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ORIGINAL ARTICLE

Isolation of Bacteria from Different Surfaces of a Hospital Wards and Clinical Laboratory in Karachi, Pakistan, With Determination of Biofilm Forming Property

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ABSTRACT

***Purpose of Study*:** The present research study is based on the isolation and evaluation of the bacteria forming biofilm which is mainly associated with hospital acquired infections and nosocomial infections. Biofilms play important role in bacterial defense and make them more resistant to available antimicrobial drugs which lead to serious health problems. Inhibition of bacterial biofilm formation is very necessary to overcome the nosocomial infections incidences because such bacteria become more pathogenic and virulent.

***Methodology*:** Collection of swab samples was carried out from the different surfaces of Emergency ER, ICU, CCU, Wards and Clinical laboratory at the Memon Medical Institute (MMI) Hospital, Karachi, Pakistan.

A total of 250 samples were collected and cultured at Mueller Hilton Agar (MHA) with the standard growth requirements for bacteria. The bacterial growth positive cultures were isolated and identified through the cultivation on the differential media like Blood agar for gram-positive and MacConkey's agar for gram-negative bacteria. Biochemical colonial and morphological evaluation of bacteria was done for the identification of bacteria on the specie level. Determination of biofilm forming property of isolated bacteria was done with the Congo Red Agar (CRA) method and confirmative analysis was done with the help of trypticase soya broth (TSB). Out of 250 swab samples 80 samples were observed with bacterial growth and 20 fungal cultures were isolated.

***Result and Conclusion*:** Out of 80 bacterial cultures, 33 were identified as *Bacillus subtilis*, 11 as *Escherichia coli* and 29 as *Staphylococcus aureus*. The number of biofilm forming isolates on CRA is 15 *Bacillus subtilis*, 4 *Escherichia coli* and 12 *Staphylococcus aureus.* Sub culturing of bacterial cultures was done on enriched CRA plates and the raise in biofilm formation was observed as 18 *Bacillus subtilis*, 5 *Escherichia coli* and 15 *Staphylococcus aureus*.

Keywords: Isolated bacteria, biofilm forming property, wards surfaces.

INTRODUCTION

A bacterium under the covering of biofilm than the planktonic inoculums express, different genetical characteristics which depends upon their locations, and make them more stable and resistant to the multiple antimicrobial and bacterial treatments like antibiotics, traditional disinfectants and others [1]. The main aspect of biofilm formation is extracellular constituent (EPS) that is secreted by and sheathes around the bacteria [2].

A bacterial biofilm is a structure or assemblage of microbial cells which are irreversibly attached to a surface and not easily removed or damaged by the formal rinsing, with disinfectants, phagocytic activity and antibiotics [3, 4]. Formation of Bacterial biofilm is predominant in natural ecosystems. It constitutes public health threats because of the unsettled resistance to actions against contamination as well as antibiotics [5]. Even with the considerations of the ultimate construction and progress of bacterial biofilms still the processes of stimulation accountable for the adaptation from planktonic to sessile cells is not clearly understood. This conversion is likely to remain a complicate and exceedingly regulated progression which results in a phenotypic transformation [6]. As described by Zobell the simple biofilm system is with liquid and solid, that stays to establish a liquid in an inheritor and allow the bacteria to inhabit the solid surface [7]. Some currently used methods are multi-well plates, generally in this technique bacteria are grown to quantify biofilms and the other technique is by adding substratum to a planktonic state culture termed as “the immersion mode” [8].

MATERIALS AND METHODS

Swab Sample Collection

A total of 250 different surfaces swab samples of intensive care unit (ICU), coronary care unit (CCU), emergency ER department, wards and Laboratory were collected from Memon Medical Institute (MMI) Hospital, Karachi, Pakistan during August 2016 to March 2017, with the help of sterile cotton swabs. Sample collection was done in a random way without any consideration of particular day timings, disinfection applied or not and type of surfaces (Table **1** and Figure **1**). Swab samples were then directly cultured on solid agar plates. The swab samples were then streaked and incubated for the growth of possible microorganisms. Only bacterial grown cultures were considered in this study for the determination of biofilm at Clinical Laboratory Memon Medical Institute (MMI) Hospital, Karachi, Pakistan.

Culture on Mueller Hinton Agar

All swab samples were cultured on Mueller Hinton Agar (MHA). MH agar media was used for the cultivation of the swab samples. The media plates were incubated with the standard bacterial growth requirements at 37°C for 24 hours. The bacterial colonies were then sub cultured for their isolation and identification.

Differentiation and Identification

Identification and differentiation of the grown bacterial cultures was done with the help of their colonial, biochemical and morphological characteristics. Differential growth media like Blood agar for gram positive and MacConkey's agar was used for the gram-negative bacteria. Identification of bacterial colonies was done on the basis of Bergey's Manual of Systematic Bacteriology. Testing of biochemical characteristics was done with the help of sugars (glucose, sucrose, maltose and lactose), TSI (triple iron sugar), IMViC (indole, methyl red, voges proskauer). Gram staining was done for the determination of morphological characteristics of isolated bacterial cultures.

Determination of Biofilm Forming Property of Bacteria

Bacterial samples were tested for their biofilm forming property with the help of media designed for identification and differentiation of bacterial colonies as biofilm and non-biofilm forming. The Congo Red Agar (CRA) method is one of the commonly used and reliable method of the evaluation of bacteria forming biofilm.

Preparation and Enrichment of Congo Red Agar

Congo Red Agar was prepared with the standard protocol (reviewed from the previously presented research literature) designed for the CRA preparation. For the preparation of CRA the Brain heart infusion agar (BHIA) 1L was supplemented with 3% (30 gm) of sucrose and 0.8 gm of Congo dye (original Congo red dye). The media was autoclaved at 121°C for 20 minutes. Isolated bacterial colonies were sub cultured

on CRA. Plates were incubated at 37°C for 24 to 48 hours and analyzed for the formation of biofilm. For maximum biofilm production additional amount of sucrose 60 gm was added with 1 L Brain heart infusion agar (BHIA).

Counter Check of Biofilm Formation by the Tube Method and Crystal Violet Staining

Confirmatory analysis of bacterial biofilm was done with the help of Trypticase soya broth (TSB) media in sterilized plastic tubes. Bacterial growth culture with black colour colonies on congo red agar were sub cultured in Trypticase soya broth (TSB) media in sterilized plastic tubes with 2% glucose. 10 ml of Trypticase soya broth (TSB) media in sterilized plastic tubes was added with a loopful of bacterial colony and incubated at 37°C for 24 to 48 hours for the formation of biofilm.

To confirm the biofilm formation the sub-cultured tubes were observed for the production of slime layer at bottom of the tubes and the adherent cells were checked with the help of crystal violet stain. The media from the plastic tubes was drained and the adherent cells were stained with the crystal violet dye for the determination of biofilm formation by the bacterial culture.

RESULTS

Swab Sample Collection

Table 1. Swab sample collection from ER, CCU, ICU, Clinical laboratory and Ward.

Departments	Surface of material							
	Bed lines	Window panes	Reception counter desk	Medical devices	Transportation try	Internal units	Collection counter	Bed side tables
Ward	20	20	-	20	-	-	-	20
ICU	15	-	10	-	-	-	-	-
Clinical Laboratory	-	-	-	-	-	20	20	-
ER	25	-	20	20	15	-	-	-
CCU	10	-	-	15	-	-	-	-

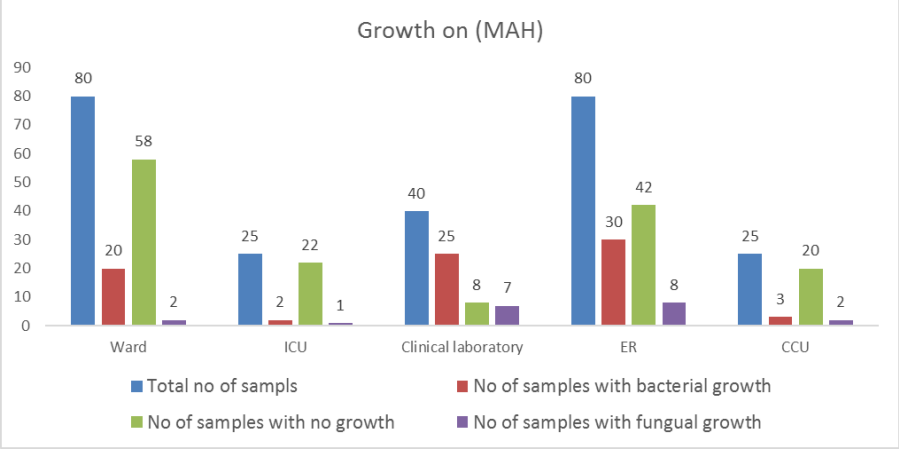

Figure 1. Cultivation of total no of swab samples on MHA with growth, without growth and fungal growth collected from ER, CCU, ICU, Clinical laboratory and Wards.

Cultivation of Swab Samples and Growth Percentage

A total of 250 swab samples were collected (Figure **2**) from the different surfaces out of which 80 samples were the bacterial growth positive samples at Memon Medical Institute Hospital. From Ward, 20 bacterial growth positive samples were collected; from ICU, 2 bacterial growth samples were collected; from Clinical Laboratory, 25 bacterial growth samples were collected; from ER, 30 bacterial growth samples were collected; from CCU, 3 bacterial growth samples were collected. A total of 20 fungal swab samples were grown whereas 150 swab samples were observed with no growth. The percentage of the bacterial samples from the total of the swab samples collected from various surfaces separately is 25% from Ward, 8% from the ICU, 62.80% from Clinical laboratory, 37.50% from ER, 12% from CCU (Table **2**). The overall bacterial growth percentage is 32%. 0.08% fungal growth was observed. Only bacterial cultures were isolated and cultivated on differential media for the differentiation and identification of cultures.

Table 2. Percentage of samples with bacterial growth, fungal growth and with no growth.

Sample side	Percentage of samples with bacterial growth	Percentage of samples with no growth	Percentage of sample with fungal growth
Ward	25%	72.5%	2.5%
ICU	8%	88%	4%
Clinical Laboratory	62.8%	20%	17.5%
ER	37.5%	52.5%	10%
CCU	12%	80%	8%

Isolation, Differentiation and Identification

Table 3. Count and identification of isolated bacterial species.

Site of Swab Sample	Name and no bacterial samples			
	Bacillus subtilis	***Escherichia coli***	***Staphylococcus aureus***	**Others**
Ward	9	3	6	2
ICU	1	0	1	0
Clinical Laboratory	9	3	10	3
ER	13	5	10	2
CCU	1	0	2	0
Total No. of Isolates (n)	33	11	29	7

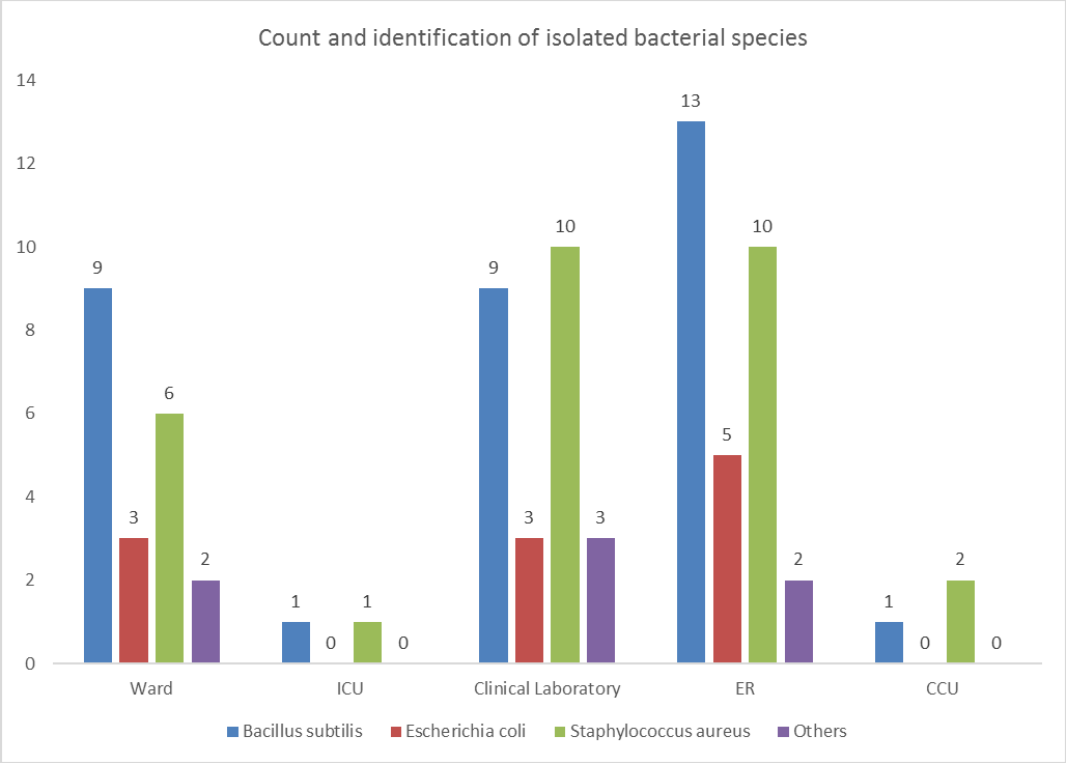

Figure 2. Count and identification of isolated bacterial species.

Table 4. Percentage of Bacteria Forming Biofilm from the Total Isolates on CRA.

Name of Organism	Total isolates	Percentage of biofilm forming isolates
B. subtilis	33	45.45%
E. coli	11	36.36%
S. aureus	29	41.37%

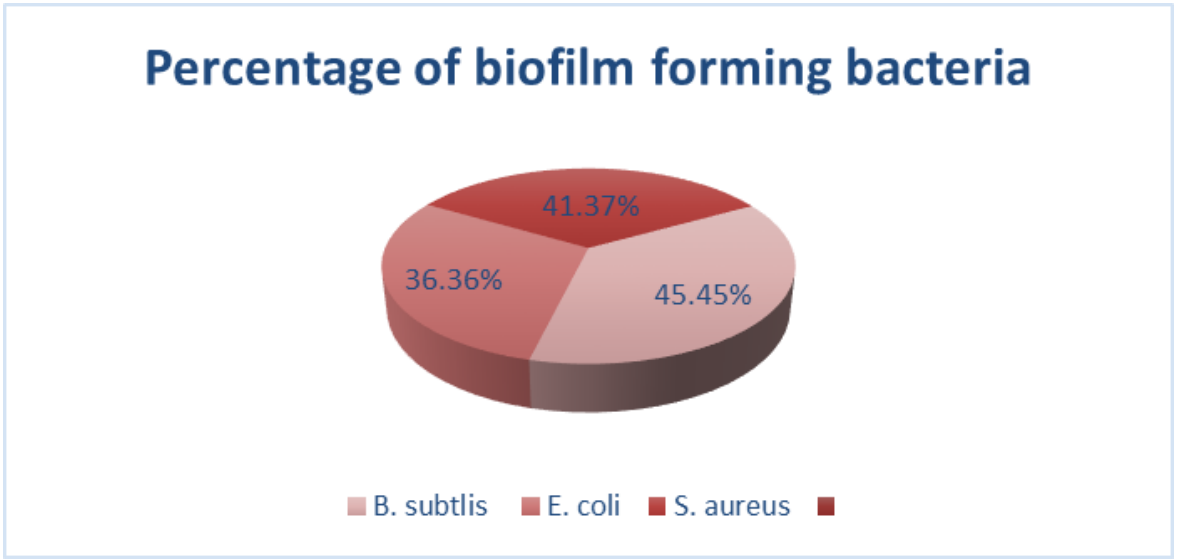

Figure 3. Percentage of biofilm forming bacteria.

The overall percentage of growth of organisms forming biofilm is from 33 isolate of *B. subtilis* 15 could form biofilm 45.45%, from 11 isolates of *E. coli* 4 were able to form biofilm 36.36% and out of 29 isolates of *S. aureus* 12 were able to form biofilm 41.37% (Table **3**). The total percentage of biofilm forming bacterial cultures out of 80 is 30 which is 37.5% (Table **4** and Figure **3**).

Bacterial Growth with Biofilm on Enriched Congo Red Agar

Table 5. Bacterial growth with biofilm on enriched CRA.

Organism	Total no of isolates	No of isolates with biofilm on CRA	No of isolates with biofilm on enriched CRA
B. subtilis	33	15	18
E. coli	11	4	5
S. aureus	29	12	15

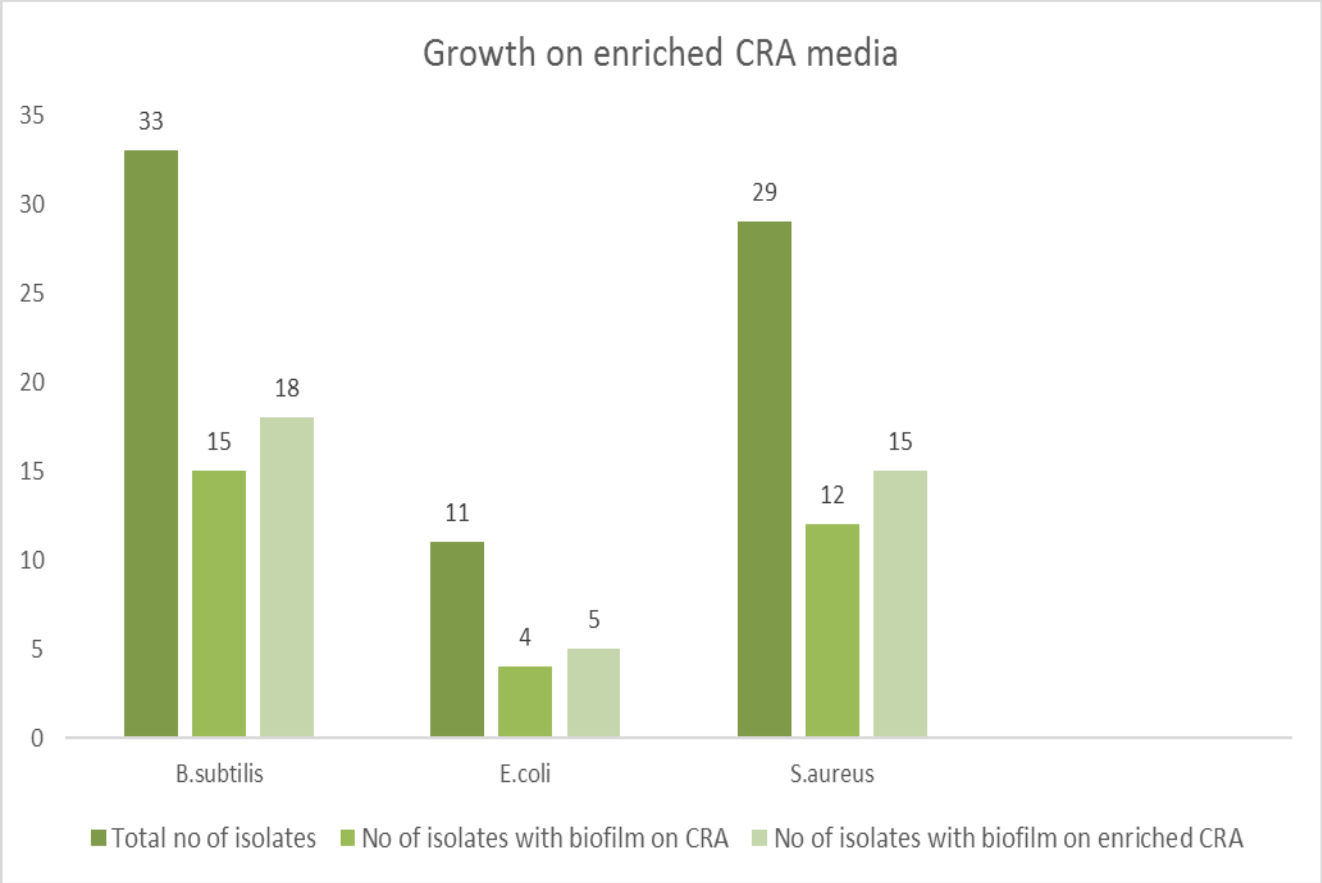

Figure 4. Bacterial growth with biofilm on enriched Congo Red Agar (CRA).

While the bacterial cultures were cultivated on CRA enriched with 60 gm of sucrose a little raise in the number of biofilm forming cultures was observed (Table **5** and Figure **4**). The difference is however not very noticeable but further researches can be done to evaluate the effect of extra nutrients or constituents in CRA media.

Confirmative Analysis of Bacterial Biofilm with the Help of Tube Method.

Table 6. Comparative analysis of bacterial biofilm with the help of tube method.

Organism	Growth on CRA	Growth on enriched CRA	Growth in plastic tube
Bacillus subtilis	15	18	18
Escherichia coli	4	5	5
Staphylococcus aureus	12	15	15

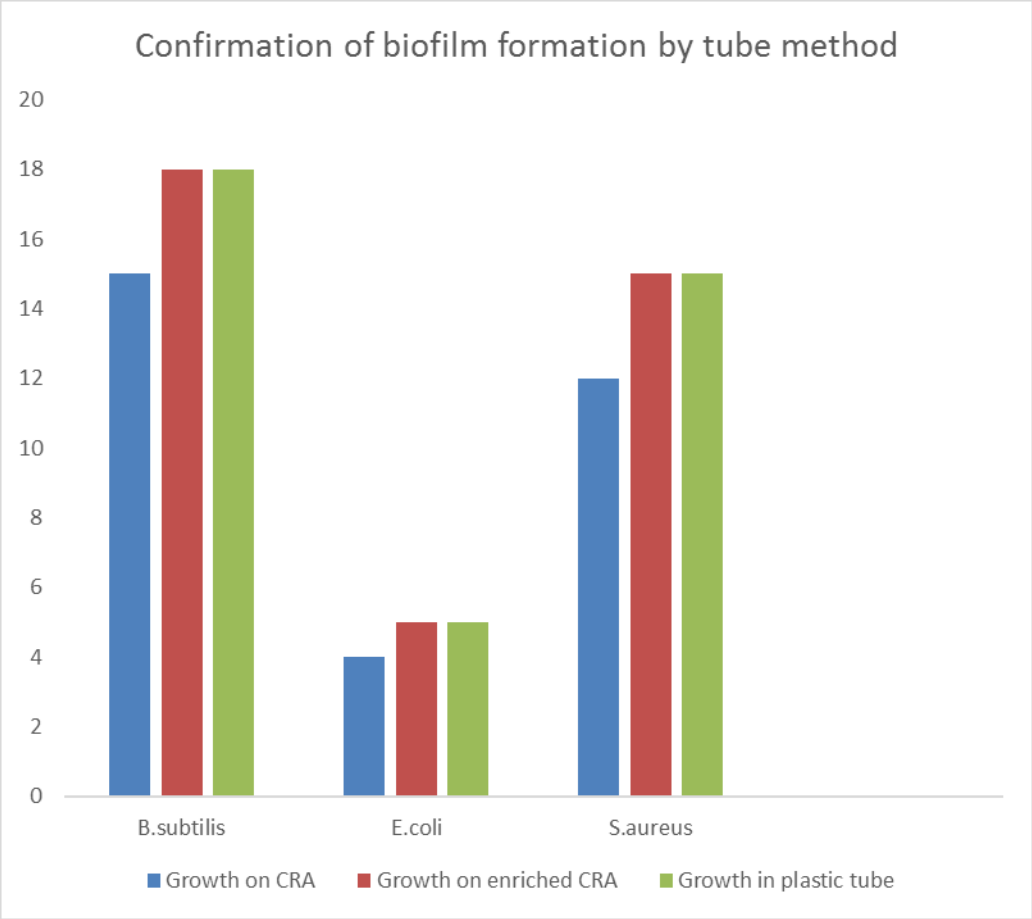

Figure 5. Bacterial growth with biofilm on enriched Congo Red Agar (CRA).

Confirmation of Biofilm Formation by Tube Method

Bacterial isolates forming biofilm were subjected to confirm their property to form biofilm and for that all cultures forming biofilm were incubated in plastic tubes with Trypticase soya broth (TSB) and stained with crystal violet stain (Table **6** and Figure **5**). All cultures forming biofilm were able to form biofilm in plastic tubes.

DISCUSSION

In this study, we tested multiple surfaces of ER, ICU, CCU, Ward and clinical laboratory. Memon Medical Institute Hospital is no doubt an improved and hygiene conscious hospital as the results were less than 50% of the total surface tested swab sample. The staff at this hospital practices a better cleaning exercise on daily basis to provide an improved and healthier environment to its patients. Still the hospital

environment is always at a high risk of microbial transportation and colonization.

The basic intention of the study was to relate the potential virulent of bacterial pathogen linked to its characteristics of biofilm formation and involvement in nosocomial infection. With the implementation of this study and observations we may confer the result that a single chance to the microorganisms' colonization can lead to a health hazards. As with the obtained results we can estimate that biofilm formation is the most defensive tool for the bacteria involved in nosocomial infections. This also puts into the pathogenesis of different types of microorganism. A microorganism is much more virulent, stable and safe from the environmental stressed conditions under the casing of biofilm composed of matrix of polysaccharide material.

A collection of 250 swab samples was obtained from the different surfaces at Memon Medical Institute Hospital. Out of which 80 samples were the bacterial growth positive samples and from Ward 20 bacterial growth positive samples were collected, from ICU 20 bacterial growth samples were collected, from Clinical Laboratory 25 bacterial growth samples were collected, from ER 30 bacterial growth samples were collected, from CCU 3 bacterial growth samples were collected. The large no. of growth sample collection from the ER and Ward indicates that these areas are at high risk of spread of infections and bacterial contamination with biofilm formation.

Another study was carried out in PNS Shifa Hospital in Karachi Pakistan in where the isolation of bacteria and fungi forming biofilm was done with the help of tube method. 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 [9].

Rendering to the CDC, the most communal pathogens causing nosocomial infections are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The common nosocomial infections are urinary tract infections (UTI), respirational pneumonia, invasive site wound contaminations, bacteremia, digestive and skin infections.

Freeman described a simple qualitative method to detect biofilm production by using CRA medium. CRA medium is prepared with brain heart infusion broth, sucrose, agar and Congo Red indicator. Black colonies with a dry crystalline consistency indicated biofilm production. Though, amongst the carbohydrates, sucrose is measured the most cariogenic (causing tooth decay) as, in addition to being fermented by oral bacteria, it is a substrate for the formation of extracellular (EPS) and intracellular polysaccharides (IPS). Therefore, while the low pH environment triggers the shift of the resident plaque microflora to a more cariogenic one, EPS promote changes in the composition of the biofilms' matrix [10]. To consider this property, the isolates were cultivated on CRA enriched with sucrose. While the bacterial cultures were cultivated on CRA enriched with 60gm of sucrose a little raise in the number of biofilm forming cultures was observed. The difference is however not very noticeable as *Bacillus subtilis* 18 from the 15 with the difference of 2 isolates, *Escherichia coli* 5 from 4 with the difference of 1 isolate and *Staphylococcus aureus* 15 from 12 with the difference of 2 isolates, but further researches can be done to evaluate the effect of extra nutrients or constituents in CRA media for the better expression of biofilm forming property of bacterial species for the *in vitro* investigation [11, 12].

CONCLUSION

This research is focused upon the isolation and control of the bacteria forming biofilm associated with nosocomial infections. Biofilms protects the bacteria from several environmental stresses and chemical changes in their surroundings. Therefore, bacteria under the biofilm are more resistant to traditional antimicrobial treatments. Swab samples from the

various surfaces of ER, Ward, ICU, CCU and clinical laboratory were collected with sterile cotton swabs and cultivated on Mueller Hilton Agar (MHA) and further tested for the property of biofilm formation on CRA. For the better enumeration of the growth positive samples the bacterial cultures were sub cultured on enriched CRA media plates. A little raise in biofilm formation by the bacterial cultures on enriched CRA shows the ability of bacteria to form biofilm increases with modification in growth media constituent like sugars, minerals, salts etc. Formation of biofilm makes bacterial pathogens in a hospital environment a serious health risk leading to increase rate of nosocomial infections.

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