

Characterization of Microbial Species in Relation With Device Associated Infection



Zoology

KEYWORDS : Antifungal; Biofilm; Medical devices; Resistance; SEM; XTT reduction.

* **Bhavna Sharma Grover**

Post Doctorate fellow, Department of Zoology, M.D. (PG) College, Sriganganagar, Rajasthan-335001, India. * Corresponding author.

Anita Chhabra

Head Of Department, Department of Zoology, M.D. (PG) College, Sriganganagar, Rajasthan-335001, India.

ABSTRACT

The formation of bacterial biofilms on medical devices is a leading cause of infections in hospitalized patients. Characterizing these biofilms and identifying the bacterial species are of major importance. We studied the formation of biofilms on the inner surface of devices using microbiological culture techniques. The bacterial species were identified as a variety of gram-negative bacilli, with a predominance of strains belonging to E.coli and Pseudomonas. The resistance pattern of isolated bacterial species were determined against selected antibiotics. Further their minimum biofilm inhibitory concentration was also determined. The bacterial species on the devices were characterized by scanning electron microscopy. Microbiological analysis of the devices is required for true identification of the causative agents of device-associated infections. This approach, combined with a routine cytobacteriological examination allows for the complete characterization of biofilm-associated species, and also may help prevent biofilm formation in such devices and help guide optimum antibiotic treatment.

1. INTRODUCTION

The increased use of implanted medical devices and associated incidences infections is a big concern. Bacterial colonization of the indwelling device can lead up to both infection and malfunction of the device [1]. Microbial biofilm are encouraged due to the non-shedding surfaces of these devices, that provide ideal substrata for colonisation by biofilm-forming microbes.

Biofilm is a complex aggregation of microorganisms growing on a solid surface and protected by self-synthesized extracellular polysaccharide matrices (EPS) composed of exopolysaccharides and a minute quantity of proteins, minerals, metals, and nucleic acids [2].

The serious medical consequences and soaring economic sequelae of device-associated infections underscore the importance of prevention. There is a desperate need to limit contamination of surfaces, equipment and implanted medical devices, and to find suitable agents for infection control that will contribute to strategies to eradicate this reservoir for infection [3]. Not surprisingly, therefore, interest in biofilms has increased dramatically in recent years. The application of new microscopic and molecular techniques has revolutionised our understanding of biofilm structure, composition, organisation, and activities, resulting in important advances in the prevention and treatment of biofilm related diseases.

Various pathogenic strains have been isolated and characterized from medical devices and chronic wounds [4]. staphylococcal and enterococcal populations from human colostrums were studied in detail for their potential virulence [5]. Difference in the susceptibility of planktonic and sessile isolates against different antibiotic was also studied to understand the mechanism.

Present work is a sincere attempt to identify and characterize such resistant pathogenic microorganism from medicinal devices and patients and study their resistance level in order to decide appropriate therapeutic measurements.

2. MATERIAL AND METHOD

2.1 Sample collection

Clinical samples were obtained from Government hospitals and various private clinics. Sample were collected and carried to laboratory in sterile capped containers. These samples were transported to laboratory for further processing.

2.2 Isolation of pathogenic strains

Each catheter lumen was flushed with 2 ml of trypticase soy broth. Broth was incubated overnight. The overnight broth culture was serially diluted with autoclaved distilled water upto 10⁻⁶ dilution and 100 µl of each dilution was spread on to Nutrient agar (HiMedia) plates and incubated overnight at 37°C. After 12-18 hours incubation the number of viable colonies were counted using total viable plate count method [6].

Colonies with visually distinguishable morphologies were randomly selected and isolated by directly streaking on Nutrient agar plates and incubated for another 12-18 hours. Primary biofilm screening was done using tube staining assay [7].

2.3 The Tube assay

Qualitative assessment of biofilm formation was determined by the tube staining assay following Christensen et al. 1982 [7], LB broth (1mL) was inoculated with 100µl of overnight culture broth and incubated for 24, 48 and 72 hours at 37°C. The tubes were decanted and washed with Phosphate Buffer Saline (PBS) (pH 7.3) and dried. Staining of dried tubes was done with 0.1% crystal violet.

Formation of biofilm was confirmed with the presence of attachment (visible film) on the wall and bottom of the tube. However, the liquid interface did not indicate biofilm formation [8].

2.4 Physical and biochemical identification of bacterial strains

Various biochemical identification methods were applied in order to identify microbial species. Gram staining, Citrate Test, Urease Test, Catalase Test, Methyl Red Test, Indole Test by Sherman, 2005 [9], were used for the determination of bacterial species.

2.5 Determination of MIC

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ampicillin, penicillin, gentamycin and erythromycin against isolated bacterial species were determined using a microbroth dilution assay using the Clinical and Laboratory Standards Institute (CLSI) guidelines. Staphylococcus and streptococcus, E.coli and pseudomonas suspension (100ml) diluted to 1x10⁶ cfu/ml was added into each well of microtiter plate. Serial dilutions of antibiotics were distributed to 96 well-microplate.

After incubation at 37 °C for 24 hours, MIC values were determined. The assay was carried out in triplicates and repeated thrice.

2.6 Effects of antibiotics on pre formed biofilm

For the estimation of antibiofilm potential of various antibiotics on mature biofilm XTT reduction assay was used. Bacterial biofilm were prepared on commercially available, pre-sterilized, flat-bottomed 96-well polystyrene microtitre plates (himedia). Biofilm of *E.coli* and pseudomonas was prepared in Tryptic soy broth (TSB) containing 0.25% glucose and Brain heart infusion broth (BHI) with 1% sucrose was used. Bacterial suspensions which were adjusted to 0.5 Mc Farland turbidity, were added into the wells of microplates that contained 150 µl BHI containing 1% sucrose for *E. coli* and pseudomonas. Plates were incubated for 1.5 h at 37°C with agitation. After the adhesion phase, the liquid was aspirated and each well was washed twice with PBS to remove loosely attached cells. 200 ul of fresh media was added to the wells and the plate was further incubated for 24 h at 37°C.

The wells were washed twice with PBS and fresh respective media containing different concentrations of antibiotics were added and the plate was further incubated for 24 h at 37°C. The effect of compound on biofilm was estimated by using a standard crystal violet assay [7] and the color change in the solution was measured with spectrophotometric readings at 490 nm.

2.7 Scanning electron microscopy

For scanning electron microscopy (SEM), biofilms were developed on glass coverslips. The coverslips were inoculated with bacterial suspension and incubated statically at 37°C for 90 min to allow adhesion. After removing non-adherent cells, the coverslips were incubated with medium at 37°C for 24 h. For antibiotic treatment groups, mature biofilms were treated with antibiotic overnight. Biofilms were washed and placed in a fixative consisting of 2.5 % (vol/vol) glutaraldehyde in PBS (pH 7.2) for 2 h. The samples were rinsed twice in PBS, dehydrated in an ascending ethanol series, treated with hexamethyl-disilazane (Himedia, india), and dried overnight. The specimens were coated with gold and observed through a FE-SEM QUANTA 200 FEG (FEI Neithelands) in high-vacuum mode.

3 RESULT AND DISCUSSION

Medical devices are responsible for a large portion of nosocomial infections, particularly in critically ill patients. Device associated infections can cause major medical and economic burden. Bacterial colonization of the indwelling device can be a preface to both infection and malfunction of the device [1,3]. Hence a proper identification of microbial species is desired. For this purpose sample collected from various clinical sources were enumerated using broth culture and diluted culture were subjected to spreading and streaking. Strains selected with observable difference in colony morphology and colour were pure cultured by quadrant streaking

Tube staining method is a quick and easy way to identify biofilm positive strains [7]. This technique was used for the identification of biofilm positive strains. Pure cultures obtained from spreading and quadrante streaking were analysed for biofilm formation. Initial screening resulted in selection of 137 biofilm positive strains with biofilm foarmentation capacity. Previously using this techniques various biofilm positive strains were isolated from several clinical samples [8, 10, 11]. Out of 137 positive strains 41 samples were taken further on the basis of moderate or high biofilm foarmentation for physical and biochemical identification.

Biofilm positive pure cultures were analyzed further for identifi-

cation of species. The morphological and biochemical characters of isolated bacterial species are presented in table 1. The morphological observations in Gram's staining resulted in the identification of numerous Gram negative bacteria.

Physical and biochemical methods applied resulted in the identification of two most prevalent species i.e. *E coli* and *Pseudomonas*. *E. coli* and *Pseudomonas* are has been isolated from various medical devices also by various authers [12, 13]. In our present study the increased number of colony counts was recorded by *E. coli*, which gave positive for indole production, glucose, lactose, and sorbitol [9]. *Pseudomonas* a gram negative bacteria was differentiated from *e coli* on the basis of negetiav catalase, MR and indole production test and positive citrate utilization. Out of the above said species *e.coli* was the most widespread (15%), with the highest occurens in collected samples followed by *pseudomonas* (7%) and some other gram negative species.

Gram negative *e.coli* and *pseudomonas* were clearly showing a higher MIC values for all tested antibiotics. Both *E.coli* and *Pseudomonas* were not only showing high average MIC but they also showed a great variation in MIC value in tested isolates. MIC values for *E. coli* varied between 8 ug/ml to 256 ug/ml and 4 ug/ml to 256 ug/ml for ampicillin and penicillin respectively with an average of 32 and 64 ug/ml (table 3). Same kind of discrepancy in MIC values were noted in *pseudomonas* isolates for ampicillin, penicillin and erythromycin, where a great variation in lowest and highest MIC values were recorded (2-1024 ug/ml, 2-1024 ug/ml and 2-256 ug/ml) (table 3).

Apart from planktonic MIC; isolated bacterial species were tested for minimum biofilm eliminating concentration (MBEC). Analysis of biofilm growth by using XTT reduction assay in presence of various antibiotic depicted a wide range of MBEC values for tested strains. *E. coli* and *Pseudomonas* biofilm showed MBEC values of 64 ug/ml and 512 ug/ml to for penicillin in average (fig. 2). Isolated strains with higher planktonic MIC also resulted in a higher MBEC values as expected. At the same time we also found some isolates that were having a lower planktonic MIC value but their MBEC values were much greater than expected. Same results were obtained for ampicillin also (fig. 1) where at the concentration of 2-4 MIC a clear biofilm removal was observed.

In order to evaluate the effect of antibiotics on bacterial biofilm growth, SEM was performed. SEM images of control tested species biofilms and of a biofilm treated with antibiotics are shown in Figure 4 and 5. Treatment of antibiotics caused lyses of cells and also reduced the density of cells in biofilm. Shrinkage of the cell membrane indicates cell lyses.

The data obtained from present work gives an idea regarding the responsible microbial species for biofilm associated infection and we can use this in deciding the required therapeutic measures.

4. ACKNOWLEDGMENT

The author is thankful to university grant commission (UGC), Project No. 15-73/11 (SA-II) 7-3-2011 for financial support.

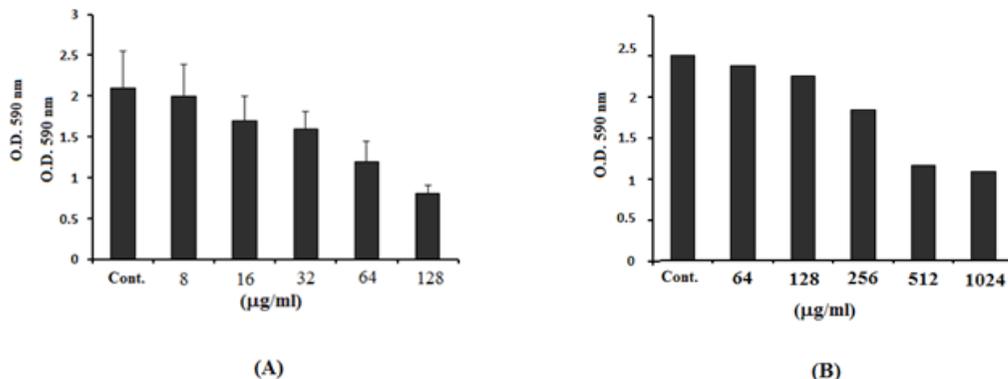


Figure 1. Effect of Ampicillin on biofilm of *E.coli* and *Pseudomonas* biofilm was quantified spectrophotometrically by XTT reduction assay.

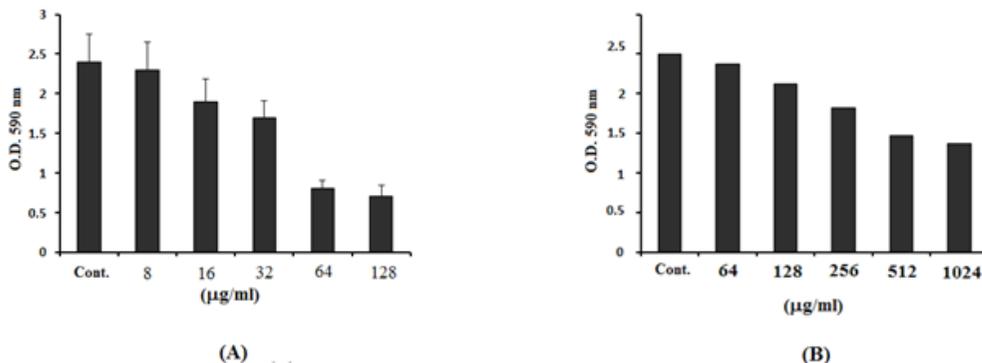


Figure 2. Effect of Penicillin on biofilm of *E.coli* and *Pseudomonas* biofilm was quantified spectrophotometrically by XTT reduction assay.

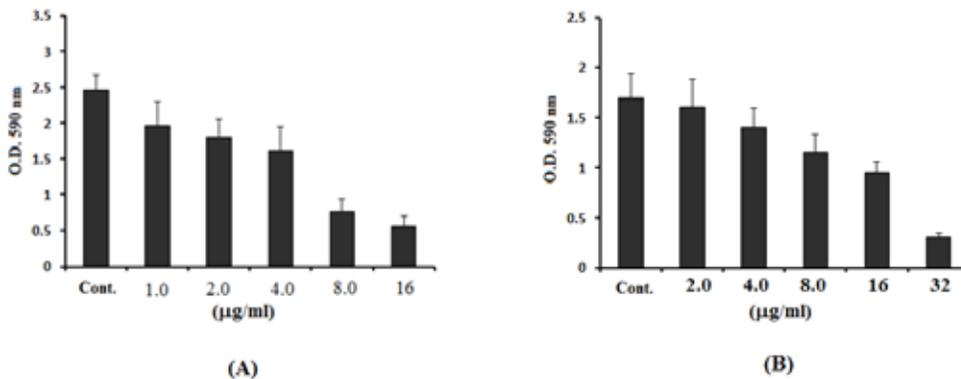
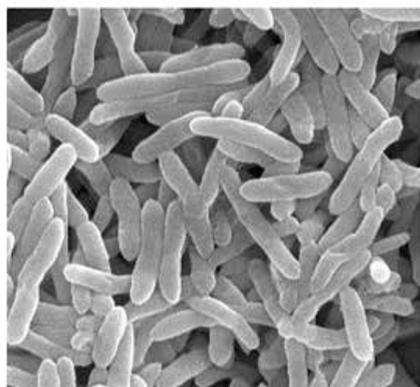
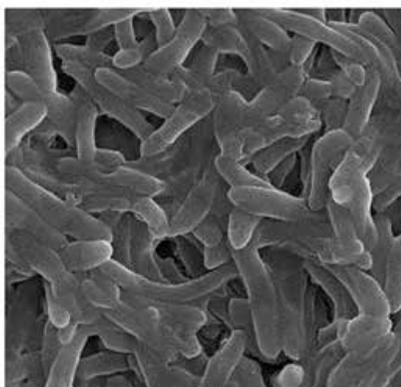


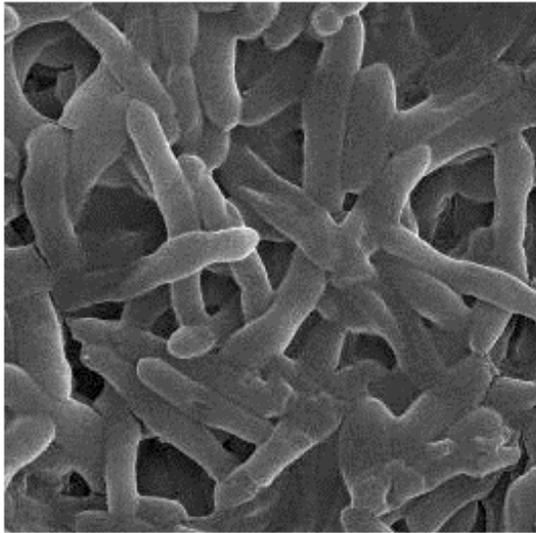
Figure 3 . Effect of Gentamycin on biofilm of *E.coli* and *Pseudomonas* Biofilm was quantified spectrophotometrically by XTT reduction assay.



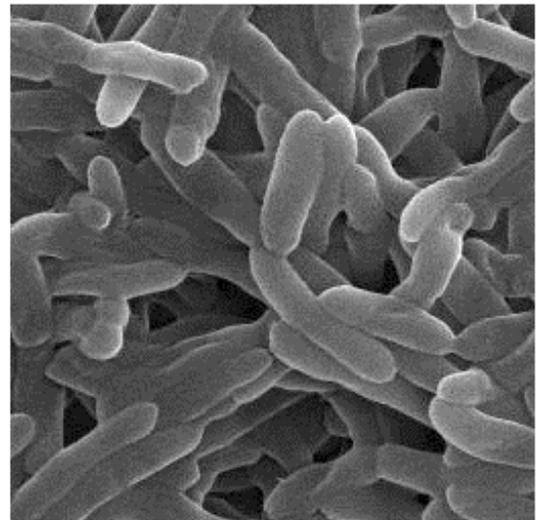
(A)



(B)

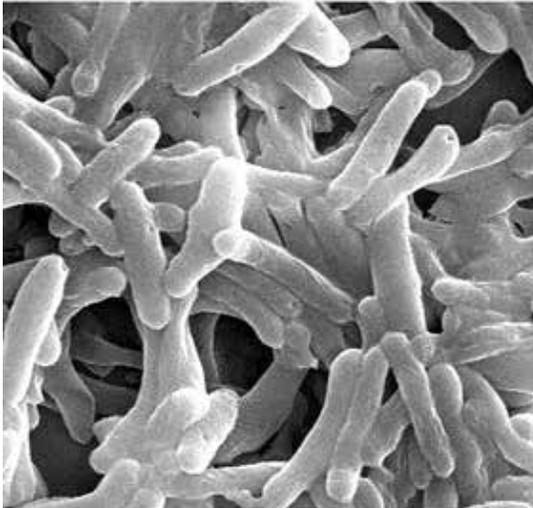


(C)

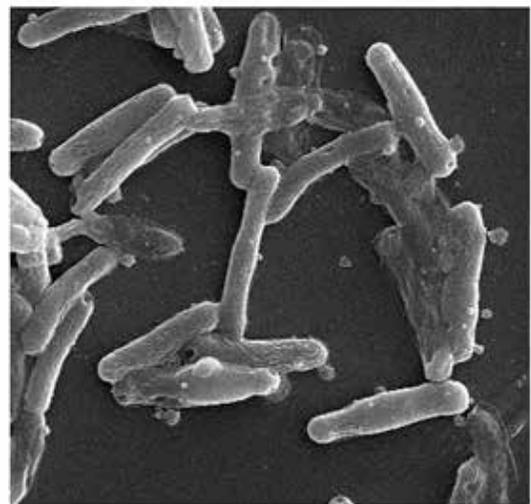


(D)

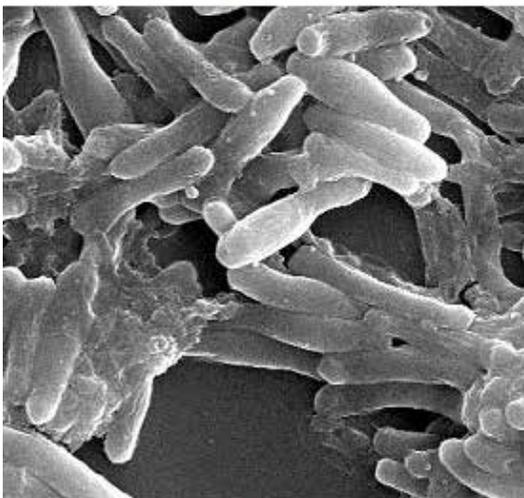
Figure 4. Scanning electron microscopy micrographs of the 48 h *E.coli* biofilms on coverslip. (A) Biofilm in the absence of antibiotics (B) Biofilm treated with Ampicillin, (C) Penicillin and (D) Gentamycin (after 24 h).



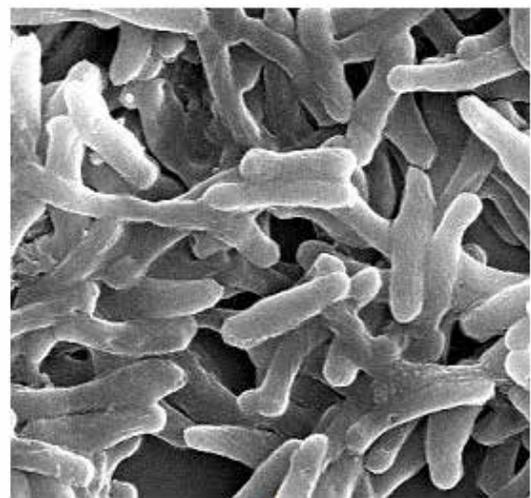
(A)



(B)



(C)



(D)

Figure 5. Scanning electron microscopy micrographs of the 48 h *Pseudomonas* biofilms on coverslip. (A) Biofilm in the absence of antibiotics (B) Biofilm treated with Ampicillin, (C) Penicillin and (D) Gentamycin (after 24 h).

Table 1:- Biochemical test for identification of bacteria isolated from clinical samples.

S. No.	Characterization	E. coil.	Pseu- domonas
1	Gram Staining	-	-
2	Motility	+	+
3	Shape	Rods	Rods
4	Catalase	+	-
5	Coagulase	-	-
6	Oxidase	-	-
7	Urease	-	-
8	Indole Production	+	-
9	Methyl red test	+	-
10	Vogus Proskaur Test	-	-
11	Citrate Utilization	-	+
12	Glucose	A/G	-
13	Mannitol	A/G	-
14	Lactose	A	-
15	Sucrose	A/G	-

Table 2: Minimum inhibitor concentration for amoxicillin, penicillin, gentamycin and Erythromycin against isolated microorganism.

S.No.	Species		Ampicillin	Penicilline	Gentamycin	Erythromycin
1	<i>E.coli</i>	MIC Range	8-256	4-256	2-32	2-256
		MIC50	32	64	8	128
2	Pseudomonas	MIC Range	2-1024	2-1024	1-32	2-256
		MIC50	256	512	16	32

REFERENCE

1. Richards MJ, Edwards JR, Culver DH, Gaines RP. Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Critical Care Medicine*. 1999; 27: 887-92. | | 2. Cos P, Tote K, Horemans T, Maes L. Biofilms: an extra hurdle for effective antimicrobial therapy. *Current Pharmaceutical Design*. 2010; 16: 2279-95. | | 3. Darouiche RO. Device-Associated Infections: A Macroproblem that Starts with Microadherence *Healthcare Epidemiology*. 2001; 33: 1567-72. | | 4. Frank DN, Wysocki A, Specht-Glick DD. Microbial diversity in chronic open wounds. *Wound Repair Regeneration*. 2009; 17:163-72. | | 5. Jiménez E, Delgado S, Maldonado A, Arroyo R, Albújar M, García N, Jarid M, Fernández L, Gómez A and Rodríguez JM. *Staphylococcus epidermidis*: a differential trait of the fecal microbiota of breast-fed infants. *BMC Microbiology*. 2008; 8: 143. | | 6. Prescott LM and Harley JP. *Laboratory Exercises in Microbiology*. 1st Edn., McGraw Hill Publ., New York, USA. 2002. ISBN: 978-0-471-42082-8. | | 7. Christensen GD, Simpson WA, Bisno AL and Beachey EH. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces *Infection Immunity*. 1982; 37: 318-26. | | 8. Mathur T, Singhal S, Khan S, Upadhyay UP, Fatma F and Rattan A. Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods. *Indian Journal of Medical Microbiology*. 2006; 24: 25-29. | | 9. Sherman N and Cappuccino JG. *Microbiology: a laboratory manual*. Sixth edition, ISBN. 2005; 81: 265-6. | | 10. Rewatkar AR and Wadher BJ. *Staphylococcus aureus* and *Pseudomonas aeruginosa*- Biofilm formation Method *Journal of Pharmacy and Biological Sciences*. 2013; 8, 5: 36-40. | | 11. Javaid U, Kaleem F, Omair M, Khalid A and Iqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. *Brazilian Journal of Infectious Diseases*. 2011; 15: 305-11. | | 12. Adedeji GB, Fagade OE and Oyelade A. Full Length Research Article Prevalence of *Pseudomonas aeruginosa* in Clinical Samples and its sensitivity to Citrus Extract. *African Journal of Biomedical Research*. 2007; 10: 183 - 187. | | 13. Phyllis DL, Hossein S, Painter T, Wu Fann, Gary WP, Wilson DA., Gillespie W, Mender A and Crystal BS. Identification of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* in Blood Cultures: a Multicenter Performance Evaluation of a Three-Color Peptide Nucleic Acid Fluorescence In Situ Hybridization Assay. *Journal of Clinical Microbiology*. 2011; 49: 2259-2261. | | 14. Ganderton , L., Chawla, J, Winters, C., Wimpenny, J , Stickler D. Scanning electron microscopy of bacterial biofilms on indwelling bladder catheters. *European Journal of Clinical Microbiology and Infectious Diseases*. 1992; 11: 789-96. |