKNOWLEDGEMENT

The researchers would like to thank the Department of Medical Technology in Far Eastern University Manila for the guidance, support and providing laboratory services, the Philippine Genome Center in University of the Philippines-Diliman for the technical assistance on the molecular analysis and Innovotech Inc. for providing the MBEC™ Assay kits.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS USED.

ARB: Antibiotic resistant bacteria; **CA:** Cetrimide agar; **EMBA:** Eosin Methylene Blue agar; **MBEC:** Minimum biofilm eradication concentration; **TSB:** Tryptic soy broth.

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Cite this article: Raquid JP, Prieto JC, Arciaga DM, Corpuz RH, Llorente SM, Mabbayad JM, Paraoan CE. Role of *Pseudomonas aeruginosa* Biofilm in the Development of Antibiotic Resistance in *Escherichia coli*. Asian J Biol Life Sci. 2018;7(2):40-6.