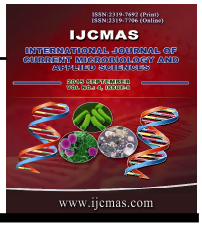
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 9 (2015) pp. 840-849**
http://www.ijcmas.com



Original Research Article

Comparison of Phenotypic Methods for the Detection of Biofilm Production in Uro-Pathogens in a Tertiary Care Hospital in India

Tayal Ruchi*, Baveja Sujata and De Anuradha

Department of Microbiology, Lokmanya Tilak Municipal Medical College and General Hospital (LTMMC & GH) Mumbai, India
**Corresponding author*

Keywords

Biofilm, Congo Red, Tissue Culture Plate, Tube method, Urinary Tract Infection

A B S T R A C T

Biofilms are surface associated bacterial communities surrounded by a matrix of exo-polymers. Biofilms contribute to the development of chronic urinary infections, refractory to antibiotic therapy. The study was conducted to analyze the prevalence of biofilm formation in mid-stream urine specimens using in vitro phenotypic detection methods and comparing these methods to choose a sensitive method with good reproducibility that can be employed in routine microbiology laboratory. A total of 137 aseptically collected, mid-stream urine specimens were analyzed. Semi-quantitative bacterial culture was performed and isolates were identified by standard biochemical tests. Biofilm formation was detected by Congo Red Agar Method (CRA), Tube Method (TM) and Tissue Culture Plate Method (TCPM). Out of 137, 37 (27%) showed biofilm formation. Maximum biofilm production was seen in *Enterococcus spp.* (71%) followed by *Escherichia coli* (27%). CRA detected biofilm production in 40.88%, TM in 37.96% and TCPM in 27% isolates. CRA and TM compared to TCPM demonstrated a sensitivity of 94.59% and specificity of 81% and 83% respectively. Detection of biofilms can be recommended for recurrent and recalcitrant infections before institution of empirical antibiotics. TCPM is a method with good reproducibility and specificity which can be used for detection of biofilms in resource limited settings.

Introduction

Biofilms are an assembly of microbial cells formed by bacterial species that are irreversibly associated with a surface and enclosed in a matrix of polysaccharide and protein material (Prakash B *et al*, 2003, Donlan RM, 2002).This confers a number of advantages such as protection from antimicrobial agents, exchange of nutrients and exchange of genetic material (Kokare CR *et al*, 2009, Harrison J *et al*, 2005).

Biofilms are responsible for nosocomial infections and chronic infections (Hall-Stoodley L *et al*, 2009, Shirtliff M *et al*, 2009). Biofilms may form on anatomical structures of the genitourinary tract and cause chronic urinary tract infection.

Studies carried out so far deal with either biofilm formation only in cases of CAUTI or biofilm formation by a particular

organism using one phenotypic method (Soto SM *et al,* 2000, Seno Y *et al,* 2005, Stickler DJ, 2008, Abdallah NMA *et al,* 2011). This study was undertaken to study the prevalence of biofilms in mid-stream urine specimens and choose the most suitable screening method, which is sensitive, easy to perform and can be routinely employed.

Materials and Methods

Sample collection

A total of 137 mid-stream, clean catch urine samples from patients presenting with complaints of urinary tract infection were used for detection of biofilm forming bacteria.

Microbiological processing

Presence of a single bacterium per oil immersion field in Gram smear of un-centrifuged urine or >5 WBCs per high power field of centrifuged urine was taken as an indication of urinary tract infection. Presence or absence of symptoms such as dysuria, frequency, incontinence, abdominal pain and suprapubic tenderness was also noted.

Urine colony count was done semi-quantitatively and a count of $>10^5$ CFU/ml was considered significant (Isenberg HD *et al,* 2010). All urine specimens showing significant bacterial growth on culture were included in the study. The pathogens were identified using routine biochemical tests.

Detection of biofilm production

Three methods were carried out for detection of biofilm production- Congo Red Agar Method (CRA), Tube Method (TM) and Tissue culture plate method (TCPM). *Staphylococcus epidermidis* ATCC 31484 & *Staphylococcus epidermidis* ATCC 12228 were used as standard positive and negative control strains respectively for biofilm production.

Congo Red Agar Method- Congo Red Agar (CRA) method is a simple, qualitative screening method to detect biofilm production. The medium used consisted of brain heart infusion broth (BHI) (37 g/l) supplemented with sucrose (50 g/l), agar No 1 (10g/l) and Congo red (0.8 g/l). First Congo Red stain was prepared as a concentrated aqueous solution and autoclaved (121ºC for 15 minutes) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55ºC. CRA plates were inoculated with the test organisms and incubated at 37°C for 24 hours aerobically. Interpretation was done using the following colour scale. (Fig. 1and 2).

Colour	Indication
Black, dry, crystalline colonies	Biofilm producer
Red/pink/Bordeaux colonies	Biofilm non-producer
Darkening of colonies without dry, crystalline morphology	Indeterminate

Tube Method - A loopful of test organisms from overnight culture plates were inoculated in borosilicate glass tubes containing 10ml of Trypticase soy broth with 1% glucose. The tubes were then incubated at 37°C for 24 hours aerobically. After incubation, the tubes were decanted and washed with phosphate buffer saline at pH 7.3 and dried. Tubes were then stained with crystal violet (0.1%) for 15 minutes. The stain was decanted and the tubes were

washed with de-ionised water and dried in inverted position.

Biofilm formation was considered positive when a visible film lined the walls and the bottom of the tube (Fig. 3). Formation of a stained layer at the air-liquid interface was considered negative for biofilm formation. The amount of biofilm formed was scored as:

Score	Interpretation
1	Weak/none biofilm production
2	Moderate biofilm production
3	High/strong biofilm production

Tissue Culture Plate Method - A loopful of isolated test organisms from overnight cultures were inoculated in 10ml of Trypticase soy broth with 1% glucose and incubated at 37°C for 24 hours. Individual wells of sterile 96 well-flat bottom polystyrene tissue culture treated plate (Sigma-Aldrich Co. LLC, USA) were filled with 200ul of the bacterial suspension corresponding to 0.5 McFarland after further dilution of 1:100 with fresh medium along with control organisms. Only broth served as a control to check sterility and non-specific binding of media. The plates were inoculated at 37°C for 24 hours. After incubation, contents of each well were removed by gentle tapping and wells were washed three times with 300 µl of sterile saline. The remaining attached bacteria were heat-fixed by exposing them to hot air at 60°C for 60 min. Then150 µl crystal violet (2%) stain was added to each well. After 15 min, the excess stain was rinsed off by decantation, and the plate was washed. 150 µl 95% ethanol was added to each well, and after 30 min, the optical densities (OD) of stained adherent bacterial films were read using a microtitre plate reader at 600nm. The OD values were calculated for all tested strains and negative controls, the cut-off value (ODc) was established. It is defined as a three standard deviations (SD) above the mean OD of the negative control: ODc=average OD of negative control + (3×SD of negative control). Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD= average OD of a strain -ODc); ODc value was calculated for each microtiter plate separately. When a negative value was obtained, it was presented as zero, while any positive value indicated biofilm production (Fig. 4). For easier interpretation of the results, strains were divided into the following categories:

1. Non biofilm producer (0) OD ≤ODc
2. Weak biofilm producer (+ or 1) = ODc<OD ≤2×ODc,
3. Moderate biofilm producer (++ or 2) = 2×ODc <OD≤4×ODc
4. Strong biofilm producer (+++or 3), 4×ODc <OD

Results and Discussion

The comparative statistical analysis for all methods was done by using 2x2 tables where applicable. Parameters like sensitivity and specificity were determined from these tables.

A total of 137 mid-stream urine specimens from patients admitted with UTI were analyzed. Gram negative organisms were isolated from 121(88.3%) specimens and Gram positive growth was seen in 16(11.7%) specimens. *Escherichia coli* was the commonest organism isolated (51.1%) followed by *Klebsiella pneumoniae* (13.1%). Among Gram positive organisms, *Enterococcus faecalis* was the predominant isolate (10.2%) (Table 1).

Out of 137, 37(27%) isolates showed biofilm formation by the gold standard tissue culture plate method. Maximum biofilm production was shown by *Enterococcus faecalis* (71%). *Escherichia coli* showed biofilm formation in 27% isolates followed by 16.7% in *Klebsiella pneumoniae* and *Enterobacter spp.* (Table 2).

Congo Red agar (CRA) detected biofilm formation in 40.88% isolates, Tube Method (TM) in 37.96% and Tissue Culture Plate Method (TCPM) in 27%. Twelve isolates were detected as biofilm producers only by CRA while seven such isolates were detected only by TM (Table 3).

When compared with TCPM, which is considered the gold standard phenotypic test for detection of biofilm formation in the present study, both CRA and TM demonstrated a sensitivity of 94.59%. TM showed higher specificity of 83% as compared to 81% shown by CRA on comparison with TCPM (Table 4).

Biofilms pose a serious problem for public health because of increased resistance of biofilm associated organisms to anti-microbial agents and the potential of these organisms to cause infections in patients with in-dwelling medical devices (Mulla SA *et al,* 2011). Bacteria in a biofilm survive antimicrobial agents at concentrations 1000-1500 times higher than those needed to eradicate their planktonic counterparts (Tenke P *et al,* 2004). Many bloodstream infections and urinary tract infections are associated with biofilm formation (Hola V *et al,* 2011). Despite good aseptic precautions, around 50% of catheterized patients develop bacteriuria in the first 10-14 days of catheterization (Noor AF *et al,* 2013).

Of the 137 specimens analyzed, Gram negative organisms were the predominant isolates accounting for 88.3% of the total growth. *Escherichia coli* was isolated from more than half the urine specimens (51.1%) followed by *Klebsiella pneumoniae* (13.1%) and *Enterococcus faecalis* (10.2%) (Table 1). This is in accordance with the studies published in Bangladesh by Noor *et al* (2013) and Behzadi P *et al* (2010). Indian studies conducted by Subramanian P *et al* (2012) and Jain *et al* (2011) also showed *Escherichia coli* and *Klebsiella pneumoniae* as the predominant uro-pathogens. Both Noor *et al* (2013) and Jain *et al* (2011) also showed *Enterococcus spp*. to be the commonest Gram positive organism isolated in their studies (6% and 5.86% respectively) similar to the present study (10%).

The maximum biofilm production was seen in *Enterococcus faecalis* where 10 out of the 14 isolates (71.4%) showed biofilm production (Table 2). This number is slightly greater than that reported by Praharaj *et al.* (2013) who found 53% of *Enterococcus spp.* isolates to be biofilm producers. In the present study, 27.1% isolates of *Escherichia coli* showed biofilm production. *Klebsiella pneumoniae and Pseudomonas aeruginosa* showed biofilm formation in 16.7% and 9.1% isolates respectively. This number is significantly lower than that reported by Subramanian *et al.* (2012a,b) (60%, 70%, and 70%). This is probably because in the present study, the samples analyzed were mid-stream urine specimens. In contrast, both studies conducted by Subramanian *et al* used only catheterized urine specimens for analysis. One isolate each of *Methicillin sensitive Staphylococcus aureus* and *Morganella morganii* found in the study showed biofilm production. No urinary isolate of *Acinetobacter spp., Proteus mirabilis* and *Klebsiella oxytoca* showed biofilm formation.

Table.1 Spectrum of organisms isolated

Organism	**Isolates**	**Percent**
Escherichia coli	70	51.1%
Klebsiella pneumoniae	18	13.1%
Enterococcus faecalis	14	10.2%
Enterobacter spp.	12	08.8%
Pseudomonas aeruginosa	11	08.1%
Acinetobacter spp.	05	03.6%
Proteus mirabilis	03	02.2%
Methicillin sensitive Staphylococcus aureus (MSSA)	02	1.5%
Morganella morganii	01	0.7%
Klebsiella oxytoca	01	0.7%
Total	**137**	**100%**

Table.2 Organism wise distribution of biofilm production

Organism	**Total Isolates**	**Biofilm producers**	**Percent**
Escherichia coli	70	19	27.1%
Klebsiella pneumoniae	18	03	16.7%
Enterococcus faecalis	14	10	71.4%
Enterobacter spp.	12	02	16.7%
Pseudomonas aeruginosa	11	01	09.1%
Acinetobacter spp.	05	00	-
Proteus mirabilis	03	00	-
Methicillin sensitive Staphylococcus aureus (MSSA)	02	01	50%
Morganella morganii	01	01	100%
Klebsiella oxytoca	01	00	-
Total	137	**37**	

Table.3 Detection of biofilm production by different phenotypic methods

Method	**Biofilm producers**	**Percent**
Congo red agar	56	40.88%
Tube method	52	37.96%
Tissue culture plate method	37	27%

Only Congo Red agar positive	12
Only Tube method positive	07
Only Tissue culture plate method positive	Nil

Table.4 Comparison of Congo Red agar method (CRA) & Tube Method (TM) with Tissue Culture Plate Method (TCPM)

CRA	TCPM		Total
	Positive	Negative	
Positive	35	21	56
Negative	02	81	81
Total	**37**	**100**	**137**

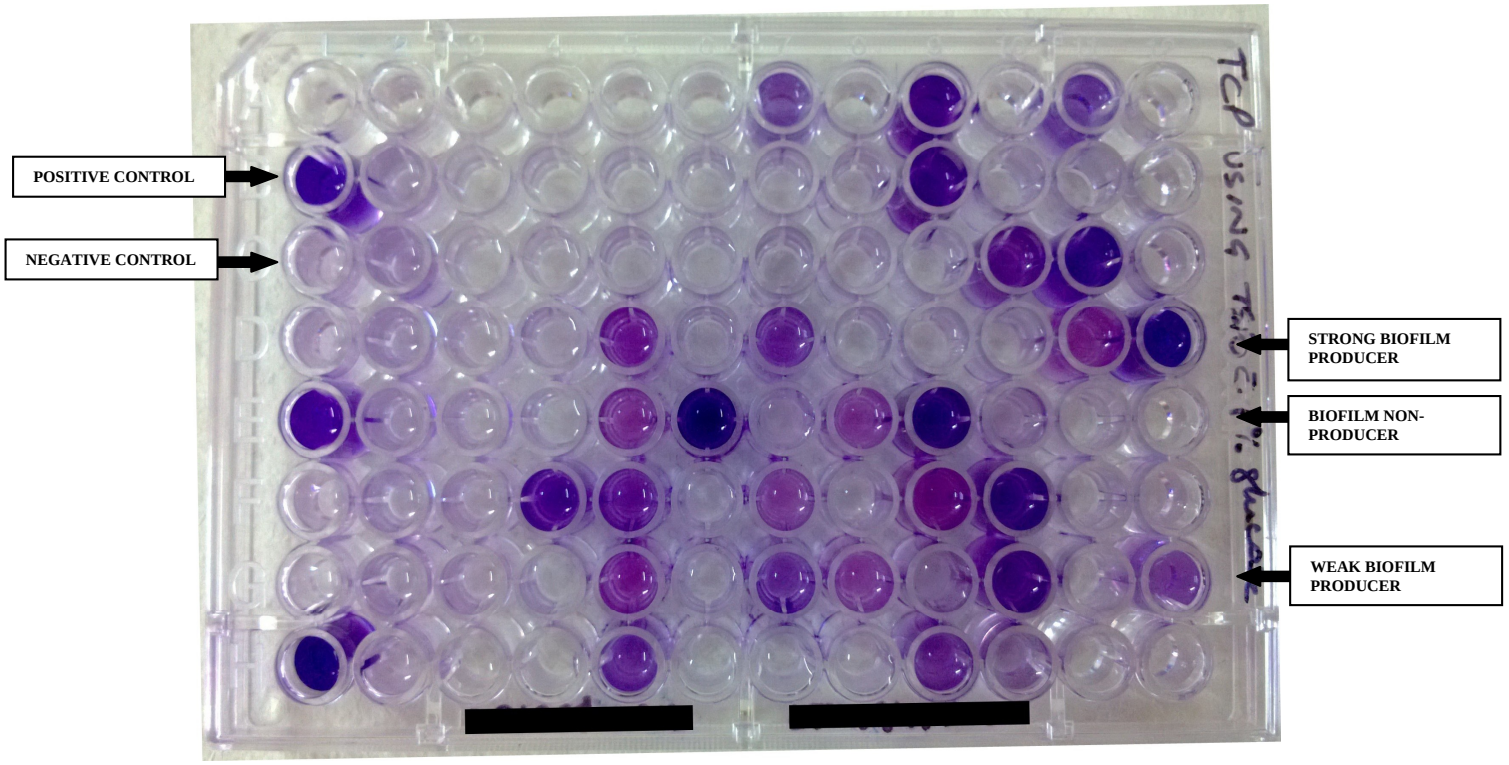
TM	TCPM		Total
	Positive	Negative	
Positive	35	17	52
Negative	02	83	85
Total	**37**	**100**	**137**

Figure.1 Congo Red agar plate showing black, dry, crystalline biofilm producing colonies of *Enterococcus faecalis*

Figure.2 Congo Red agar plate showing pink, moist, non-biofilm producing colonies of *Klebsiella pneumoniae*

Figure.3 Borosilicate glass tubes showing biofilm formation

Figure.4 Tissue culture plate showing biofilm formation



Congo Red Agar (CRA), Tube method (TM) and Tissue Culture Plate method (TCPM) were selected because of their ease of performance in routine laboratory settings. 137 clinical isolates from urine were tested for their ability to form biofilms using these three *in vitro* methods. Congo Red agar method detected biofilm formation in 56 (40.88%) isolates, Tube method in 52 (37.96%) isolates and Tissue Culture Plate method in 37 (27%) of the urinary isolates (Table 3). This pattern of biofilm detection is similar to the one demonstrated by Turkyilmaz S *et al* (2006) who detected biofilm formation in 61.1% samples by Congo Red agar method, 55.5% by Tube method and 50.5% by Tissue culture plate method in isolates of *Staphylococcus aureus*.

Twelve isolates were detected as biofilm producers by Congo Red agar method but not by Tube method or Tissue Culture Plate method (Table 3). This observation is similar to the one made by Subramanian P *et al* (2012) who showed that the Congo Red method was a more rapid and more sensitive method than the other phenotypic methods like the Tube method. Moreover, the subjective errors during reporting are seen more with Tube method as compared to Congo Red agar method. The Tube method showed seven isolates as being biofilm producers which were not detected by either Congo Red agar method or Tissue Culture plate method (Table 3). This can be explained by the use of the three colour scale of Freeman *et al.* (1989) in this study instead of the four colour scale developed by Satorres *et al.* (2007). Therefore, a few biofilm producers may have been missed. Subramanian *et al.* (2012) also missed two isolates by Congo Red agar method which were detected by Tube method. The Congo Red agar method is rapid, sensitive and reproducible and has the advantage that colonies remain viable on the medium. It is also not subject to inter-batch variation of media and observer subjectivity which sometimes affects the reproducibility of the Tube method (Freeman *et al* 1989). However, TCPM was the most specific test for detection of biofilm formation in the present study. The test is easy to perform and assesses biofilm forming capacity of an isolate both qualitatively and quantitatively. Subjective error is overcome in this method because the reading of biofilm formation is done using an ELISA reader.

Out of a total of 137 urinary isolates which were tested for biofilm production, 37 (27%) were detected by TCPM. CRA detected 35 of the 37 biofilm producers thus demonstrating a sensitivity of 94.59% and a specificity of 81% (Table 4). Studies conducted in India by Praharaj *et al.* (2013) and Golia *et al.* (2011) demonstrated similar findings with specificity around 80%. TM detected 35 out of 37 biofilm producers detected by TCPM (gold standard test for the present study) demonstrating a sensitivity of 94.59% and a specificity of 83%. Similar findings have been reported in other studies conducted by Golia *et al.* (2011), Praharaj *et al.* (2013) and Hassan *et al.* (2011). (Sensitivity= 61% to 100%, Specificity= 66% to 100%).

In another study conducted by Abdallah *et al* (2011), it was found that the highest percent of biofilm formation was detected in CoNS (57.1%) isolates followed by *Pseudomonas* (50.0%), *Klebsiella spp.* (44.4%), *Staphylococcus spp.* (42.9%), *E. coli* (31.6%) and *Enterococci* (28.6%) while least biofilm forming was *Proteus* (16.7%) and *Enterobacter* (0.0%). This study compared biofilm formation in mid-stream and catheterized urinary specimens. Hence, the biofilm production pattern could be vastly different from the present study where only mid-stream specimens have been analyzed.

To conclude, biofilms are a major cause of recurrent and recalcitrant urinary tract infection (UTI), leading to increased morbidity in the patient, increased duration of hospital stay and increased economic burden and drain on resources. Uncomplicated UTI has rarely been studied for biofilm formation over the entire spectrum of uro-pathogens. The role of biofilms in conversion of uncomplicated UTI to chronic UTI due to partial clearance of the infection needs to be studied in greater detail. Of the three phenotypic methods used to detect biofilm formation, Tissue culture plate method (TCPM) is a method with good reproducibility and good specificity. This method can be used routinely in the microbiology laboratory to detect biofilm formation.

Acknowledgement

The authors would like to thank the Department of Microbiology, Seth G.S. Medical College & K.E.M. Hospital, Mumbai, India for contributing the standard strains used as controls for the phenotypic tests.

References

Abdallah NMA, Elsayed SB, Mostafa YMM and El-gohary GM 2011. Biofilm forming bacteria isolated from urinary tract infection, relation to catheterization and susceptibility to antibiotics. Int J Biotechnol Mol Biol Res., 2(10):172-178.

Behzadi P, Behzadi E, Yazdanbod H, Aghapour R and Akbari Cheshmeh M 2010. A survey of urinary tract infections associated with three most common uropathogenic bacteria. Maedica;5:111-115.

Donlan RM 2002. Biofilms: Microbial life on surfaces. Emerg Infect Dis., 8(9):881-890.

Freeman J, Falkiner FR and Keane CT 1989. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol., 42:872-874.

Golia S, Hittinahalli V, Karjigi SK and Reddy KM 2012. Correlation between Biofilm Formation of uropathogenic *Escherichia coli* and its Antibiotic Resistance pattern. J Evol Med Den Sci;1(3):166-175.

Hall-Stoodley L and Stoodley P 2009. Evolving concepts in biofilm infections. Cellular Microbiol.., 11(7):1034–1043.

Harrison J, Turner R, Marques L and Ceri H 2005. Biofilms. American Scientist., 93(6): 508-516.

Hassan A, Usman J, Kaleem F, Omair M, Khalid A and Iqbal M 2011. Evaluation of different detection methods of biofilm formation in the clinical isolates. *Braz J Infect Dis.*, 15(4):305-311.

Hola V and Tenke P. 2011 eds. The Formation of Poly-Microbial Biofilms on Urinary Catheters, Urinary Tract Infections. ISBN: 978-953-307-757-4, InTech, Available from: http://www.intechopen. com/books/urinary-tract-infections/the-formation-of-poly-microbial-biofilms-on-urinary-catheters

Isenberg HD and Garcia LS 2010. Urine cultures. In: Garcia LS eds. Clinical Microbiology Procedures Handbook. 3rd ed. Washington (DC): American Society for Microbiology; Pg.1-18.

Jain S, Soni R, Bhuyar G and Shah H 2011. Prevalence of Uropathogens in Various Age Groups and Their Resistance Patterns in a Tertiary Care

Hospital in Central India. NJIRM., 2(4):7-10.

Kokare CR, Chakraborty S, Khopade AN and Mahadik KR 2009. Biofilm: Importance and Applications. Indian J Biotechnol., 8:114-168.

Mulla SA and Revdiwala S 2011. Assessment of biofilm formation in device associated clinical bacterial isolates in a tertiary care hospital. Ind J Patho Microbiol., 5(3): 561-564.

Noor AF, Shams F, Munshi SK, Hassan M and Noor R 2013. Prevalence and antibiogram profile of uropathogens isolated from hospital and community patients with urinary tract infections in Dhaka City. J Bangladesh Acad Sci., 37(1):52-63.

Praharaj I, Sistla S and Parija SC 2013. Virulence factors in Clinical and Commensal isolates of *Enterococcus species*. Ind J Path Microbiol., 56(1):24-30.

Prakash B, Veeregowda BM and Krisnappa G 2003. Biofilms- A survival strategy of bacteria. Curr Sci., 85(9):1299-1307.

Satorres SE and Alcaraz LE 2007. Prevalence of ICAA and ICAD genes in *Staphylococcus aureus* and *Staphylococcus epidermidis* strains isolated from Patients and Hospital Staff. Cent Eur J Public Health., 15(2):87-90.

Seno Y, Kariyam R, Mitsuhata R, Monden K and Kumon H 2005. Clinical implications of biofilm formation by *Enterococcus faecalis* in the urinary tract. Acta Medica Okayama;14(3):79-87.

Shirtliff M and Leid JG 2009. The Role of Biofilms in Device-Related Infections. Springer Series on Biofilms,Vol. 3.

Soto SM, Marco F, Guiral E and Vila J 2000. Biofilm Formation in Uropathogenic Escherichia coli Strains: Relationship with Urovirulence Factors and Antimicrobial Resistance. Clinical Management of Complicated Urinary Tract Infection. Dr. Ahmad Nikibakhsh (Ed.), ISBN: 978-953-307-393-4, InTech, Available from: http://www.intechopen.com/books/clinical-management-of-complicated-urinary-tractinfection/biofilm-formation-in-uropathogenic-escherichia-coli-strains-relationship-with-urovirulence-factors

Stickler DJ 2008. Bacterial biofilms in patients with indwelling urinary catheters. Nature., 5(11): 509-608.

Subramanian P, Shanmugam N, Sivaraman UD, Kumar S and Selvaraj S 2012(a). Antibiotic resistance pattern of biofilm forming uropathogens isolated from catheterized patients in Pondicherry, India. AMJ., 5(7):344-348.

Subramanian P, Shanmugam N, Sivaraman UD, Kumar S and Selvaraj S 2012(b). The Isolation and the Biofilm Formation of Uropathogens in Patients with Catheter associated Urinary Tract Infection (UTIs). J Clin Diag Research., 6(9):1478-1482.

Tenke P, Riedl CR, Jones GL, Williams GJ, Stickler D and Nagy E 2004. Bacterial biofilm formation on urologic devices and heparin coating as preventive strategy. Int J Antimicrob Agents., 23(1):67-74.

Turkyilmaz S and Ezkiizmirliler S 2006. Detection of Slime Factor Production and Antibiotic Resistance in Staphylococcus strains Isolated from Various Animal Clinical Samples. Turk J. Vet Anim Sci., 30:201-6.