

Biofilm Formation by Environmental Microbes Isolated from Hospitals in Karachi, Pakistan

Aneela Shaheen ^{a*}, Rakhshanda Baqai ^b

^a*Department of Microbiology, University of Karachi, Karachi-75270, Pakistan*

^b*Dadabhoy Institute of Higher Education, Main campus Karachi, Karachi, Pakistan*

^a*Email: aneelashaheen84@gmail.com*

^b*Email: r_baqai@yahoo.com*

Abstract

The purpose of this study was to isolate and identify the microbes from hospital environmental samples and to evaluate the potential of biofilm formation by isolated microbes. For this, 125 surface swabs of different environmental samples were taken from PNS SHIFA hospital, Karachi, Pakistan. Bacteria and fungi were isolated and identified by culture plate method. Trypticase soy broth (TBS) media was used for biofilm development by microbes in plastic tubes. Developed biofilm in tubes was visualized with crystal violet staining method and then biofilm forming potential was estimated by measuring the optical density through spectrophotometer. Total 202 microbes including 126(62.38%) bacteria and 76(37.62%) fungi were isolated and identified. Among environmental samples, hospital ward curtains and medical trays were highly contaminated with bacteria and fungi (with 26% each of total assemblage respectively). *Staphylococcus aureus* was in highest abundance followed by *Candida albicans* with 28.7% and 15.8% of total assemblage of isolation respectively. Moreover; *Staphylococcus aureus* followed by *Candida albicans* also found to have highest potential to form biofilm with 30.25% and 23.52% of total assemblage of biofilm formation respectively which clearly indicates that *Staphylococcus aureus* and *Candida albicans* may recognized as major agents of hospital acquired infection and can relate with enhanced potential of biofilm formation. Highest abundance and biofilm formation potential of bacteria and fungi in combination can also underline a direct extensive and striking interaction between prokaryotic and eukaryotic cells in biofilm.

Keywords: Biofilm; Bacteria; Fungi; Hospital acquired infection.

* Corresponding author.

1. Introduction

In natural environments, microorganisms occur mostly as surface attached cells rather than as planktonic or free floating cells [1, 2, 3, 4]. In response to stressed conditions, microbes develop survival strategy for which they tend to attach to available surfaces and form biofilms. Not all microbes attached to surfaces can form a biofilm. Establishment of biofilm from a planktonic culture through surface attachment is a continuum process. Microorganisms attached to a substrate produce extracellular polymeric substances (EPS), exhibiting an altered phenotype compared with corresponding planktonic cells, especially regarding growth, gene transcription, protein production and intercellular interaction within biofilms [5, 6, 7]. It can be formed by different type of micro-organisms, including bacteria, viruses, fungi and others can be form on almost any biological or inanimate surface [8] and due to biofilm development, they are able to survive on dry surfaces for extended periods [9].

In the hospital environment, surface like indwelling medical devices and prostheses, water lines and tubing on endoscopes and on wounds [10, 11] microorganisms colonize on all abiotic and biotic surfaces available in form of biofilms, making them as a source of infection for hospitalized individuals and create an important public health problem [12, 13, 14]. Pathogen causing nosocomial infection may also be acquired from patients (own's skin, gut, respiratory flora) as well as hospital staff (surgical or clinical staff) or contact with surrounding environmental sources [15, 16, 17]. That is why biofilm is considered to be involved in 65% of nosocomial infection [18, 19, 20].

Neither dryness of microbes on to hospital surfaces has been studied nor attachment of bacteria to that surfaces and formation of biofilms studied in detail. Vickery et al. [19], tried to study them in detailed and took the several hospital surfaces samples after cleaning and bleach disinfection by cutting the materials out of the hospital environment and they found the viable *meticillin-resistant Staphylococcus aureus (MRSA)* in biofilm on three surface samples. Some other research findings have occurred that biofilm attachment may appears to be loosened by some biocides but it is difficult to remove bacteria from biofilms through cleaning with disinfection [21]. Furthermore, surface attached bacteria have reduced susceptibility to antibiotics and may also have reduced susceptibility to biocide. Such findings are clear justification for failures of adequate biocides in hospital disinfection, contributing to failures in hospital cleaning [22].

A biofilm changes the lifestyle of microbes in such a way that it directly enhances their virulence. Such surface attached microbes have remarkable involvement in chronic bacterial infections and cannot easily be exterminated by conventional therapy as biofilm can be up to 1000 folds more resistant to antibiotic treatment than the same organism growing planktonically [1, 23, 24, 25]. Biofilm infections can be caused by a single microbial species or by a mixture of bacterial or fungal species [26]. Infectious diseases caused by biofilms are dental caries (caused by acidogenic Gram-positive cocci *Streptococcus* sp.), periodontitis (Gram-negative anaerobic oral bacteria), otitis media, or middle ear infection (non-type able *Haemophilus influenza*), chronic tonsillitis (by various species), cystic fibrosis pneumonia (*Pseudomonas aeruginosa*, *Burkholderia cepacia*), endocarditis (streptococci, staphylococci), necrotizing fasciitis (Group A streptococci), musculoskeletal infections (Gram-positive cocci), osteomyelitis (various species), biliary tract infection (enteric bacteria),

infectious kidney stones (Gram-negative rods), bacterial prostatitis (*Escherichia coli* and other Gram-negative bacteria). Organisms like *P. aeruginosa*, Staphylococci and *E. coli* are associated to such hospital acquired infections that are caused by foreign body materials, such as contamination of implants, catheters, contact lenses and prostheses [27, 28].

Among fungi, *Candida* species are known as most recognized nosocomial pathogen and associated with hospital acquired infection with mortality rate of 35%. US National Nosocomial Infections Surveillance System rank *Candida* species as the fourth most common cause of bloodstream infection, behind coagulase-negative staphylococci, *Staphylococcus aureus* and enterococci [29]. Diseases that are associated with *Candida* species biofilms: nosocomial pneumonias and urinary tract infections [30].

Various model systems have been used to characterize the overall properties of biofilms. Mostly these model systems were used to study bacterial biofilms. First and simplest model used to study biofilm was to grow the adherent population on rough surfaces e.g. small discs cut from catheters. Then growth of adherent cells were monitored quantitatively by a colourimetric assays [31]. One another method using similar principal has been used to study the formation of biofilms on denture acrylic strips [32]. Now days, 96 well microtiter plates are used for rapid processing of large numbers of samples even for antifungal agent susceptibility testing for biofilms [33]. Microorganisms were grown in trypticase soy broth (TSB) for biofilm formation [34]. The most widely used method for biofilm formation in microtiter plates is the crystal violet (CV) staining method [35], which only measured biofilm biomass at the bottom of the well. CV is a basic dye that stains both living and dead cells in the extracellular matrix of biofilms by binding to negatively charged surface molecules and polysaccharides [36]. Later, the method was modified for enhancing the efficiency and to quantify the biofilm biomass in whole well by solubilization of dye by addition of acid [37, 38].

In this regard, we aimed to study our local hospital environment for availability of pathogenic bacteria and to the best of our knowledge; no study has been carried out in Karachi hospital with reference to biofilm forming potential as survival strategy. So we also aimed to develop a low cost in-vitro method that enables quantitation of total amount of biofilm produced by microbes. For this purpose we isolate and identify the common bacterial and fungal strains contaminating the hospital environment and to determine microbes potential to form biofilms. For biofilm formation, a differential CV staining method was modified by replacing the microtiter plates with sterile cheap plastic tubes, enabling the phenotypic biofilm formation in tubes.

2. Materials and methods

2.1 Sample collection

Total 125 surface swab samples were taken from different places of PNS SHIFA hospital located in Karachi, Pakistan. All samples were taken with help of TRANSWAB (M40 Compliant, medical wire, UK) having amies medium for transportation of aerobes and anaerobes. Out of 125 total samples, 40 samples were taken from different medicine trays, 35 samples were taken from hand surface of ward boys and nurses, 30 samples were taken from the patient's bed linens and 20 samples were taken from hospital's curtains surface. All swab

samples were stored in ice box and transported to the laboratory within 2 hours.

2.2 Isolation and identification of microorganisms

Collected swab samples were subjected for culturing on bacterial cultural media: nutrient agar and fungal cultural media: Sabroud dextrose agar (SDA) for propagation of bacteria and fungi. After inoculation, nutrient agar plates were placed in inverted position and incubated at 37°C for 24 hours whereas SDA were placed upward position and incubated at room temperature for one week. After incubation, bacterial colonies were isolated and identified on the basis of bergey's Manual of Determinative Bacteriology [39]. After one week, isolated colonies of fungi from SDA plates were mounted by the use of 10% Potassium hydroxide (KOH) with lactophenol cotton blue. All of isolated bacterial strains were then preserved and stocked in nutrient broth containing 20% (vol/vol) glycerol and store at freezing temperature while fungal isolates were stored on SDA slants.

2.3 Biofilm production by tube method and quantification with Crystal Violet staining

Trypticase soy broth (TSB) media in sterilized plastic tubes were used for production of biofilm. Under aseptic conditions, 02 ml trypticase soy broth (TSB) media were inoculated with loopful of colonies from culture media plates. TSB tubes were incubated at 37° C for 24 hours. After 24 hours, 2% glucose was added to each tube of TSB and re-incubated at 37° C for next 24 hours. After incubation TSB growth medium was discarded from tubes and washed three times with Phosphate buffer saline (PBS having 7.3 pH) under aseptic condition to eliminate the unbound bacteria. To evaluate the formation of biofilm, remaining attached bacteria were fixed with 2 ml of 99% methanol and tubes were left for 15 minutes. Then tubes were emptied and left to dry. To visualize the biofilm production from microorganisms, cells attached to plastic tubes in form of biofilm were stained with 0.2 ml of 2 % crystal violet for 5 minutes. Excess stain was rinsed off by placing tubes under running tap water. Airs dried the tubes in inverted position and dye that adherent cells were solubilized with 1.5 ml of 33% glacial acetic acid. The optical density of each tube was determined at 570 nm wavelength by using spectrophotometer to quantify the biofilm formation by microbes. The blank (negative control) was determined for each tube by measuring the optical density of a tube filled with only PBS.

3. Results

Out of 125 different environmental samples from hospital, total 202 bacterial and fungal strains were isolated and identified by culture plate method according to Bergey's manual of determinative bacteriology [39] and Practical Mycology: Manual for identification of fungi [40]. Bacteria were in abundance in different environmental samples of hospital with 62.38 % of total isolation assemblage followed by fungi with 37.62 %. Among environmental samples, hospital ward curtains and medical trays were highly contaminated with bacteria and fungi (with 26 % each of total assemblage). The numbers of isolated bacteria and fungi in each set of hospital environmental samples are shown in figure 1. which clearly indicating the highest number of bacterial and fungal isolation in hospital ward curtains and medical trays set of samples.

Among isolated bacteria and fungi Staphylococcus aureus followed by Candida albicans were occurred

frequently with 28.7% and 15.8% of total assemblage of isolation as shown in figure 2.

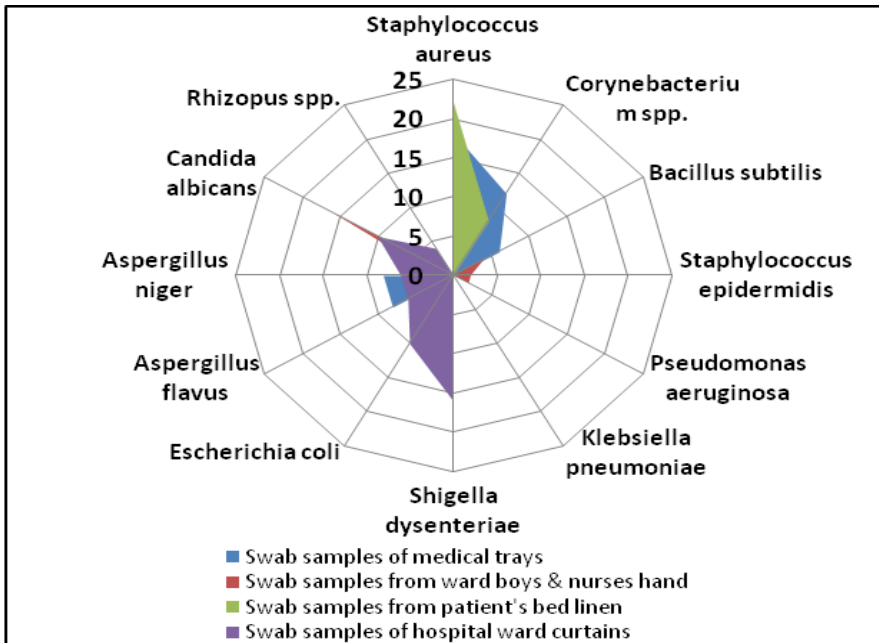


Figure 1: Comparative isolation of microbes from hospital different environmental samples

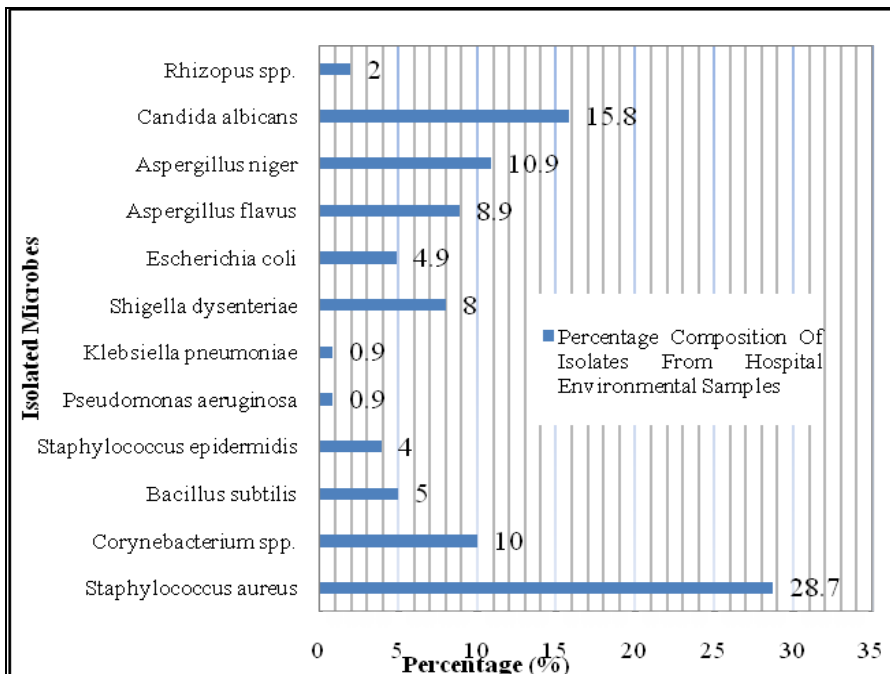


Figure 2: Total percentage composition of microbes isolates from environmental samples of hospital (%)

Isolated bacteria and fungi were then tested for their ability to form biofilm in trypticase soy broth (TSB) media as shown in figure 3 where enhanced growth is seen in some tubes and some showed less growth in TSB media. Then biofilm forming potential was visualized by crystal violet staining method and indicated the clear picture of positive potential of isolated strains to form strong and moderate, weak or no biofilm. Figure 4 shows the clear picture of microbe's potential for biofilm formation as indicated a purple color biofilm formation on bottom and wall of tubes which were formed, washed, stained with crystal violet.



Figure 3: Growth of microbes in TSB media after 24 hours of incubation, promoting to form biofilm (right) and comparative TSB media tubes after 48 hours of incubation with 2 % glucose, indicating enhanced growth and no growth of microbe in TSB tube (left)



Figure 4: Biofilm formation by microbe having positive potential for biofilm formation (right) and clear tube indicating microbe have no potential to form biofilm (Left) after both tubes stained with crystal violet.

These biofilms were then solubilized and then quantify the biofilm forming potential by measuring the optical density of crystals violet color on 570 nm wavelength to differentiate the microbes in strong, moderate, weak or no biofilm forming microbes. It should be noted here that among isolated bacteria and fungi *Staphylococcus aureus* followed by *Candida albicans* were also have strong potential to form biofilm with 30.25% and 23.52% of total assemblage of isolated microbes to form biofilm as presented in figure 5. Together these results indicate a direct interaction of such empowering potential of microbes to form biofilm with survival of microbes in extreme condition such as hospitals etc.

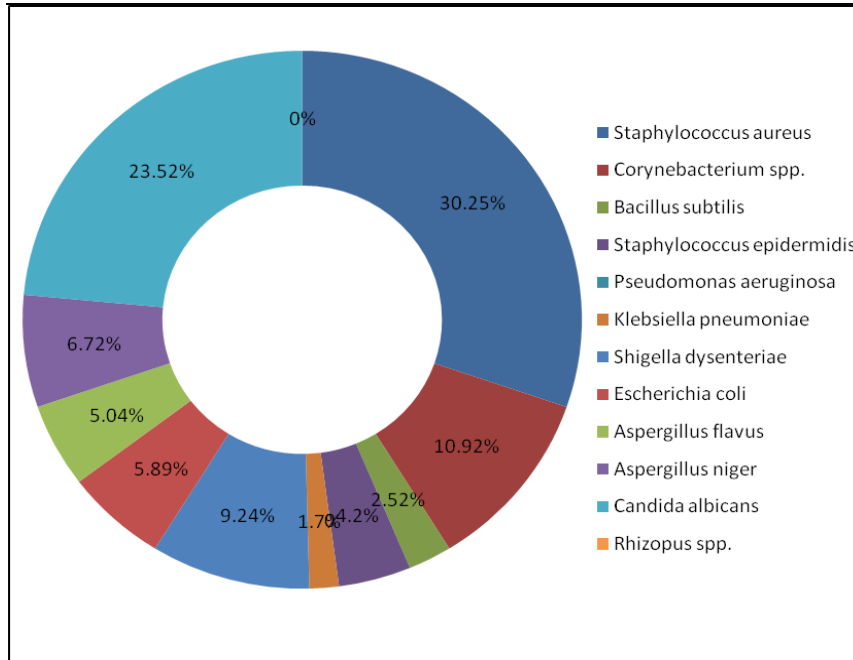


Figure 5: Comparative percentage Potential of biofilm formation by microbes isolated from hospital environmental samples (%)

4. Discussion

Increased prevalence in secondary infections (mostly by bacteria and fungi) in hospitalized patients throughout the world enlightens the urgent need to assess the causes of hospital acquired infections. One of the main highlighted finding by scientists worldwide is biofilm formation by surface attached bacteria. Although it is a general public ideology that hospital environment is not a enriched providing media for microbial growth but It was find out by the world wide scientists that microbes are likely to be attached on hospital dry surfaces and they survive for unusually long periods (weeks to months) in developed biofilms that may take major role on the prevalence of contaminations with pathogens and number of bacteria that are on hospital dry surfaces. This has important implications, particularly for hospital outbreak investigation [41]. Coagulase negative *Staphylococcus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas* species, *Klebsiella* species, and *Enterococcus* species are generally known to cause nosocomial infections, and may be the common cause of colonization in indwelling medical devices even responsible for biofilm production [27, 42, 43].

Similar findings were also noticed in present study as out of total 202 microbial isolates, 126(62.38%) bacteria

and 76(37.62%) fungi were isolated and identified in hospital environmental surface samples. *Staphylococcus aureus* was highly positive followed by *Candida albicans* with 28.7% and 15.8% of total assemblage of isolation. Moreover; *Staphylococcus aureus* followed by *Candida albicans* also have a highest potential to form biofilm with 30.25% and 23.52% of total assemblage of isolated microbes. These reports clearly indicate that biofilm forming potential is the main survival strategy for these microbes in hospital stress environment and may become a source of hospital acquired infections. Potential of microbes to form biofilm were monitored by Tube method (TM). Although this method is slightly more laborious and slower than the traditional 96-well microtiter plate staining procedure, but it is cost effective than other techniques that are used to determine the spatial location of biofilms.

The samples collected for this study, some were in direct use and some that were in-direct use of hospitalized patient. Among environmental samples, hospital ward curtains and medical trays were highly contaminated with bacteria and fungi (with 26% each of total assemblage respectively). According to Curran et al [44] giving the reason that considering the presence of staphylococci as normal skin flora, the microorganisms can reach catheters through skin abrasions and catheter provides a favorable environment for bacteria to form biofilm. Biofilms provide a mixed bacterial community where the horizontal transfer of resistance genes and extensive interspecies interactions may take place in these communities. Some studies indicated that fungi can regulate the antibacterial action and bacteria can control the activity of antifungal agents in these biofilms. Such supporting interspecies interactions might explain the enhanced antimicrobial resistance of these mixed-species biofilms and potential of microbes to survive for longer periods on hospital dry surfaces. Presence of *C. albicans* in a biofilm increased the resistance of slime-negative staphylococci to vancomycin and *Candida* resistance to fluconazole was enhanced in the presence of slime-producing staphylococci [26].

So there must be step forward to take necessary action to overcome microbial growth in hospital environment. There should be proper use and selection of biocides and detergents with the best all round performance which are able to control surface attached microbial growth on surfaces. Proper cleaning of all surfaces should be occurred on regular basis. Further research is also required to evaluate the prevalence and composition of biofilms in situ on hard and soft hospital surfaces. Vickery et al [17] giving more suggestion to overcome the prevalence of microbes and their biofilm forming potential and develop in-vitro models that represents the microbes and biofilm present in hospital environment, harnessing surface science to develop a hospital environment that reduces the chance of biofilm formation such as microfibre or automated room disinfection technology and surface modification.

5. Conclusion

Staphylococcus aureus and *Candida albicans* have highest level of survival strategy due to biofilm that is why they may become major agents of hospital acquired infection and their emergence as important nosocomial pathogens can relate with enhanced potential of biofilm formation. Highest abundance and biofilm formation potential of bacteria and fungi in combination can also underline a direct extensive and striking interaction between prokaryotic and eukaryotic cells in biofilm.

6. Recommendations

As Tube method for biofilm formation is cheap and cost effective so it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories. There are many limitations in study which need to be covered for full understanding of the prevalence and composition of biofilm developed by microbes. Followings are some recommendations, which should be studied in future;

- To understand the hydrophobic characteristics of microbe for biomaterial, adhesion assay should also be performed. Findings can be helpful for selections of biomaterial uses generally in hospital. This may limits the spread of microbes from such sources and decrease the cause of secondary infections.
- Further research is also required to evaluate the genotypic characteristics of biofilm forming microbes, to suppress the major genes responsible for biofilm formation.
- To develop the in-vitro model that fully represents the microbes and biofilm relationship presents in hospital environments.
- Moreover, antibiotic susceptibility of isolated microbes should be performed which will be useful for the best selection of disinfectant.
- Selection and use of the best disinfectant that have highest efficiency performance to kills every type of microbes on small concentration basis.

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