Journal of Research in Biology

An International Scientific Research Journal

Original Research

Detection of biofilm formation in urinary isolates: need of the hour

Authors:
Saha R[1*], Arora S[1], Das S[1], Gupta C[1], Maroof KA[2], Singh NP[1] and Kaur IR[1].

Institution:
1. Department of Microbiology, University College of Medical Sciences and Guru Teg Bahadur Hospital, Dilshad Garden, Delhi – 110095, India.

2. Department of Community Medicine, University College of Medical Sciences and Guru Teg Bahadur Hospital, Dilshad Garden, Delhi – 110095, India.

Corresponding author:
Rumpa Saha.

ABSTRACT:

The purpose of the study was to estimate biofilm (BF) formation in urinary catheterized patients, by comparing three methods i.e. Tissue culture plate method (TCP), Congo Red Agar method (CRM) and Tube method (TM) and to study the antimicrobial resistance pattern in BF producing and non BF producing isolates. A total of 130 urinary catheterized patients were taken as the study group. From one milli litre of urine sample isolates $\geq 10^2$ colony forming units per milli litre were screened for the detection of BF by TCP, TM and CRM. Antibiotic sensitivity test for both BF producing and non BF producing bacterial and fungal isolates were done as per CLSI guidelines. From 130 urine samples in our study group, 55 samples grew microorganisms of significance, of which 11 samples were poly-microbial in nature. Of these biofilm production was seen in 49 microorganisms (89.09%) by any of the three methods used. TCP method picked up 69% of biofilm producers as compared to TM and CRM which picked up only 36% and 27% biofilm producers respectively. Our study reveals TCP method as the more dependable one as compared to TM and CRA methods for the quantitative biofilm detection, so it can be recommended as a screening method in laboratories.

Keywords:
Biofilm, biofilm detection, Congo Red Agar.

Abbreviations
BF - Biofilms; TCP - Tissue Culture Plate; CRM - Congo Red Method; TM - Tube Method; CLSI - Clinical Laboratory Standard Institute; CAUTI - Catheter associated Urinary Tract Infection; CLED - Cysteine Lactose Electrolyte Deficient; BHIB - Brain Heart Infusion Broth; TSB - Trypticase soy broth; ELISA - Enzyme linked immunosorbent assay; MHA - Muller Hinton Agar; MIC -Minimum Inhibitory Concentration; ATCC - American type culture collection; GPC -Gram positive cocci; GNB - Gram negative bacilli.

Web Address:
http://jresearchbiology.com/documents/RA0395.pdf.

Article Citation:
Saha R, Arora S, Das S, Gupta C, Maroof KA, Singh NP and Kaur IR.
Detection of biofilm formation in urinary isolates: need of the hour.
Journal of Research in Biology (2014) 4(1): 1174-1181

Dates:
Received: 01 Dec 2013 **Accepted:** 08 Feb 2014 **Published:** 17 Feb 2014

Journal of Research in Biology

INTRODUCTION

Indwelling urinary catheters play an essential part in the management of disorders of the urinary tract, especially in the elderly and disabled patients. These urinary catheters serve as a portal of entry for microorganisms leading to Catheter Associated Urinary Tract Infections (CAUTI). Many of these microbes colonize and adhere to the artificial surface of the indwelling catheters, which then forms biofilms. Biofilms are communities of microorganisms which are embedded within a matrix of extracellular polymeric material and display an altered phenotype. Based on the type and length of the stay of a gadget, composition of microorganism in a biofilm may vary from one to numerous. The same is true for urinary catheter biofilms where number of organisms is directly proportional to length of exposure**.**

Microorganisms commonly isolated from indwelling urinary catheters are *Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Proteus mirabilis* and *Candida* sp (Donlan, 2001).

Biofilms carry important clinical repercussions as they provide a niche for survival of microbes, by conferring protection to microbes from drying, mechanical damage and other influences from external environment, human immune system and antimicrobial agents (Costerton *et al.,* 1995; Mah and Toole, 2001). High antimicrobial concentrations are required to inactivate organisms growing in biofilms and resistance may often increases thousand folds. (Stewart and Costerton, 2001)

Moreover biofilms act as a persistent source of infection or may provide reservoir for new infections. The biofilms often leads to crystalline material blocking the catheters and induce complications like painful distension of the bladder, urolithiasis, reflux of infected urine resulting in pyelonephritis and sometimes urinary leakage around the outside of the catheter causing the patient to become incontinent, thus leading to failure of medical device.

There are different methods for the estimation of biofilm formation including Tissue culture plate method, Tube method, Congo Red agar method, bioluminescent assay, light or fluorescence microscopic examination, confocal laser scanning microscope and piezoelectric sensor (Mathur *et al.,* 2006).

There is paucity of data in Indian literature regarding biofilm formation in urinary catheterized patients. This study was undertaken with the aim to estimate biofilm formation in urinary catheterized patients, to compare three methods i.e. Tissue culture plate method (TCP), Congo Red method (CRM) and Tube method (TM) for biofilm production and to study antimicrobial resistance pattern in biofilm producing isolates.

MATERIALS AND METHODS

The study was done over a period of one year from April 2008 – March 2009 at department of Microbiology, of our tertiary care hospital after obtaining clearance from Institutional Ethical Committee. A total of 130 urinary catheterized patients were taken as study group who gave informed consent to the work. One ml of urine samples were collected from catheter with aseptic precautions and the samples were immediately sent to the Microbiology laboratory. The samples were plated on Cysteine Lactose Electrolyte Deficient (CLED) medium. The age, sex, days of catheterization of the patients were noted. Isolates were identified by standard microbiological procedures. The presence of $\geq 10^2$ c.f.u./ ml in aseptically collected urine was taken as significant bacteriuria (Winn *et al.,* 2006)**.** The cultures were maintained on nutrient agar slopes, Enterococci were maintained on brain heart infusion slopes and Candida species were maintained on Sabouraud's Dextrose Agar (SDA) slopes. Control strains used for biofilm production in the study were: *S. epidermidis* ATCC

35984 (strong biofilm producer), *S. epidermidis* ATCC 35983 (moderate biofilm producer) and *S. epidermidis* ATCC 12228 (non biofilm producer), *Acinetobacter baumannii* ATCC 19606 and *Candida albicans* ATCC 90028.

Biofilm formation was detected by the following three methods:-

Tissue culture plate method (Christensen *et al*., 1995):

Isolates from freshly subcultured plates were inoculated in trypticase soy broth (TSB) with 1% w/v glucose and incubated for 18 hours at 37˚C in stationary conditions and then diluted to 1:100 with fresh TSB. Individual wells of sterile polystyrene 96 well flat bottom microtitre plates were filled with 200µl aliquots of diluted culture. Un-inoculated TSB served as a control to check sterility and non specific binding of media**.** Control strains were also inoculated in triplicate. The microtitre plate was incubated for 24 hrs at 37˚C. After incubation contents of each well was removed by tapping the plates. After washing the wells for four times with 200µl of phosphate buffer saline (PBS pH 7.2), the floating planktonic bacteria were removed. The biofilms thus formed in plates were fixed using 2% w/v sodium acetate for 10 minutes and tainted with 0.1% w/v crystal violet for 30 minutes. After washing thoroughly with de-ionized water to remove any excess stain, the plates were dried. Micro-ELISA auto-reader at the wavelength of 540 nm was used to measure the Optical Density (OD) of the stained adherent micro-organisms. The OD_{540} value of sterile medium, fixative and dye were averaged and subtracted from all test values. The mean OD_{540} value from a control well was deducted from all test OD_{540} values. These OD_{540} values were considered as an index of bacteria adhering to surface and forming biofilms. Experiments were performed in triplicate. Interpretation of biofilm production was done according to the criteria of Stepanovie *et al.,* (2007). (Table 1)

Table 1. Interpretation of biofilm production

Average OD value	Biofilm production
$\leq OD_{540C}$/ $OD_{540C} < \sim \leq 2x\ OD_{540C}$	Non/weak
$2x\ OD_{540C} < \sim \leq 4x\ OD_{540C}$	Moderate
$> 4x\ OD_{540C}$	Strong

Tube method:

A quantitative method was used as described by Christensen *et al.,* (1982). Ten milli litre of BHI broth with 1% w/v glucose was taken in test tubes and was inoculated with loop full of microorganism from overnight culture plates and incubated at 37˚C for 24 hrs. The tubes were washed with PBS (pH 7.3) after decanting the culture. The dried tubes were then stained with crystal violet (0.1% w/v) for 30 minutes after fixing with sodium acetate (2% w/v) for 10 minutes. Through washing was again done with de-ionized water to remove excess stain. Tubes were then kept in inverted position for complete drying. Biofilm formation was detected by the presence of visible film on the wall and bottom of the tube. Ring formation at the liquid culture interface was taken as negative. The amount of biofilm formation was scored according to the results of control strains and graded as 0, 1, 2 and 3 denoting absent, weak, moderate and strong biofilm formation respectively. Experiments were performed in triplicate.

Congo red agar method (Freeman *et al.,* 1989):

Congo red media was prepared as a concentrated aqueous solution of 0.8 g/l of Congo red and autoclaved separately from other medium constituents [brain heart infusion broth (37 g/l), sucrose (50 g/l), agar (10 g/l)]; then added when agar gets cooled to 55˚C. The required microbial strains were inoculated on the prepared media and incubated aerobically for 24 hrs at 37˚C. Growth of black colonies with a dry crystalline consistency was taken as positive biofilm production; pink colonies with occasional darkening at the centre of the colonies were non biofilm producers. Black colonies without dry crystalline colonial morphology indicated indefinite results. The experiment was performed in triplicate and repeated for three times.

Table 2. Comparison of biofilm production by three methods – TCP, TM and CRM

Isolate	TCP (%)	TM (%)	CRM (%)	No BF producer (%)
Gram positive organism n-12	11(91.66)	2(16.66)	2 (16.66)	1 (8.33)
Staphylococcus aureus n = 8	7	1	2	1
Enterococcus sp n = 4	4	1	0	0
Gram negative organism n-37	24(64.86)	17(45.94)	12 (32.43)	4 (10.81)
Escherichia coli n = 20	13	11	5	3
Klebsiella sp n = 7	4	3	3	1
Citobacter sp n = 2	1	0	1	0
Proteus sp n = 2	1	1	2	0
Acinetobacter sp n = 2	2	1	0	0
Pseudomonas sp n = 4	3	1	1	0
Candida sp n-6	3 (50)	1(16.66)	1 (16.66)	1 (16.66)
Candida albicans n = 2	1	0	0	1
Candida tropicalis n = 4	2	1	1	0
Total n = 55	38(69.09)	20(36.36)	15 (27.27)	6 (10.90)

Antimicrobial susceptibility testing was done on Muller-Hinton agar (MHA) for both biofilm producing and non biofilm producing bacterial isolates by Kirby Bauer disk diffusion method as per Clinical and Laboratory Standards Institute guidelines (CLSI, 2006)**.** The antibiotic panels used were 25µg Cotrimoxazole, 30µg Cefotaxime, 30µg Vancomycin, 300 units Nitrofurantoin, 10µg Norfloxacin, 120µg High level gentamicin, 30µg Tetracycline, 30µg Amikacin, 10µg Gentamicin, 10µg Imipenam, 100µg Piperacillin; 10µg Tazobactam and 300 units Polymyxin B . Antibiotics discs were procured from HiMedia Laboratories Pvt. Ltd, India.

Antifungal susceptibility profile of BF forming and non biofilms forming Candida isolates was done by determining MIC for Amphotericin B, Itraconazole and Fluconazole by microdilution method as described by CLSI guidelines (CLSI, 2008)**.** *Candida albicans* ATCC 90028 were used as control.

Statistical Analysis:

Data entered in MS Excel and SSPS 17.0 were used for data analysis. Chi square test was used to compare proportions between various groups. Sensitivity, Specificity and predictive values were calculated using the standard formulae.

RESULTS

Among 130 urine samples from our study group, 55 samples grew microorganisms of significance of which 11 samples were polymicrobial in nature. Of these biofilm production was seen in 49 microorganisms (89.09%) by any of the three methods used. All sets of polymicrobial organisms were biofilm producers. All comparisons were done keeping TCP as gold standard. The different organism isolated and their biofilm producing capacity is compared in Table 2.

TCP method picked up 69% (38) of biofilm producers as compared to TM and CRM which picked up only 36% (20) and 27% (15) of biofilm producers respectively. This difference was found to be highly significant ($x^2 = 17.55$, $P < 0.001$). Table 3 shows sensitivity and specificity of TM and CRM. By TCP method, the number of strong biofilm producers were 20

Table 3. Diagnostic parameters TM and CRM for Biofilm detection

Parameters	TM	CRM
Sensitivity	34.21%	21.05%
Specificity	58.82%	58.82%
Positive Predictive Value	65.00%	53.33%
Negative Predictive Value	28.57%	25.00%

Table 4. Comparison of antimicrobial resistance pattern of BF producer with non BFproducers

Antimicrobial agents	BF producer (%)	Non BF producer (%)
Staphylococcus aureus n =8	**n= 7**	**n=1**
Cotrimoxazole	6(85.71)	1 (100)
Cefotaxime	5(71.42)	1 (100)
Vancomycin	0	0
Nitrofurantoin	3(42.86)	0
Norfloxacin	6(85.71)	0
Enterococcus n-4	**n=4**	**n = 0**
Vancomycin	1 (25)	-
High level Gentamicin	4(100)	-
Nitrofurantoin	2 (50)	-
Norfloxacin	4(100)	-
Tetracycline	4(100)	-
Gram negative organism n=33	**n= 21**	**n = 12**
Amikacin	15 (71.43)	6 (50)
Gentamicin	15 (71.43)	6 (50)
Cotrimoxazole	18 (85.71)	6 (50)
Imipenam	7 (33.33)	1 (8.33)
Piperacillin-Tazobactam	15 (71.43)	5 (41.67)
Norfloxacin	17 (80.95)	8 (66.67)
Nitrofurantoin	13 (61.90)	4 (33.33)
Pseudomonas n = 5	**n = 4**	**n = 1**
Amikacin	3 (75)	1 (100)
Gentamicin	3 (75)	1 (100)
Imipenam	3 (75)	0
Piperacillin-Tazobactam	2 (50)	0
Polymyxin B	0	0
Norfloxacin	3 (75)	0
Candida spp n = 6	**n=3**	**n=3**
Fluconazole	2 (66.67)	1 (33.33)
Itraconazole	3 (100)	2 (66.67)
Amphotericin B	0	0

and the same by TM and CRM was 3 and 14 respectively and this difference was found to be highly significant ($x^2 = 21.4$, $P < 0.001$, d.f = 2). (Figure1). When degree of biofilm production was compared, TM showed similar detection rate with TCP for moderate biofilm producers, but the same is not true for strong biofilm producers. This difference was also highly significant.($x^2 = 21.06$, $P < 0.001$, d.f = 1). Figure 2 shows colonies of biofilm and non biofilm producers on Congo Red medium.

The antimicrobial resistance pattern of the biofilm producing isolates is given in Table 4. Among the gram negative organism, the resistance was more for biofilm producers as compared to non biofilm producers however it was not significant except for Cotrimoxazole ($x^2 = 4.911$, $P = 0.0266$).

Biofilm production has also increased significantly with the days of catheterization ($x^2 = 16.88$, $P < 0.001$) (Figure 3).

DISCUSSION

More than 40% of all healthcare associated infections are due to CAUTI. Eradication of biofilm based catheter related infection is often challenging because they exhibit increased resistance to antimicrobial therapies by various mechanisms (Douglas, 2003).

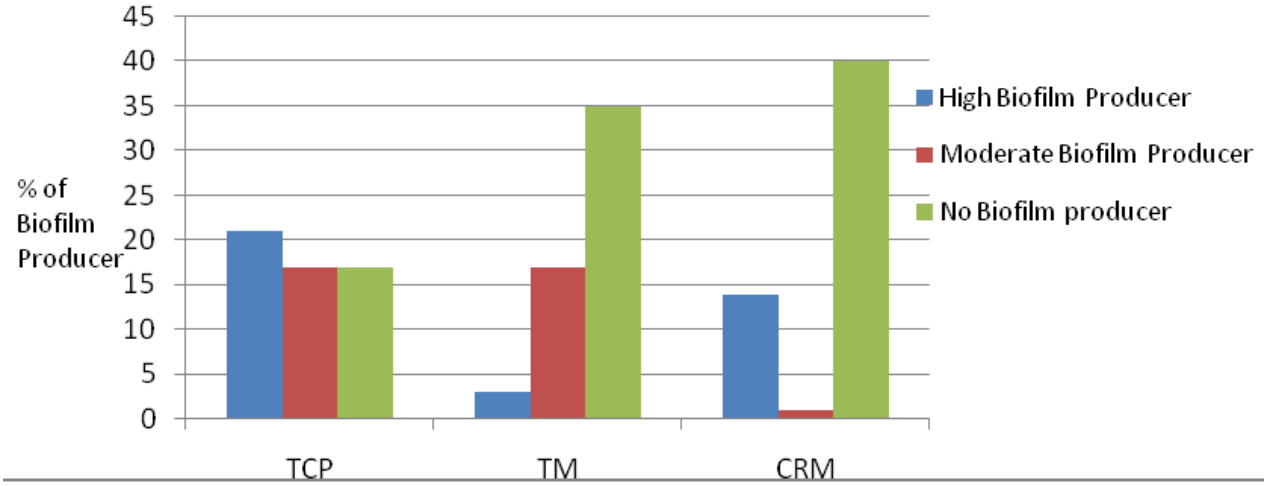

Figure 1 Degree of biofilm formation by TCP, TM and CRM

In this study we evaluated 55 isolates by three different screening methods for their ability to form biofilms. In our study we have found that TCP method detected biofilm formation in 69% of isolates. We have used 1% sucrose in BHI for growing biofilms in microtitre plate. Addition of sugar increases the biofilm production; as reported by other authors (Mathur *et al.,*2006; Bose *et al.,* 2009 ; Hassan *et al.,* 2011).

Overall TCP method detected maximum biofilm producers. The ability to detect biofilm production of Gram Positive Cocci (GPC) was less for TM and CRM method as compared to TCP method whereas TM and CRM picked up greater number of biofilm producers among the Gram negative bacilli (GNB). This difference was however not significant (x^2 = 197, *P* = 0.1226, d.f = 2).

Figure 2. Colonies of biofilm and non biofilm producers on Congo Red agar medium

TM detected 36% of isolates as biofilm producers while 63% isolates were identified as non biofilm producers. TM is only 34.21% sensitive, 58.82% specific for biofilm detection. This is not consistent with the findings of Mathur *et al.,* 2006; Bose *et al.,* 2009 from India, who reported higher sensitivity and specificity for Tube method. In our study, this method correlated well with TCP for identifying moderate biofilm producers (30.90% i.e. 17 / 55), but detection rate for high biofilm producer was very low (5.45% i.e. 3/55). This difference may be due to the inter-observer variability in the reading of results, resulting in low sensitivity and specificity in our study.

Only 27% isolates were identified as biofilm producers by CRM similar to Ruzicka *et al.*, 2004 who detected 43.5% of biofilm producers by this method. This was higher in comparison to the 3-6% detection rate by other workers from India and Pakistan (Mathur *et al.*, 2006; Bose *et al.*, 2009; Hassan *et al.*, 2011). The sensitivity and specificity, however, remained low (21.05% and 58.82% respectively). Surprisingly, in this study CRM outscores TM in the detection of high

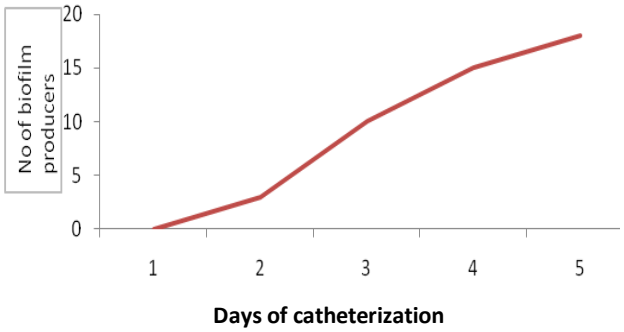

Figure 3. Relationship of Biofilm production with duration of catheterization.

biofilm producers. CRM detected 25.45% (14/55) while TM detected 5.45% isolates as high biofilm producers and this difference was highly significant. CRM is a comparatively easier method and also over-rules inconsistency by observation which could possibly explain such finding.

The antimicrobial susceptibility pattern of microbes isolated from clinical samples has important implications especially in clinical settings as it helps clinicians to decide treatment protocol for patients and also help hospital infection control team to formulate hospital antibiotic policies. As biofilms form significant reservoir of infection, it is essential to find antibiogram for biofilm producing isolates. In our study, we found that biofilm producing gram negative isolates were more resistant to antimicrobial agents as compared to non biofilm producing isolates. However comparison could not be done in case of *Enterococci* sp as all the isolates produced biofilm and in case of *Staphylococcus aureus* and *Pseudomonas* sp there were unequal distribution of biofilm producing and non biofilm producing isolates. More antimicrobial resistance among biofilm producers has also been seen in other studies (Hassan *et al*., 2011; Ruzicka *et al*., 2004). Some of the non biofilm producing strains were also resistant to antimicrobial drugs. The enhanced survival of drug resistant pathogens may be due to the widespread injudicious use of broad spectrum antibiotics in our setup, which is a tertiary care hospital.

CONCLUSIONS

The ability of microorganisms to form biofilms on the medical devices is a challenge for the clinicians because biofilm associated microorganisms are much more resistant to antimicrobial agents, which may result in treatment failure. Therefore effective treatment strategies should be explored to deal such infections. Our findings indicate that TCP is a suitable and reproducible method for the screening of biofilm producers in health care setups.

REFERENCES:

Bose S, Khodke M, Basak S and Mallick SK. 2009. Detection of biofilm producing staphylococci: need of the hour. J Clin Diagn Res., 3(6):1915-1920.

Christensen GD, Simpson WA, Bisno AL and Beachey EH. 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun. 37(1): 318-26.

Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM and Beachey EH. 1985. Adherence of coagulase negative Staphylococci to plastic tissue cultures: a quantitative model for the adherence of Staphylococci to medical devices. J Clin Microbiol., 22(6): 996-1006.

CLSI. 2006. Performance standards for antimicrobial susceptibility testing, Sixteenth information supplement. CLSI document M-100-S16 (M7). Wayne PA: Clinical and Laboratory Standards Institute. 26(3)

CLSI. 2008. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. Approved Standard, 3rd edn. CLSI document M27-A3. Villanova, PA: Clinical and Laboratory Standards Institute.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR and Lappin-Scott HM. 1995. Microbial Biofilms. Annu Rev Microbiol., 49: 711-745.

Donlan RM. **2001.** Biofilm formation: A clinically relevant microbiological process. Clin Infect Disease. 33 (8): 1387-1392.

Douglas LJ. **2003.** Candida biofilms and their role in infection. Trends Microbiol., 11(1): 30-36.

Freeman DJ, Falkiner FR and Keane CT. **1989.** New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol., 42(8):872-874.

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.

Mah TF and O'Toole GA. **2001.** Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol., 9(1): 34-39.

Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T and Rattan A. **2006.** Detection of biofilm formation among the clinical isolates of staphylococci: An evaluation of three different screening methods. Indian J Med Microbiol., 24(1):25-29.

Ruzicka F, Hola V, Votava M, **Tejkalová R, Horvát R, Heroldová M and Woznicová V**. **2004.** Biofilm detection and clinical significance of *Staphylococcus epidermidis* isolates. Folia Microbiol (Praha). 49(5): 596-600.

Stepanovic S, Vukovi D, Hola V. Bonaventura GD, Djukić S, Ćirković I and Ruzicka F. 2007. Quantification of biofilm in microtitre plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. APMIS. 115(8): 891-899.

Stewart PS and Costerton JW. **2001.** Antibiotic resistance of bacteria in biofilms. Lancet. 358(9276): 135 -138.

Winn W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P and Woods G. 2006. Editors Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6 th ed. Philadelphia: Lippincott Williams and Wilkins.