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In vitro and molecular identification of Biofilm producing multidrug resistant nosocomial isolates from the surfaces of hospital environment

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Abstract

The hospital environment and the fomites are the major sources of nosocomial infection. The majority of infections are transmitted directly or indirectly by environmental contamination. Nosocomial bacteria cause a serious problem in the health care system globally. The emergence of antibiotic resistance among nosocomial bacteria is increasing leading to treatment failure and prolonged hospital stays. Among the antibiotic resistance nosocomial pathogens, a high prevalence of biofilm producers was increased. This poses a threat to the hospital environment to treat the patient effectively. So, the present study has identified the biofilm-producing multidrug-resistant nosocomial isolates from the hospital environmental surfaces. Totally 60 bacterial isolates were identified from the different environmental surfaces of the acute care hospital, Cuddalore. Six different species were identified based on their morphological and biochemical characteristics. All the isolates were screened for the antibiotic resistance pattern. Among the six different species of isolates, *Micrococcus* spp. showed the maximum number of resistance to the studied antibiotics (83%) followed by *Staphylococcus aureus* (60%) and *Proteus* spp. (53%). Most of the isolates showed resistance to Imipenem (78%), Meropenem (73%) and Cefuroxime and Erythromycin (49%). And Levofloxacin, Amikacin and Vancomycin were showed susceptible by the maximum isolates. Biofilm production of the isolates was studied. Totally 68% of the isolates showed biofilm production and 31% showed no biofilm production. Among the 31 biofilm-producing isolates 8 showed the maximum amount of strong biofilm production. And the two randomly selected isolates were

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identified by the 16srRNA sequencing analysis. It was confirmed as *Staphylococcus aureus* and *Micrococcus luteus*. Monitoring the development of biofilms in the hospital setting is essential for halting the spread of illnesses that are multidrug resistant. By detecting and describing biofilm producers, the burden of hospital-acquired infections can be reduced, and the spread of pathogens can be stopped.

Key words : Nosocomial pathogen, Hospital surface, Biofilm, Multidrug-Resistant.

Nosocomial infections were mainly transmitted by the inanimate objects of the hospital environment and healthcare workers. Nowadays hospital-acquired infections are spread more and lead to the emergence of multidrug-resistant pathogens[15]. Hospital environments, the hands of healthcare workers and hospitalized patients are the predominant sources of transmitting nosocomial pathogens[14]. Certain criteria in the hospital environment are noted to cause nosocomial infections such as load of pathogens, appropriate temperature and the environment's surface. Hence, gram-positive bacteria grow on the dry surface and gram-negative bacteria adhere on moist surfaces[2]. Fomites or the inanimate objects of the hospital environment are the major reservoirs of nosocomial pathogens. Proper disinfection procedures of hospitals can control the fomites-based outbreaks of nosocomial infections[20]. Infants and bedridden hospitalized patients are the major concern of infecting fomites associated with nosocomial infections[13]. ESKAPE pathogens are considered to cause antibiotic resistance by the following ways, enzyme inactivation, changing permeability, synthesis of biofilm and efflux pumps. It also affects the treatment failure and diagnosis of the diseases[23].

Due to the inappropriate usage of antibiotics, enteric bacteria are resistant to the antibiotics and become multi-drug resistant pathogens[3]. *Staphylococcus aureus* possess multidrug resistant determinant and causes nosocomial infections. It showed resistance to chloramphenicol, erythromycin, cephalexin and tetracycline[28].

The hydrolytic enzymes generated by the biofilm producers will cause the infection, whether it is connected to a device or not, to adhere to new surfaces[25]. To control infections brought on by biofilm producers, antibiofilm substances or antibiotic combinations that reduce the effectiveness of attachment and colonisation on the surface should be employed[16]. According to one study, 90% of the bacteria that cause chronic wounds also create biofilm. It results in treatment failure and increases the likelihood that the final antibiotics won't work[9].

The rise in antibiotic resistance and persistent recurring infections, especially in diabetic patients, have been linked to numerous nosocomial and harmful bacteria. Many chronic infections, including diabetic foot ulcers, cellulitis, and necrotizing fasciitis, have been linked to the biofilm formation, which

results in the emergence of multidrug resistant strains and the failure of treatment[10].

It has been noted that polymicrobial communities made up of pathogenic and nosocomial bacteria can be found in affected tissues and pathogenic and nosocomial bacteria can be found in affected tissues as well as in the natural environment as producers of biofilms[22]. AMR that develops in biofilms is brought on by things like altered microbial metabolism, poor drug penetration, and increased drug damage because of secretions in the biofilm matrix. Exchange of AMR genes occurs as a result of biofilm formation in a multi-microbial environment.

Study design :

The present study was conducted in an acute care hospital in Cuddalore. It offers services in various specialities including paediatrics, cardiology, neurology, psychiatry and plastic surgery. In addition to inpatient services, it offers 24-hour emergency medical care and a pharmacy and is equipped to provide a wide range of diagnostic testing. The hospital also hosts a blood bank. The procedures of the study were reviewed and accepted by the hospital management. Samples were collected from the six wards of the hospital includes Operation Theatre (OT), Orthopaedics Surgery (OS), Wound and emergency Unit (WEU), Intensive care unit (ICU), Dialysis Unit (DU), Special Ward (SW) from March, 2022 to September, 2022.

A total of 60 bacterial strains were isolated from the different inanimate surfaces of the hospital environment in the acute care hospital, Cuddalore. All the isolates were identified in the Department of Microbiology, Annamalai university. The isolates were stored as glycerol stocks at -20^{o}C and were regularly sub cultured in Nutrient agar slants for routine use.

Selection of multidrug resistant nosocomial isolates :

A total three of bacterial pathogens such as, Streptococcus pneumoniae (ATCC - 19615), Streptococcus pyogenes (ATCC - 49619), Pseudomonas aeruginosa (ATCC - 9027) were purchased from the American Type Culture Collection (ATCC), USA, for the study. The bacterial cultures were sub cultured, maintained on Nutrient agar slants, and stored in a refrigerator at 4 (°C) for further experiments. All the bacterial isolates were tested for their susceptibility to 16 different antibiotics by the kirby- Bauer disk diffusion method in accordance with clinical and Laboratory Standards Institute guidelines (CLSI, 2012) [20]. Bacterial inoculum was prepared by inoculating a loopful of organisms in 5 ml of Nutrient broth and incubated at 37 °C for 12 hours till moderate turbidity was developed. The turbidity was matched with the 0.5 Mc Farland standard and then used to determine bactericidal activity. The suspension was then inoculated onto a Muller-Hinton agar plate. The gram-negative isolates were tested against antibiotics Levofloxacin (5mcg), Amikacin (30mcg), Ceftriaxone (30mcg), Erythromycin (13mcg), Ceftazidime (30mcg), Gentamicin (120mcg), Meropenem(10mcg), Clindamycin (2mcg). Gram positive isolates were tested against the antibiotics, Ciprofloxacin (5mcg), Cefepime (30mcg), Cefazolin (30mcg), Imipenem (10mcg), Amoxiclav (30mcg), Cotrimoxazole (5mcg), Piperacillin (100mcg), Cefuroxime

(30mcg). Then, all plates were incubated at 37°C for 18–24 h, aerobically. After overnight incubation. the susceptibility pattern of the isolates was determined by comparing with the standard chart. Multi-Drug Resistant isolates were screened by showing resistant to three to more antimicrobial categories [21]. Susceptible to one agent in three or more antibiotic classes, atleast one in all or minimum two classes, all antibiotic classes were denoted as MDR, XDR and PDR.

Quantitative detection of biofilm : Tube method :

Biofilm formation among the bacterial isolates was determined by the tube method. In this method, each 24 hrs culture of bacteria in a tube of trypticase soy broth was emptied and washed with phosphate buffer saline. The tubes were air-dried and blotted with crystal violet for 20 min, after which the tubes were rinsed of excess stain with distilled water. Each tube was air-dried in an inverted manner. Positive biofilm development was documented when a visible purple layer covered the bottom of the tube. The isolates were categorized into non-biofilm, moderate, and high biofilm-producing organisms respectively when there is no visible film lining, medium intense film lining and intense film lining[29].

Biofilm formation assay with TCP method:

Wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid (Tarsons, India) were filled with 200µl tryptic soy broth containing NP24 and NP64 bacterial suspensions individually, further, the inoculum of each bacterium was adjusted to a concentration of 10^8cfu/ml (equal to 0.5 McFar-land standard). Negative control wells contained broth only. The plates were covered and incubated aerobically for 48hrs at 37°C. Then, the content of each well was washed three times with 250µl of sterile physiological saline. The plates were shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 200µl of 99% methanol per well, and after 15 min plates were emptied and left to dry. Then, plates were stained for 5 min with 0.2 ml of 2% crystal violet used for Gram staining per well. Excess stain was rinsed off by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent cells was resolubilized with 160µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 570 nm by using a microplate reader (Biotek Elx808, WI, USA). All strains were classified into the following categories: non-adherent (0), weakly (+), moderately (++), or strongly (+++) adherent, based upon the ODs of bacterial films (Stepanović et al., 2000). We defined the cut-off OD (OD_C) for the microtiter-plate test as three standard deviations above the mean OD of the negative control. Strains were classified as follows: Non-adherent: $OD \leq ODc$; weakly adherent: $ODc < OD \leq 2 \times ODc$; moderately adherent: $2 \times ODc < OD \leq 4 \times ODc$; strongly adherent: $4 \times ODc < OD$.

Confocal laser scanning microscopy (CLSM) analysis :

CLSM was used to visualize the bacterial adhesion formed on glass microscopic slides after 48hrs incubation. Slides were incubated on broth containing conical flask, after incubation slides were fixed with 2% (v/v) glutaraldehyde (HiMedia, India) in

phosphate-buffered saline at pH 7.4 (137mM NaCl, 3mM KCl, 10mM Na_2HPO_4, and 2mM KH_2PO_4), for 15 min. Excess fixative was removed by washing the films with PBS for 15min. The bacterial films were then stained with 0.01% (w/v) acridine orange (HiMedia, India) in PBS for 15min, which was followed by washing with PBS for 30 min to remove excess stain. The stained films were visualized by CLSM with an Olympus LSMGB200 CLSM (Olympus Optical Co. Ltd., Tokyo, Japan). The CLSM used an argon ion laser at 488 nm for excitation and a 605–632nm bandpass filter for emission. Images were captured at 10X magnification and processed using Olympus LSMGB200 CLSM bundled programs (Rice *et al*., 2005).

Molecular identification of bacteria :

DNA isolation procedure: DNA extraction was performed using 2mL bacterial culture, collected at the mid-exponential growth phase using the Roche Kit (Germany) according to the manufacturer's instructions

Primers: Universal set of the Eubac primers (Gurtler and Stanisich, 1996)

27F - 5' - AGAG TTTG ATCM TGGC TCAG -3'
1492R - 5' - TACG GYTA CCTT GTTA CGAC TT -3'

PCR amplification : The PCR was done on a thermal cycler (Eppendorf) with 50µl reaction mix. The reaction mix contained 10× amplification buffer (5µl), 1.5mM $MgCl_2$ (5µl), 1µl of each forward and reverse primer, 1µl dNTP and 0.25µl Taq polymerase. After an initial denaturation at 95°C for 1min, amplification was carried out with 35 cycles of 35s at 94 °C, 40s at 55 °C, 2min at 72 °C followed by a final extension for 8min at 72°C. The PCR products were analyzed by electrophoresis using 1.2% agarose gel (Genei).

DNA Sequencer*:* The PCR product was purified using the Qiagen PCR purification kit and then sequenced on an ABI Prism 377 automatic sequencer (Applied Biosystems, CA, USA).

Phylogenetic tree reconstruction*:* The evolutionary distances were computed using the maximum Neighbor-Joining method. The evolutionary analysis was conducted using MEGA 11 [27] and the tree topologies were evaluated by bootstrap analyses based on 1,000 replicates and phylogenetic trees were inferred using the neighbor-joining method.

Totally 60 bacterial isolates were identified from the different environmental surfaces of the acute care hospital, Cuddalore. Fig. 1. Shows the distribution of bacterial isolates. The isolates belonged to six different genus *Escherichia coli Staphylococcus aureus Bacillus* spp. *Micrococcus* spp. *Proteus* spp. *and Salmonella* spp.

In this study, Multi drug resistant isolates were identified based on the selected broad-spectrum antibiotics including Carbapenem drugs by disc diffusion assay. Later they were screened for their biofilm-forming ability by tube method and Tissue culture plate method. By the results of antibiotic resistant and biofilm production selected isolates were confirmed by PCR and the sequence were submitted in NCBI for the future reference.

Antibiotic-resistant pattern of the isolates was found out by the disk diffusion

method. Totally ten antibiotics were used for the test. Table-1. Showed the frequency of multidrug resistant pathogens among the tested isolates. Among the six different species of isolates, *Micrococcus* spp. showed the maximum number of resistance (83%) followed by *Staphylococcus aureus* (60%) and *Proteus* spp. (53%).

Fig. 2. Shows that the efficacy of biofilm production among the isolates. Biofilm production was identified by the Tube method and Tissue culture plate method. Among the tested isolates strong biofilm production was noted in the *Staphylococcus aureus* and *Micrococcus* spp. The maximum number of non-biofilm producers were identified in the *E. coli, Proteus* spp. and *Salmonella* spp. Maximum weak biofilm production was identified in *Proteus* spp. and *Salmonella* spp. Table. 2 showed the number of biofilm and non-biofilm producers among the 60 isolates used in the study. Totally 68% of the isolates showed biofilm production and 31% showed no biofilm production.

Antibiotic-resistant pattern of the biofilm-producing isolates were tested with the ten broad spectrum antibiotics. Fig. 3 showed the antibiotic resistant pattern of the biofilm producers. Most of the isolates showed resistance to Imipenem (78%), Meropenem (73%) and Cefuroxime and Erythromycin (49%). And Levofloxacin, Amikacin and Vancomycin were showed susceptible by the maximum isolates.

Fig. 4 & 5 showed biofilm production's efficacy by tube method and its CLSM image. Among the 31 biofilm-producing isolates 8 showed the maximum amount of strong biofilm production. And the two randomly selected isolates were identified by the 16srRNA sequencing analysis. It was confirmed as *Staphylococcus aureus* and *Micrococcus luteus*.

Fig. 6 & 7. Showed the phylogenetic tree of *Micrococcus luteus* and *Staphylococcus aureus*. And the Table-3 showed accession number of sequenced isolates.

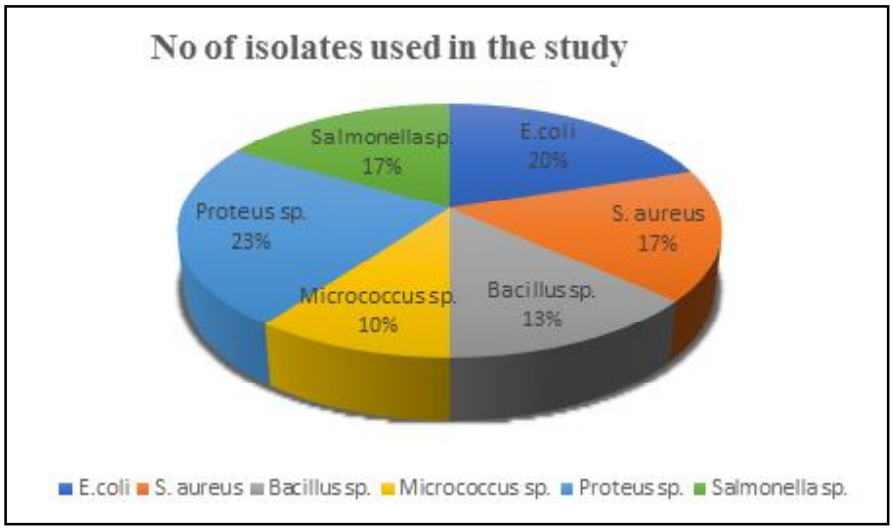

Fig. 1: Distribution of the isolates used in the study

Table-1. Frequency of MDR pathogens among the isolates

Isolates	No.of Isolates	(%) MDR
E. coli	12	6(50)
S. aureus	10	6(60)
Bacillus spp.	8	3(37)
Micrococcus spp.	6	5(83)
Proteus spp.	14	8(57)
Salmonella spp.	10	3(30)
Total	60	31

Table-2. Number of Biofilm and Non-biofilm producers of the Isolates

Isolates	Biofilm producer	Non-Biofilm producer
E.coli (12)	5	7
S. aureus (10)	10	0
Bacillus spp. (8)	6	2
Micrococcus spp. (6)	6	0
Proteus spp. (14)	8	6
Salmonella spp. (10)	6	4
Total	41	19

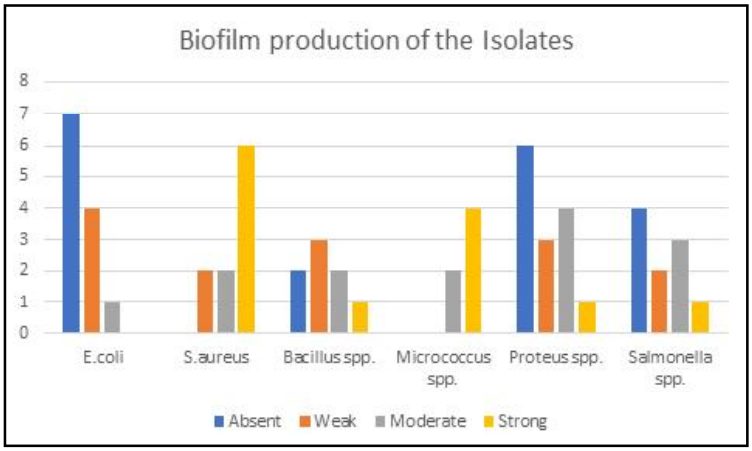

Fig. 2. Biofilm producing ability of the Isolates

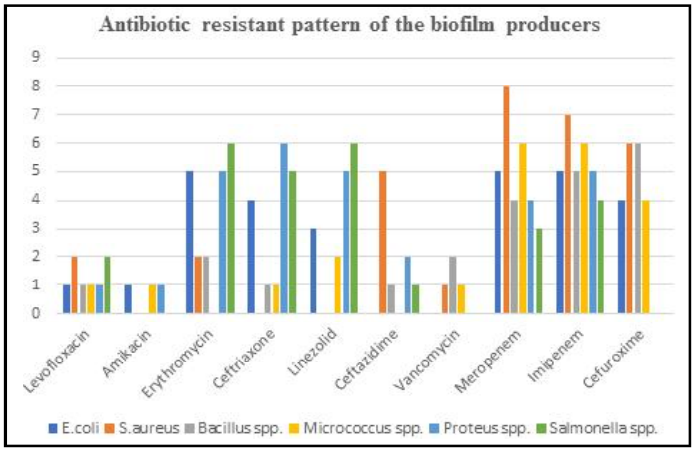

Fig. 3. Antibiotic-resistant pattern of the biofilm producing isolates.

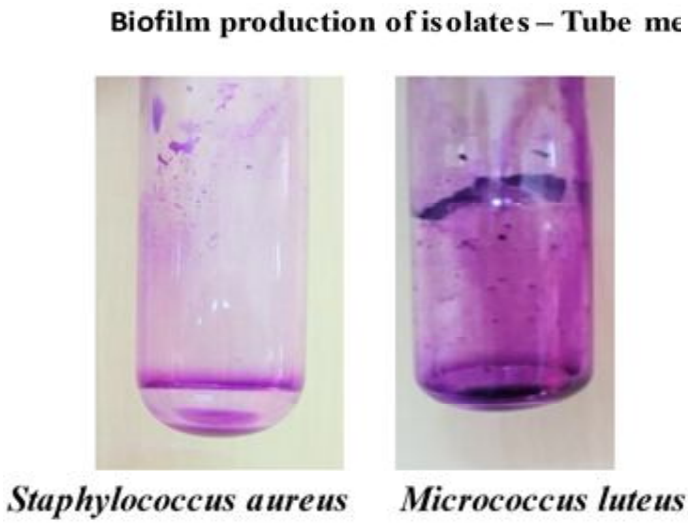

Fig. 4. Identification of Biofilm by Tube Method

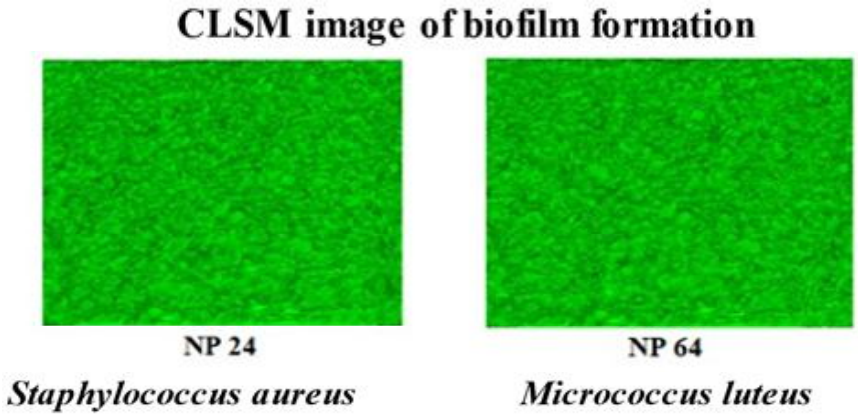

Fig. 5: CLSM images of biofilms formed after 48hrs incubation on microscopic glass slides

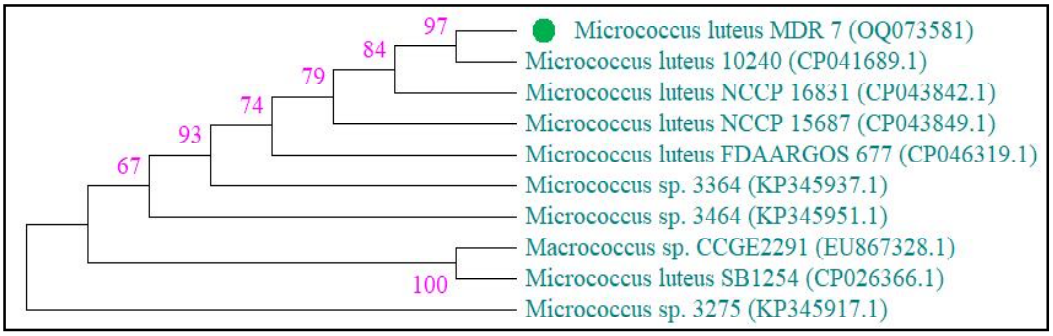

Fig. 6. Phylogenetic tree of *Micrococcus luteus*

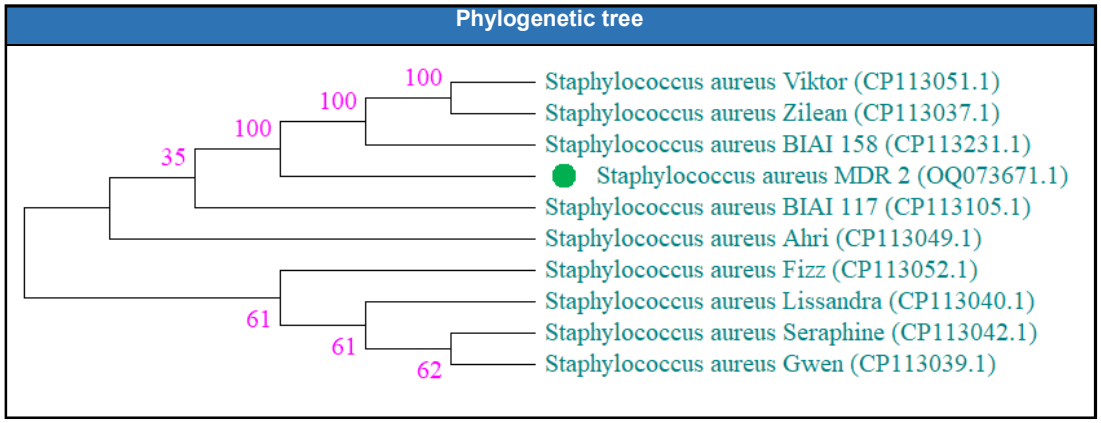

Fig. 7. Phylogenetic tree of *Staphylococcus aureus*

Table-3. Molecular identification of biofilm producers

S. no.	Bacteria	Accession No.
1.	*Micrococcus luteus*	OQ073581
2.	*Staphylococcus aureus*	OQ073671

In this study, the isolates from the hospital surfaces were screened for their biofilm-forming ability and antibiotic-resistant pattern. The resistance develops by various methods like restricted penetration of antibiotics into biofilms, decreased growth rate and expression of resistance genes[10]. Most of antibiotic-resistant strains produced biofilm. This could confirm the biofilm-producing ability may also confer antibiotic resistance.

The tube adherence assay followed in our study is very simple to perform and a reliable test used as a general screening method for the detection of biofilm-producing organisms. The study conducted by Rewatkar A.R *et al.,* concludes that the Tube method is a more qualitative and reliable method as compared to Congo Red Assay (CRA), more so for strongly biofilm-producing isolates. Our study also concluded that the tube method was more reliable to identify the biofilm producers.

The ability of many staphylococci to create biofilm makes it simpler for bacteria to attach to surfaces and medical equipment. Three-quarters of our isolates (37%) formed biofilms, according to the biofilm formation test. It should be kept in mind that in our investigation, the influence of biofilm formation on antibiotic resistance was not detected because some strains were discovered to be sensitive to specific antibiotics despite the fact that they formed biofilms. This could be explained by the fact that we performed the susceptibility assay using planktonic colonies[24]. Similarly, 68% of the multidrug-resistant isolates were biofilm producers in the present study.

In this investigation, isolates of *P. aeruginosa* from southwestern Iran had higher levels of carbapenem resistance than isolates from numerous other nations, including China (37.2% for imipenem). 2019 by Hu *et al*.[11]

The high resistance rate of 52.1% and 49.5% against imipenem and meropenem, respectively, was another crucial finding of the current study. The prevalence of carbapenem resistance varied widely between investigations,[5] ranging from 17.5% to 100.0%.

Resistance to ampicillin and other beta-lactam drugs in enteric organisms is mediated by β-lactamases. The introduction of new β- lactams with different activity spectra has led to a selection of different genes and mutations that confer resistance to these drugs in enteric bacteria[4,17].

E. coli isolated from the UTI showed resistance against Cloxacillin (92.5%), amoxicillin (90.8%), ampicillin (90.8%), erythromycin (75.8%), cotrimoxazole (70.0%), streptomycin (70.0%) and tetracycline (68.3%) while 85.8% and 84.2% [1].

The present study stated that 83% of the isolates were multi-drug resistant. Among that 68% were biofilm producers. By using modern molecular tools, this association between antibiotic resistance and biofilm development could be further examined. It is essential for developing effective techniques for treating and preventing infections as well as comprehending the processes of infection and antibiotic resistance in healthcare settings. Preventative methods and the creation of medications from natural resources are required to prevent the growth of biofilm on hospital surfaces and medical devices.

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