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ANTIBIOTIC SUSCEPTIBILITY STUDY ON GRAM POSITIVE BACTERIA BIOFILMS

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Abstract

Bacteria in a biofilm have greater capability in overcoming antibiotic attack with biofilm bacteria being 10 to 1,000-fold more antibiotic resistant as compared to their planktonic counterparts. As a consequence, infections involving biofilms present particular problems to manage effectively. Current studies of biofilms in the presence of antibiotics have been performed under full aerobic conditions and after 24-48 hours of biofilm formation. Neither of these two parameters reflects the in-vivo state of the biofilms. Biofilm formation inside the body require longer periods and might be weeks before the clinical symptoms indicate the presence of infection, furthermore there are few places in the human body where a biofilm is exposed to the levels of oxygen present in atmosphere. These local environment differences are likely to influence antibiotic activity. Earlier studies have confirmed that planktonic cells show different responses to antibiotics under different environments. This research aims to study the process of biofilm formation by Staphylococcus and Enterococcus bacteria and their response to antibiotics (Vancomycin, Teicoplanin, Linezolid, Dalbavancin, Telavancin, Daptomycin and Tigecycline) using different concentrations under different environmental conditions, thus reflecting the in-vivo conditions. Microtiter plate based system was used to grow biofilms; biofilm biomass and activity of biofilm bacteria were estimated using the Crystal violet and MTT assay respectively. Minimum inhibitory concentrations (MIC) of planktonic cells were analyzed experimentally. Biofilms were tested with different concentrations of antibiotics that were calculated with reference to their planktonic counterpart at each environment. Experimental results revealed significant effect of antibiotics to the planktonic cells but no significant variations were observed for their biofilms. However some significant variances were found in the antibiotic activity at different experimental environment; further improvements in the methodology must be done in order to validate these results.

Keyword: : Biofilm, Antibiotics, Staphylococcus, Enterococcus

1.INTRODUCTION

Biofilm is the community of microorganisms irreversibly attached to a surface, producing extracellular polymeric substances[1].There is now widespread recognition of the contribution of biofilms to human infection. Cases of biofilm infection include the common examples of device-related infections such as those associated with catheters, artificial joints and heart valves, intra uterine devices and stents. Persistent biofilms are regarded as the main cause of chronic infections in urinary, gastrointestinal and respiratory tract without any involvement of external material[2]. These biofilms exhibited increased resistance to the antibacterial drugs which have made them very difficult to treat. Almost every study done for the susceptibility testing of bacterial biofilms have been carried out in the fully aerobic conditions. This fully aerobic condition does not resemble to the environment inside human body. Human body has different level of oxygen concentrations at different parts. This study manly focused to study the susceptibility of biofilms mimicking the environment inside human body to eight different antibiotics namely; Vancomycin, Teicoplanin, Linezolid, Dalbavancin, Telavancin, Daptomycin and Tigecycline. These antibiotics were chosen because of their common use in the clinical application to treat *Staphylococci* and *Enterococci*. However Dalbavancin and Televancin were replaced by Amikacin and Gentamycin in the

experiments as the suppliers were unable to provide them timely. In order to mimic the environmental conditions inside human body the experiments were carried out in aerobic, anaerobic and micro aerophillic environments. Furthermore the time for bacterial cell to form active biofilm was estimated via crystal violet and MTT assay and antibiotic test was performed for the same time period biofilms. This study was expected to predict the in vivo success or failure of particular antibiotic therapies, and also the efficacy of antibiotic therapy at particular environment.

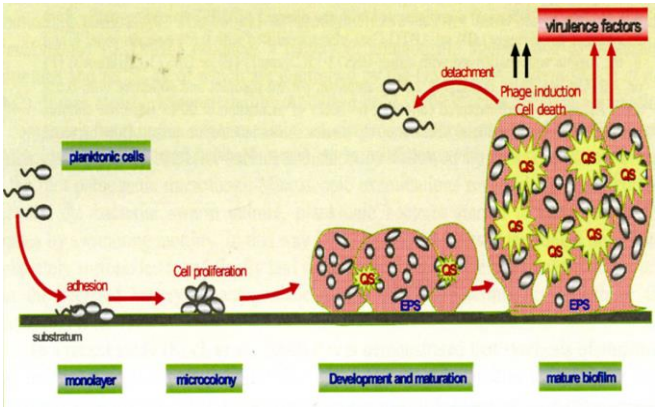

Figure 1 Sequential Developmental stages of biofilm (Hoiby et al. 2010)

2.MATERIALS AND METHODS

This chapter provides the details on the materials used to carry out this study along with the detailed description of the methodology followed during this study.

2.1.Bacterial Isolates

Clinical isolates were randomly collected from hospital inpatients. These isolates were cultivated and identified prior to this study and were obtained from Charing-cross Hospital, London. Species used in this study were:

*Staphylococcus aureus***:** 12 isolates

Methicillin Resistant *S.aureus:* 12 isolates

Vancomycin Sensitive *Enterococcus***:** 12 isolates

Vancomycin Sensitive *Enterococcus***:** 12 isolates

2.2.AntibacterialDrugs

Eight drugs (Amikacin, Daptomycin, Rifampicin, Tigecycline, Teicoplanin, Daptomycin, Gentamicin, Vancomycin) were used to test the antibiotic susceptibility of planktonic and biofilm cells.

2.3.Growth Media

It was used for isolation of bacterial samples. It was prepared according to manufacturer's instruction by adding 7ml of horse blood in 100ml of autoclaved molten Nutrient Agar medium (Oxoid, UK).

Brain Heart Infusion (BHI) broth Medium:

This was prepared according to manufacturer's instructions (oxoid, uk) and was used for biofilm formation.

2.4.Microtiter Plates

96-well flat-bottom cell culture microtiter plates (Nunc, Denmark) were used for in-vitro biofilm formation. 96-well flat-bottom non cell culture microtiter plates (Nunc, Denmark) were used for Crystal Violet assay and for determining the MIC of planktonic cells.

2.5.Gas Packs

Anaerobic gas packs and Campylobacter packs (oxoid, UK) were used to generate anaerobic and microaerophillic environments respectively in air-tight bags for the growth of bacteria.

2.6.Solutions

33% Glacial acetic acid, Isopropanol, Methanol (fsicher Scientific, UK)

2.7.Stains

1% Crystal Violet, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Fromazan; (Sigma-Aldrich, USA)

2.8.Centrifuge

Centaur 2; Henderson Biomedical Ltd., UK

2.9.Microtiter plate reader

Multiskan Spectrum Microtiter plate reader; Thermo Electron Corporation

2.10.In vitro formation of biofilms:

Clinical isolates were grown on the blood agar medium and were incubated at 37°C in aerobic environment for 18 hours. After the incubation time the plates were checked for presence of contamination. Isolated colonies from the agar plates were inoculated into 10ml BHI broth and incubated overnight at 37°C in aerobic environment. At the end of incubation period each culture was centrifuged for 8 minutes at 3000rpm and the supernatant discarded. The pallet was then re-suspended into 10ml of sterile distilled water and vortex making sure the all cells were separated and washed thoroughly. This mixture was again centrifuged and re-suspended as described above. The re-suspended mixture was used to create inocula of 10ml bacterial suspension with density corresponding to MacFarland 4 (0.66± 0.02 at 520nm). 100µl of this standardized solution was then inoculated into each of the wells of 96 well cell culture microtiter plates and placed on a rotator at 80rpm for two and half hours.

After this two and half hour time, the inoculation solution from each well was removed using a multichannel pipette making sure not disturbing the bottom of wells. 100µl of sterile distilled water was added to each well running the water down the wall sides and aspirated out without disturbing the bottom. Visual inspection now showed the very thin monolayer of bacterial cells on the bottom of the wells. Gently 100µl of BHI broth with 1% glucose was added to each well and incubated in respective environments.

2.11.Changing the growth Medium at 5 and 10 days.

After five days incubation the growth medium for the 10 and 15 days biofilm cultures were changed. The growth medium was gently aspirated out with help of multichannel pipette and each well washed with sterile distilled water taking care that the biofilms were not disturbed. 100µl of BHI broth was added to each well.

2.12.Crystal Violet Assay:

Crystal Violet staining was performed for the determination of total biomass. Culture medium was removed from the wells of microtiter plates after their respective incubation period, and then washed with 100µl of sterile distilled water ensuring all residual medium was removed without disturbing the biofilms. Distilled sterile water was added to the wells very carefully by pressing the tip of pipettes half way up the side of wells and then gently dispersing the pipette trigger. The plates were then gently tapped to remove excess water and allowed to dry at 37°C for 10 minutes. Visual inspection after this revealed no remaining fluids and the wells were completely dry.

100µl of methanol was then gently added to each well as water was added in washing process and was left for 10 minutes to penetrate the biofilms. Methanol was then aspirated out and the plates were inverted and tapped to remove excess methanol. The plates were then kept at 37°C for 10 minutes to dry completely. 80µl of 1% Crystal Violet was then added to each well and then allowed to penetrate for 10 minutes, after which it was aspirated out. The wells were then washed in triplicate or four times with sterile distilled water as required until visual inspection revealed any crystal violet in aspirated water. The plates were then inverted, tapped and then left for 10 minutes at 37°C to dry.

The bound crystal violet to the biofilms was then leeched out by adding 100µl of 33% acetic acid to each well and left for 10 minutes to penetrate with gentle tapping every 2 minutes. At exactly 10 minutes 80µl of resultant acetic acid and crystal violet solution was aspirated out ensuring not to touch the bottom and was transferred to new non tissue culter 96-well microtiter plate. The plate was then read on plate reader at 570nm.

2.13.MTT Assay

After the respective incubation periods, 10µl of freshly prepared MTT solution was added to each well and the plates were incubated at 37°C for two hours in their respective environment. After incubation time the plates were rotated in a rotator for 20 minutes at 180 rpm after which the growth medium was gently aspirated out without disturbing the bottom. 100µl of isopropanol (100%) was then added to each well to solubilize the formazan for 30 minutes with gentle tapping from sides in each 5 minutes. The plates were then read at microtiter plate reader at wavelength of 570nm.

2.14.Determination of MIC of Planktonic Cells.

The antibacterial drugs used are listed in the materials sections. 1ml of sample which was used for the

formation of biofilm was added to 10ml of sterile distilled water which was the inoculum used for the MIC determination of planktonic cells. 200µl of antibacterial drug were placed in the first well of each row at required concentrations which was calculated as per the EUCAST MIC break points datasheet. 100µl double strength BHI solution was then added to the remaining wells of the plate. Double dilutions were then done by aspirating the 100µl of from the first wells using multi-channel pipette and transferring it to the adjacent well. The solutions were mixed well using the pipettes before transferring to the adjacent well. This process was done 11th column. 100µl from the 11th column was discarded and 12th column was left as control. 100µl of inoculated sterile distilled water was added to the wells, diluting the double strength media to working level with first well with highest concentration. The 12th well now contains no antibacterial drug which was the positive control. The plates were then incubated at 37°C for 24 hours in respective environments. After the incubation periods the plates were read in plate reader at wavelength of 570nm.

2.15.Antibiotic test on Biofilms:

After formation of 5 days old biofilms they were treated with three different concentration of each drug which is summarized here as high, moderate and low. The concentration of each drug in each category is listed below. All Concentrations are in µg/ml.

Biofilms were formed as described previously. After the incubation of five days growth medium was gently aspirated out and the biofilms was washed with sterile distilled water. Antibacterial drugs were diluted to required concentrations in the medium, and 100µl of it was added to each well. The plates were then incubated in the respective environment for 24 hours. After the incubation time MTT assay was performed to determine the antibiotic activity on biofilms.

Table 1: Concentration of Antibiotics used

Antibiotic	**High Conc.**	**Moderate Conc.**	**Low Conc.**
Amikacin	100	300	1000
Daptomycin	20	200	2000
Gentamicin	100	300	1000
Linezolid	40	200	800
Rifampincin	100	300	1000
Teicoplanin	100	300	1000
Tigecyline	10	100	1000
Vancomycin	100	300	1000

3.RESULTS

This chapter summarizes the outcomes from the data gathering phase. The data were collected and analyzed as to represent it in convenient way.

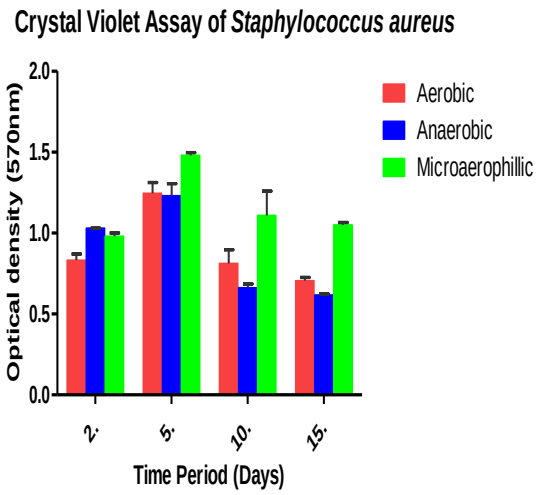

Figure1: *Crystal Violet assay of Staphylococcus aureus in aerobic, anaerobic and microaerophillic environments at different time interval.*

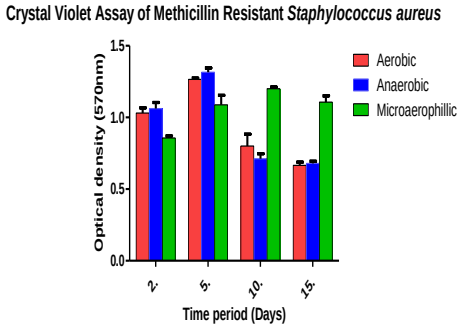

Figure 2: Crystal Violet assay of Methicillin Resistant Staphylococcus aureus in aerobic, anaerobic and microaerophillic environments at different time interval.

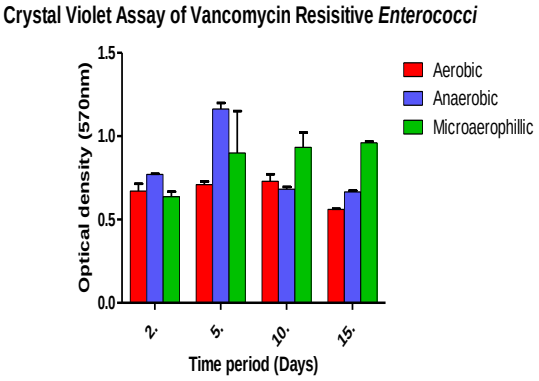

*Figure 3: Crystal Violet assay of Vancomycin Sensitive Enterococcus in aerobic, anaerobic and*microaerophillic environments at different time interval.

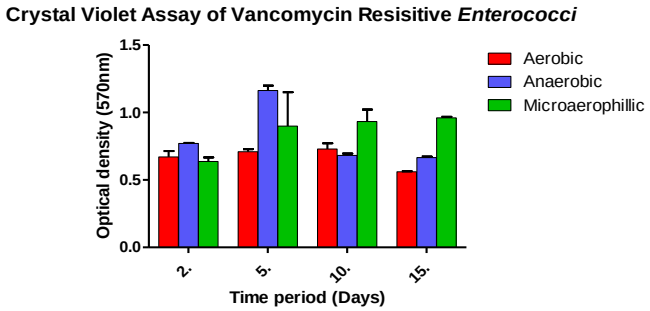

Figure 4: Crystal Violet assay of Vancomycin Resistive Enterococcus in aerobic, anaerobic and microaerophillic environments at different time interval.

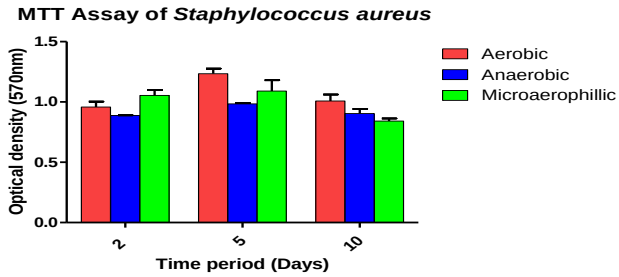

Figure 5: MTT assay of Staphylococcus aureus in aerobic, anaerobic and microaerophillic environments at different time interval.

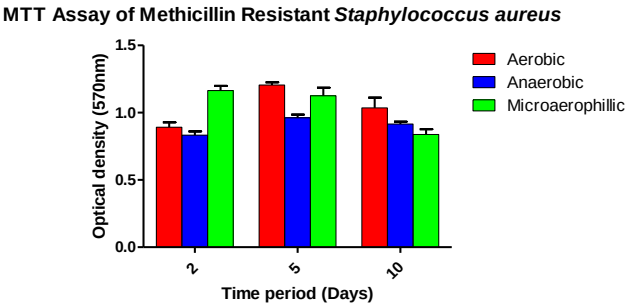

Figure 6: MTT assay of Methicillin Resistant Staphylococcus aureus in aerobic, anaerobic and microaerophillic environments at different time interval

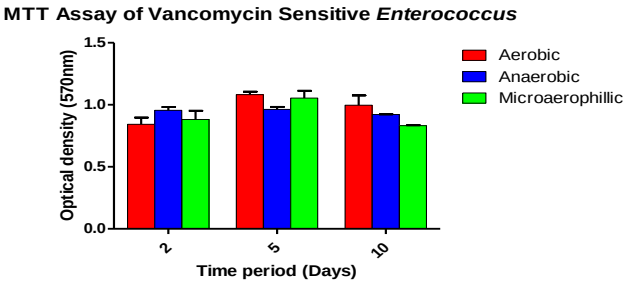

Figure 7: MTT assay of Vancomycin Sensitive Enterococci in aerobic, anaerobic and microaerophillic environments at differet time interval.

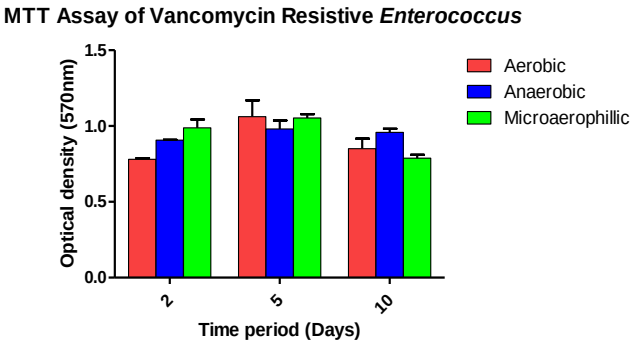

Figure 8: MTT assay of Vancomycin Resistive Enterococci in aerobic, anaerobic and microaerophillic environments at different time interval

3.1.Staphylococcus

Biofilm formation of S. aureus cells varied according to the incubation time period (Figure 1). S. aureus biofilms had maximum biomass at 5 days time period which were significantly different (P < 0.05) from other time periods. Biomass in different environments was seen to have little differences which were found to be statistically insignificant (P> 0.05). MRSA biofilms also showed variations in biomass when compared at different time periods and environments (Figure 3). Experimental observations showed that biomass of MRSA biofilms was increased from 2days to 5 days' time period but this increase was not found to be statistically significant (P > 0.05).

Viability of S. aureus (Figure5) and MRSA (Figure 6) biofilms cells were compared at different time periods and environments. Experimental variations were seen in terms of different time periods and environments. Biofilms of both S. aureus and MRSA cells were observed to be more active at 5 days' time, however the statistical analysis revealed no significant difference (P > 0.05). Similarly the variations found in activity of biofilm cells at different environment were also found to be statistically insignificant (P> 0.05) for both S. aureus and MRSA.

3.2.Enterococcus

Biofilm formation of VSE and VRE were compared at different time periods and environments as shown in figure 3 and figure 4 respectively. The experimental observations showed little variations in biofilm with respect to incubation time and environment. Statistical analysis confirmed that there were no significant variations in formation of biofilm with respect to time and environment (P >0.05).

Metabolic activity of VSE (Figure7) and VRE (Figure 8) when compared at different time periods and environment gave similar results as their crystal violet assay. The variations in activity were observed to be very low experimentally. This was confirmed by statistical analysis which revealed no significant difference (P > 0.05)

3.3.MIC of planktonic cells

MIC of planktonic cells were determinedby the colorimetric measurement at 570nm which revealed that optical density greater than 0.32 showed turbidity. The results are listed below in the table for each species.

Table 2: MIC of planktonic S. aureus

Antibiotics Anti biotics	Aerobic	Microaero phili c	Anaerobic
Amikacin	8	11	14
Daptomycin	2	5	5
Gentamicin	3	5	7
Linezolid	4		
Rifampincin	<0.125	<0.125	<0.125
Teicoplanin	4	4	4
Tigecyline	1	1	1
Vancomycin	4	4	4

Table 3: MIC of planktonic MRSA

Antibiotics	Aerobic	Microaero phili c	An- aerobic
Amikacin	18	21	24
Daptomycin	2	4	4
Gentamicin	5	7	8
Linezolid	4		
Rifampincin	<0.125	<0.125	<0.125
Teicoplanin	4	4	4
Tigecyline	0.5	1	1
Vancomycin	4	4	4

Table 4: MIC of planktonic VSE

Antibiotics	Aerobic	Microaero phili c	An- aerobi c
Amikacin	64	64	64
Daptomycin	4	4	4
Gentamicin	>128	>128	>128
Linezolid	5		
Rifampincin	222	230	241
Teicoplanin	4	2	2
Tigecyline	1	1	1
Vancomycin	6	4	4

Table 5: MIC of planktonic VRE

Antibiotics	Aerobic	Microaer ophilic	An- aerobic
Amikacin	264		
Daptomycin			
Gentamicin	>128	>128	>128
Linezolid	4		
Rifampincin	>512	>512	>512

Teicoplanin	4	2	2
Tigecyline	2	1	1
Vancomycin	6	4	4

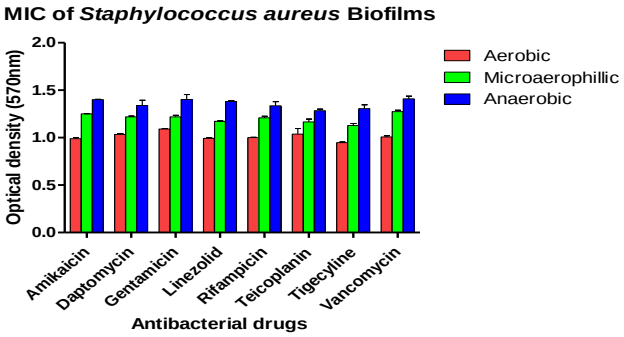

Figure 9: Antibiotic activity of s. aureus biofilms in aerobic anaerobic andmicroaerophillic environment. (Drug concentration: Highest)

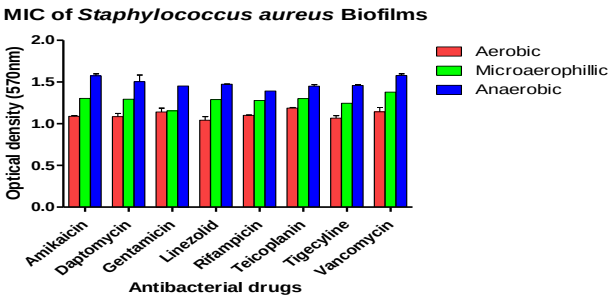

Figure 10:Antibiotic activity of s. aureus biofilms in aerobic anaerobic and microaerophillic environment. (Drug concentration: Moderate)

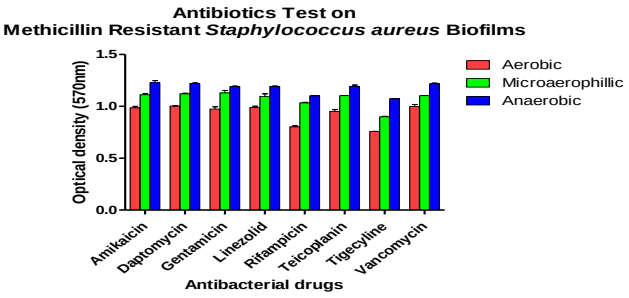

Figure 11:Antibiotic activity of MRSA biofilms in aerobic anaerobic and microaerophillic environment. (Drug concentration: High)

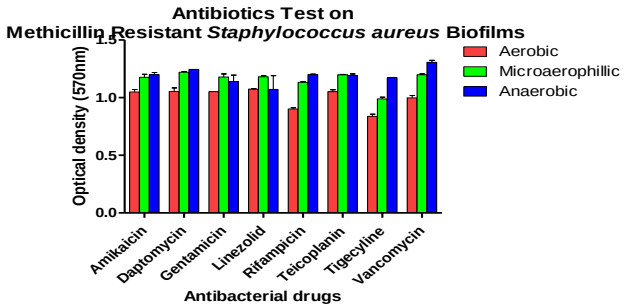

Figure 12: Antibiotic activity of MRSA biofilms in aerobic anaerobic and microaerophillic environment. (Drug concentration: Moderate)

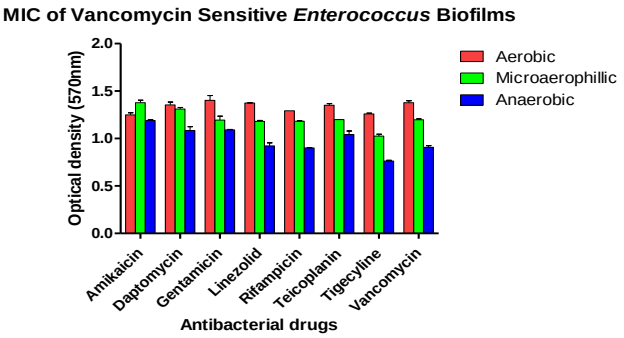

Figure 13: Antibiotic activity of Vancomycin Sensitive biofilms in aerobic, anaerobic and microaerophillic environment. (Drug concentration: High)

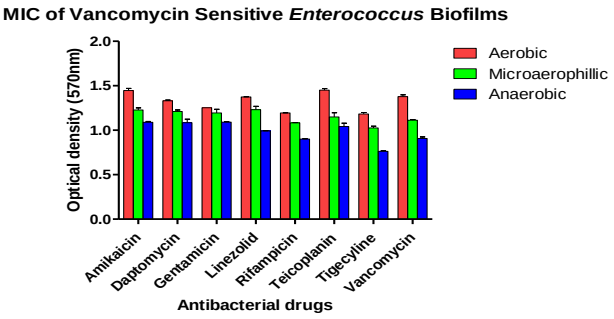

Figure 14: Antibiotic activity of Vancomycin Sensitive biofilms in aerobic anaerobic and microaerophillic environment. (Drug concentration: High)

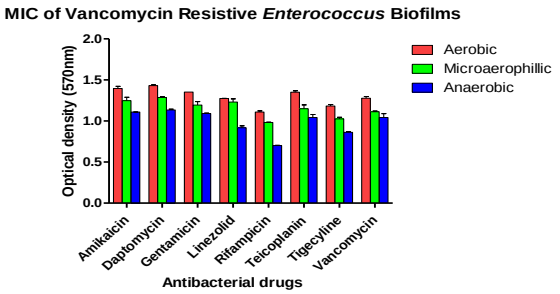

Figure 15: Antibiotic activity of Vancomycin Sensitive biofilms in aerobic anaerobic and microaerophillic environment. (Drug concentration: High)

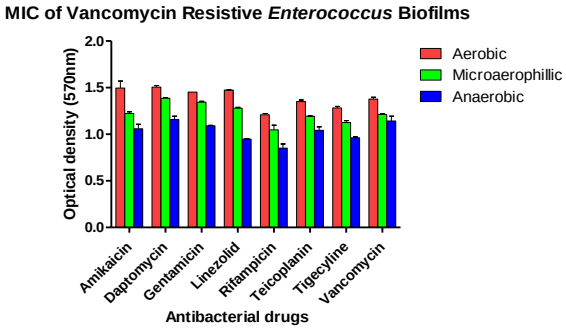

Figure 16: Antibiotic activity of Vancomycin Sensitive biofilms in aerobic anaerobic and microaerophillic environment. (Drug concentration: High)

3.4.Staphylococcus

When treated with the antibiotic drugs for period of 24 hours, the response of S. aureus (Figure 9 & Figure 10) and MRSA (Figure11 and Figure 12) biofilms was observed to be similar. Both S. aureus and MRSA showed resistivity to all the antibiotics used at every concentration. When compared at different environmental conditions both S. aureus and MRSA showed highest drug activity at aerobic conditions which was confirmed by statistical analysis ($P < 0.05$).

3.5.Enterococcus

After the exposure of biofilms to the antibiotics over the time period of 24 hours , the viability test of VSE biofilms (Figure 13 & Figure 14) and VRE biofilms (Figure 15 and 16) showed gradual decrease in viability form aerobic environment to anaerobic environment. The difference were found to be statistically significant ($P < 0.05$) which confirmed the maximum drug activity at anaerobic environment.

4.DISCUSSION

This chapter analyses the results for relevant conclusions by comparing them with previous studies. The analytical comparison of findings with the related previous studies will be helpful to validate the results and create scope for further studies.

4.1.Comparison of biofilm formation at different time period in different environment (Crystal Violet Assay)

The results (see section) showed that biofilm formation by S. aureus and MRSA in all environments increased from two days' time period to 5 days' time. Previous studies have also supported the fact that there is increase biofilm biomass with increase in incubation time[3], [4]. However the biomass of 10 days biofilm of S. Aureus and MRSA decreased from that of 5 days and remained to be similar at 15 days' time period. Though various environmental and physiochemical factor have been identified (Heydorn et al, 2000), nutrient availability can be accounted to clarify the decrease of biomass in 10 and 15 days incubation period[5]. Former studies complying similar microtiter plate based methodology was done with change of medium in every 24 hours for incubation of more than 10 days[4]. The change of medium provides biofilm cells with fresh nourishment, as well removes the accumulated toxic materials resulting to better proliferation of cells[4], [6]. In this particular study the growth medium in the microtiter plates were only changed at every 5 days interval. This possibly resulted in nutrition lack for the longer period incubation biofilms which resulted in the constant crystal violet assay result for 10 and 15 days biofilms.

The formation of biofilms as per their biomass was also analyzed in aerobic, microaerophilic and anaerobic environment, and was found to have no significant difference. Study on availability of oxygen and their effect on staphylococcus biofilms have revealed that MRSA cells forms significantly high biofilm under microaerophilic environment as compared to aerobic and anaerobic environment[7]. Study from Carmton et al , concluded that significant amount of S. Aureus biofilm formation only in aerobic environment ;however the result from Stepanovic et al disagreed to that of Carmton et al, as this study concluded there was no

significant difference in staphylococcal biofilm formation under aerobic and anaerobic condition[7], [8].

Also the biomass of VSE and VRE were analyzed using crystal violet assay (fig. 3 and 4). The result showed no significant changes in the biomass neither as per their incubation period nor with respect to environment. From this study it seems that *Enterococci* biofilms achieve its maturity within time period of 2 days. Maturity of enterococcal biofilms within the time period of 24 hours is supported by the study performed by Liu and his coworkers (2010).

The fact that there was no significant difference in formation of biofilm in different environments by all species may also be due to the method used to generate these environments. Air tight plastic bags were used with their respective gas packs for generation of anaerobic and microaerophilic environments. However, the indicators were used to ensure the desired environments was achieved but could not be constantly monitored if it was achieved through out study period or not. Other possibility might be that the plastic bags were not efficiently air tight to maintain the desired environments.

Crystal violet assay procedure involves the steps of several washing. Probability of damaging the biofilms while performing the washing step was negligible. However maximum precaution was taken to ensure that there was no experimental error. Various studies have also suggested that crystal violet assay is not reproducible as compared to other assays (Broschat et al, 2005, peters et al, 2008), but did not seem to have effect in this study as it was performed under duplicates which did not showed significant difference.

4.2.Comparison of Viability biofilm cells at different time intervals in different environment (MTT Assay)

The results showed that all the bacterial biofilms used in the study showed no significant increase or decrease in their viability (see results Section). This result was quiet contradicting because increased biomass is the consequence of high metabolic activity, which was not seen in case of S. Aureus, in this study. Bopp and Lettieri, concluded that MTT assay required many procedural steps that enhanced the chances of variability in result[9]. Similar doubting results were obtained as the MTT assay reading for particular strain performed in duplicates showed variability beside this, different experimental errors might have occurred. In this study the viability results at different environment have no significant difference; however various studies have revealed different conclusions. A study conducted by Hoiby et al, suggest that higher the oxygen concentration higher is the protein synthesis which results into high metabolic activity[10].

The result of the current study concludes that there is no effect of oxygen concentration in viability of biofilm cells. However this can only be validated after the conformation of no experimental errors in different procedural steps like washing, and generation of different environmental conditions occurred.

4.3.MIC Determination of planktonic cells

MIC obtained from the experiment (see results section) was found to be near to the MIC break points set by EUCAST. Due to restriction of time MIC for Linezolid was only determined for two isolates of each species in aerobic environment. However study from Gunderson et. Al (2002) have concluded that there was no apparent difference between killing of MRSA and VRE in aerobic and anaerobic environment.

It was found by the experiment that S.aureus and MRSA cells were highly susceptible to rifampicin (<0.125µg/ml) in all environmental conditions. Similarly all planktonic species were susceptible to tigecyline. Gentamicin and Amikacin were seemed to be comparatively resistant to VSE and VRE, but these findings were supported by EUCAST MIC breakpoints. Overall result of MIC breakpoint determination from this current study is supported from the EUCAST. However, only two isolates from each species was were used for MIC determination. For more independent and efficient data for this study MIC for all the isolates must be determined at least in triplicates which was not permitted by time constraints.

4.4.Antibiotic test on biofilms

4.4.1.Staphylococcus

The analysis of result revealed that both S.aureus and MRSA were resistant to all the antibiotics used. Among all the drugs Rifampicin and Tigecyline were found to have maximum activity. Raad and co-worker (also have similar findings with rifampicin when exposed to biofilms for time period of 24 hours[11]. Similarly study

from Rose and Poppens) agreed with the findings that Rifampicin and tigecyline had more activity as compared to Vancomycin[12]. However study from both Raad et al and Rose and Poppens suggested that, S. aureus and MRSA biofilms produced resistant to rifampicin after prolonged treatment. Rifampicin was found to have the most bactericidal effect on staphylococcus planktonic cells whereas for biofilms the activity was not significant at the concentration as high as 1000µg/ml. This supports the study carried out by Tote et al, that concluded MIC of S. aureus biofilms was more than 1000 times greater as compared to their planktonic cells[13].

While comparing the drug activity at different environmental conditions maximum activity was seen in aerobic environment. This finding supports the previous studies that biofilms are increasingly susceptible to antibiotics in active presence of oxygen[10], [14].

4.4.2.Enterococcus

VRE and VSE biofilms were also found to be resistant to all the antibiotics tested. Study from Canton and his co-workers support the findings for Vancomycin and Linezolid[15]. Whereas the results of Daptomycin against VRE and VSE biofilms contradicted with the study done by Canton and his co-workers. Various other studies also suggested that Daptomycin is efficient against VSE and VRE biofilms as it penetrated the biofilms and showed effective reduction in bacterial growth[16], [17]. While comparing the antibiotic activity at different environmental conditions high metabolic activity was seen in aerobic conditions. This can be correlated with the study carried by Eliana Drenkard which suggested that variability in oxygen concentration results in different metabolic activity of biofilm cells when treated with antibiotics[18]. Study from Jensen et alsuggested that resistant shown is due to active efflux of drug molecules through pumps present on the surface of resistant bacteria[19]. Furthermore Chopra and Roberts suggested that this efflux is an energy dependent process[20]. Maximum drug activity was seen in anaerobic environment for VRE and VSE biofilms this might be due to the low activity of efflux pumps particularly at anaerobic environment as its energy dependent. However further investigation is required to authenticate this comparative analysis.

5.CONCLUSION

The findings from the study disagreed with the hypothesis. There were no significant variations in the biomass and viability of biofilms under different environmental conditions. This might possibly be due to the errors in maintaining the desired incubation environments. Though study produced significant results for the antibacterial susceptibility of planktonic cells, antibiotics did not have considerable effect on biofilms. Biofilms exhibited resistant to the antibiotics with the concentrations as high as 1000 times to their planktonic counterparts. The facts that all antibiotics are unable to exhibit significant effect on biofilms indicate the need for multiple drug therapy. Use of drugs combination for varying period of time increased the biofilms penetration and decreased emergence of resistance towards used drugs[12]. Microtiter based model used in the study for growing the biofilms has proven to be easy and fast method. However, use of XTT assay for determination of viability over MTT would be more convenient and time saving without hindering the results efficiently.

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