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Study Of MIC & MBEC Of Planktonic & Sessile Form Of Biofilm Strains Of Urinary Catheter(> 1m In-Situ)With Nitrofurantoin In A Tertiary Care Hospital

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Abstract: *Biofilms are complex communities of single or multiple spp of microorganisms that develop on abiotic & biotic surfaces.Since biofilms contaminate catheters,ventilators & medical implants,they act as a source of disease in humans.They also represent a barrier of eradication as the physiological state of bacterial cells within biofilm confers high level resistance to antibacterial substance. In this study; >1 month urinary catheters in-situ have been selected as clinical samples due to their prompt availability with pre-formed biofilms on most of them. During evolution of biofilm increasingly resistant sub-populations appear with time. Drug sensitivity of planktonic form of a potential biofilm-producing organism is determined by minimum inhibitory concentration(MIC)value, while minimum biofilm elimination concentration (MBEC) value denotes the drug concentration required to eliminate highest resistant sub-population within the biofilm.So MEBC is variable depending upon maturation of biolfilm with colonisation of highly resistant sub-populations.In this study an attempt has been made to compare the MIC value of planktonic form with MBEC value of sessile form of same organism in preformed biofilm.Nitrofurantoin has been choosen for this study as the drug is widely used as urinary antiseptic.*

Key words*: Biofilm, quorum-sensing,N-acyl homoserine lactone, ppGpp, sigma factor, MIC,MBEC*

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I. Introduction:

The United states National Institute of Health reports that 80% of chronic infections are biofilm related [1] . The urinary bladder resists infection by the periodic passing of urine, which washes out unattached pathogen & sloughing of colonised uroepithelial cells on glycosaminoglycan(GAG)mucous layer.Urinary catheter-associated infection increases by approximately 10% each day the catheter is in place.The microorganisms form biofilm on catheter in-situ.During periodic removal of catheter ; portion of biofilm detach,planktonic form arises & ascend upward to act as a persistant source of infection.

The new definition of a biofilm is; a microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other,are embedded in a matrix of extracellular polymeric substances(slime) that they have produced & exhibit an altered phenotype with respect to growth rate & gene transcription [2].Immobilised bacterial cells within a biofilm are called sessile, whereas those free floating in the aquatic environment are called planktonic.The close proximity of cells within the biofilm allows plasmid transfer.Biofilms are formed preferentially in very high shear environment(i,e rapidly flowing milieus)exeed Raynolds number of 5000. Bacterial two-component signal transduction systems (TCSTS) consist of 2 regulatory proteins ,a cytolasmic membrane-associated sensor receives specific signal from environment & a response regulator that mediated the internal response of bacterial cells. Sensor & response regulator proteins exchange information by transferring a phosphate group.TCSTS can sense a variety of signals including quorum-sensing signal molecule.The term 'quorum sensing' is used to describe the phenomenon where accumulation of a diffusible LMW signal molecule (called autoinducer)enables individual bacterial cells to sense the minimal number or 'quorum' of bacteria,that has been achieved for a concerted response to be initiated [3] . The signalling molecule N-acyl homoserine lactone (AHL) is mainly responsible for structural complexity of biofilm [4] by gm-ve bacteria. LuxI & LuxR protein families function as AHL synthases & transcripyional regulators respectively.In Gm+ bacteria quorum-sensing molecules are small modified peptides. S.aureus & S.epidermidis produce autoinducing peptides (AIP) via the agr regulatory locus [11].

The enhanced antibiotic resistance [5] of biofilms has been attributed to the presence of EPS matrix,a slow growth rate, spatial heterogenecity & biofilm-specific drug tolerant physiologics including the presence of persister cells & small colony variant.Complex slime secreted by bacteria,provide a barrier to immune cells like phagocytes.When gm-ve bacteria is subjected to nutrient limitation or to antimicrobial agents, the growth rate decreases & gene expression markedly altered.This is essential for long term survival of cells & is partly mediated by alternative sigma factors (σ^s)encoded by rpoS gene.Positive regulation of this expression results in σ^s synthesis being activated by guanosine 3´-diphosphate(ppGpp).The cells producing ppGpp are more resistant to biocids & antibiotics [6].The oxidative stress response (to H_2O_2) involves the production of neutralising enzymes to prevent cellular damage, with the SOS defence system response coming into operation when DNA damage take place [7].

The small subpopulation of bacteria or Persisters (pocket of surviving organisms in biocide treated biofilms) do not have any specific resistance mechanisms.Hyperresistance in these persisters is a transient phenotypic switch & on reculturing, they convert to wild-type with a new population of persisters [8]. Furthermore sublethal treatment with a biocide could induce the expression of multidrug efflux pumps & efflux mutants.Thus mar (multiple antibiotic reflux) expression is greatest within the depth of biofilm; where growth rate is lowest [9].Lewis [10] has proposed that the inactivation of a cell does not occur as a result of direct action of a biocide, but rather from programmed cell death(PCD). Persisters of such a programme are then regarded as cells deficient in PCD,that grow rapidly in presense of exudates released from lysed community cells.

Nitrofurantoin is employed for prophylaxis of UTI when catheterisation or instrumentation of lower urinary tract is performed. The drug, in orally tolerated doses, attain antibacterial concentration only in the urinary tract & may be considered as a form of local therapy. Reactive nitrofurantoin metabolites causes DNA strand breakage of bacteria, also interfere with protein synthesis.Resistance to nitrofurantoin develops slowly & no cross resistance with any other antimicrobial agent is known.The drug is well-absorbed orally(bioavailability 90%),plasma t1/2 30-60 min. Antibacterial concentrations are not attained in blood or tissues.

The intrinsic activity of antimicrobial drugs in vitro is usually expressed in terms of minimum inhibitory concentration(MIC); less commonly by minimum bactericidal concentration (MBC). These values are derived by titration of the drug against a standard inoculums of pure culture of isolated bacterial spp. in broth or on agar plates. MIC is the lowest antimicrobial concentration that inhibits the visible growth after a 16-20 hrs of incubation for most common pathogens.MBC is assessed from broth titrations by subculture of those dilutions of antibiotic below the MIC & defined as the lowest concentration that kills 99·9% inoculums(i.e, a fall in viable count of at least 3log $_{10}$CFU/ml).MIC tests the sensitivity of the bacteria in their planktonic phase.The MIC is of limited value in determining the true antibiotic susceptibility of bacteria in their biofilm phase.The minimum biofilm eliminating concentration (MBEC) assay,on the other hand ,allows direct determination of drug sensitivity in biofilm phase (sessile) of bacterial development.

II. Aim & Objectives:

Aim of this study is to develop appropriate antimicrobial policy for management of biofilm mode of bacterial infections in urinary tract.The objective of this study is to demonstrate any difference of antimicrobial concentrations to inhibit planktonic form of biofilm isolates & sessile form within catheter surface biofilm.

III. Materials & methods:

A total of 50 consequetive, non-repeative, urinary Foley catheter (Proximal parts) were collected over a period of 1 yr at Institute of Postgraduate Medical Education& Research(IPGME&R);from patients attending at urology OPD.The study included the patients of all age groups & both sexes,having in-situ urinary catheterisation for >1 month with a history of chronic UTI for any cause,preoperative & postoperative cases,road traffic accidents etc.

Catheter samples kept in dessicator for >2months;assuming that biofilm strain will survive in stressful environmental condition.

For broth dilution method; the proximal part of catheters washed with NS several times, aseptically transferred into Brain Heart Infusion(BHI) broth in test tubes & incubated overnight. 100 mg Nitrofurantoin dissolved in phosphate buffer solution (pH 8·0,0·1 mol/L) according to CLSI guideline. Stock solutions are prepared. Following concentrations of nitrofurantoin are made by serial dilution from stock solution; 200µg/ml,100µg/ml, 50µg/ml, 25µg/ml,12·5 µg/ml 6·25 µg/ml, 3 µg/ml, 1·5 µg/ml. 20 ml of Brain heart infusion broth (BHIB) is poured to all dilution tubes Including a control tube (medium without antibiotic).Two sets of such tubes of BHI broth with nitrofurantoin were prepared . In one set loopfull from overnight growth of catheter-containing BHI (planktonic form) inoculated & are incubated for 48 hr at 37 ◦C, then examined with naked eye for turbidity. To confirm naked-eye reading & to demonstrate cidal as well as static effects , loopful of broths are streak-inoculated on to nutrient agar plate. The subculture from the tube containing the

MIC, formed no more than a few colonies derived from inoculated bacteria that have survived exposure to the antibiotic. Subcultures from the tubes containing less than the MIC gave hazy growths & S/C from tubes containing cidal concentrations (MCC) would give no growth.In the other set,the catheter pieces serially transferred aseptically from lower to higher concentrations of nitrofurantoin-BHIB & incubated overnight, then scraped & subcultured on nutrient agar to get MBEC value of sessile form of bacterial strains.In each step,the catheter pieces scraped to cross-check the viability of biofilm organisms by subculturing on blood agar.A sterility check was also included for each broth dilution in every batch of tests.

For agar dilution method; serial dilutions of nitrofurantoin(50 µg/ml 20 µg/ml 10µg/ml 5µg/ml 2·5 µg/ml) are mixed with 20 ml of nutrient agar, autoclaved(at 121◦C for 15 min) & poured into plates. Nitrofurantoin tolerates autoclave temperature. From overnight growth in BHIB; strains, standerdised with 0·5 McFarland turbidity (working inoculums 10^7 cfu/ml) were inoculated on each plate (as 'spot') containing an antibiotic dilution. After overnight incubation,plates were checked for visible growth /no growth & MIC of planktonic forms determined accordingly.Hazy growth or 1 /2 colonies on the 'spot' is ignored with the agar dilution method.

IV. Result:

Urinary pathogens are said to be susceptible to nitrofurantoin in MIC value 32 µg/ml or less. At the concentration achieved in urine (100 µg/ml), nitrofurantoin is bactericidal.

With a 100mg oral dose of nitrofurantoin plasma levels are typically <1 µg/ml, while in urine it reaches 200 µg/ml [12].

MIC often difficult to interprete, so it is convenient to describe a series of MIC results by defined criteria.This criteria are the range ,the MIC_{50} & MIC_{90} values.This may be obtained by arranging the MIC results from lowest to highest. MIC value of the strain that appears 50% up the series refers to MIC_{50} & value that appears 90% up the series refers to MIC_{90}

Result of MIC versus MBEC of planktonic & sessile forms respectively with nitrofurantoin (broth dilution method):

Biofilm isolates	Total number of biofilm isolates	MIC of planktonic forms	MBEC of sessile forms	Interpretation
E.coli	10	N=8,[3µg/ml] N=2,[6·25µg/ml]	N=8,[12·5µg/ml] N=2,[6·25µg/ml]	MBEC>MIC MBEC=MIC
Klebsiella spp.	8	N=4,[25µg/ml] N=3,[6·25µg/ml] N=1,[1·5µg]	N=4,[200 µg/ml] N=3,[50µg/ml] N=1,[50µg/ml]	MBEC>MIC ,, ,,
Proteus mirabilis	5	N=2, [25µg/ml] N=3,[12·5µg/ml]	N=2, [200µg/ml] N=3, [50µg/ml]	MBEC>MIC ,,
Pseudomonas aeruginosa	12	N=1,[>200µg/ml] N=11,[>200µg/ml	N=1,[>200µg/ml] N=11,[>200µg/ml	No cut off point obtained for both MIC &MBEC
Citrobacter spp.	7	N=6[25µg/ml] N=1[3µg/ml]	N=6, [200µg/ml] N=1, [50µg/ml]	MBEC>MIC ,,
Staphylococcus aureus	8	N=6[25µg/ml] N=1,[6·25µg]	N=6, [200µg/ml] N=1, [100µg/ml]	MBEC>MIC ,,

N= total number of sensitive isolates to a particular drug concentration

Interpretation: the antibiotic susceptibility of sessile organisms in their biofilm state tested by MBEC assay is significantly higher than the MIC of same organism in their plaktonic form. But Pseudomonas strains yielded different result,both planktonic & sessile forms showed resistance to nitrofurantoin in all dilutions.

Result of MIC determination of biofim strains(planktonic forms)with nitrofurantoin(agar dilution method):

Biofilm isolates	Number	MIC 2·5µg/ml	MIC 5µg/ml	MIC 10µg/ml	MIC 20µg/ml	MIC 50µg/ml
E.coli	10	N=6	-	-	N=4	-
Klebsiella spp.	8	-	-	N=2	N=5	N=1
Proteus mirabilis	5			N=1	N=4	-
Pseudomonas spp.	12	-	-	-	-	N=1
Citrobacter spp.	7	N=1	N=1	-	N=5	-
Staphylococcus spp.	8	-	-	N=2	N=4	N=2

Interpretation: Among the biofilm isolates (planktonic)E.coli strains were more sensitive to nitrofurantoin. Pseudomonus spp. were mostly either insensitive or with much higher MIC value.

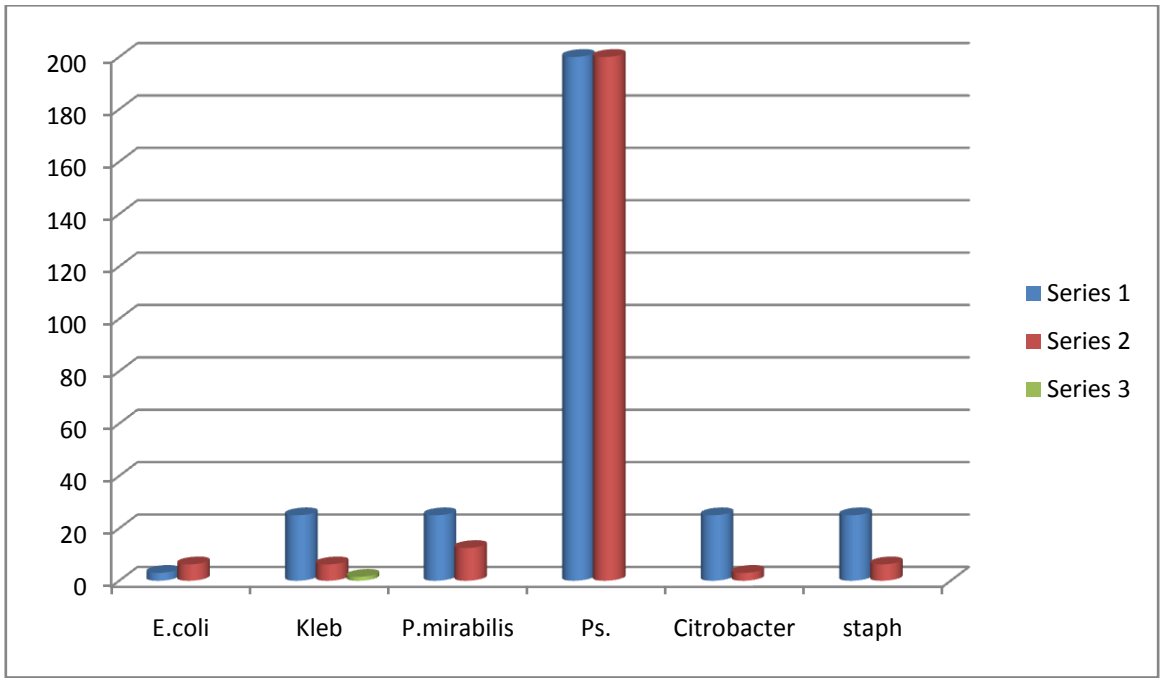

Fig: Graphical representation of MIC of the planktonic forms

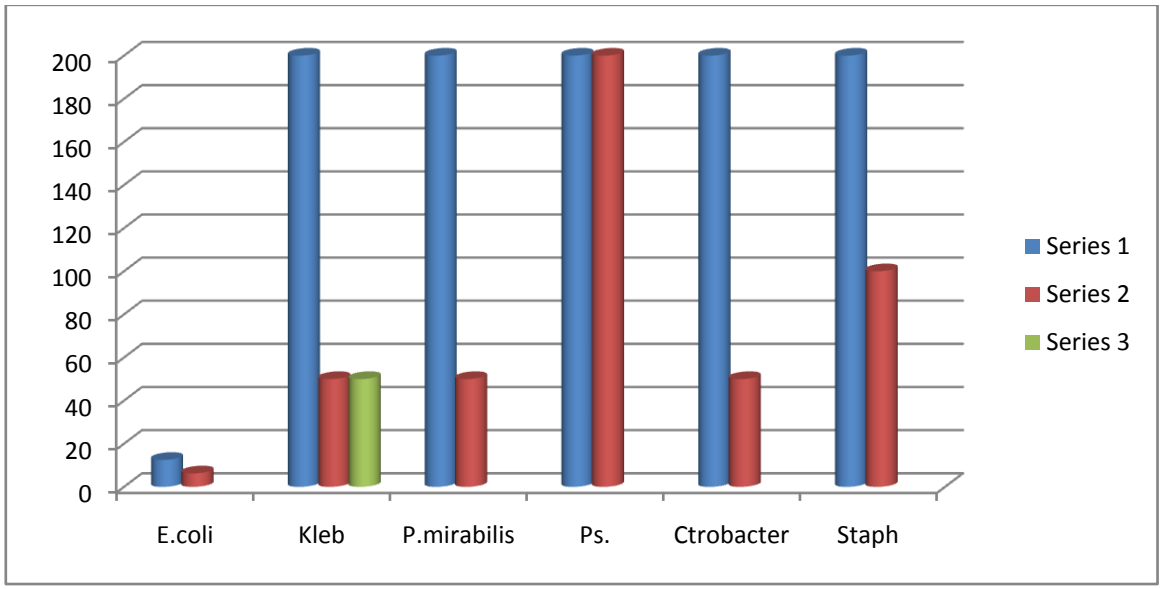

Fig: Graphical representation of MBEC value of sessile form of biofilm isolates

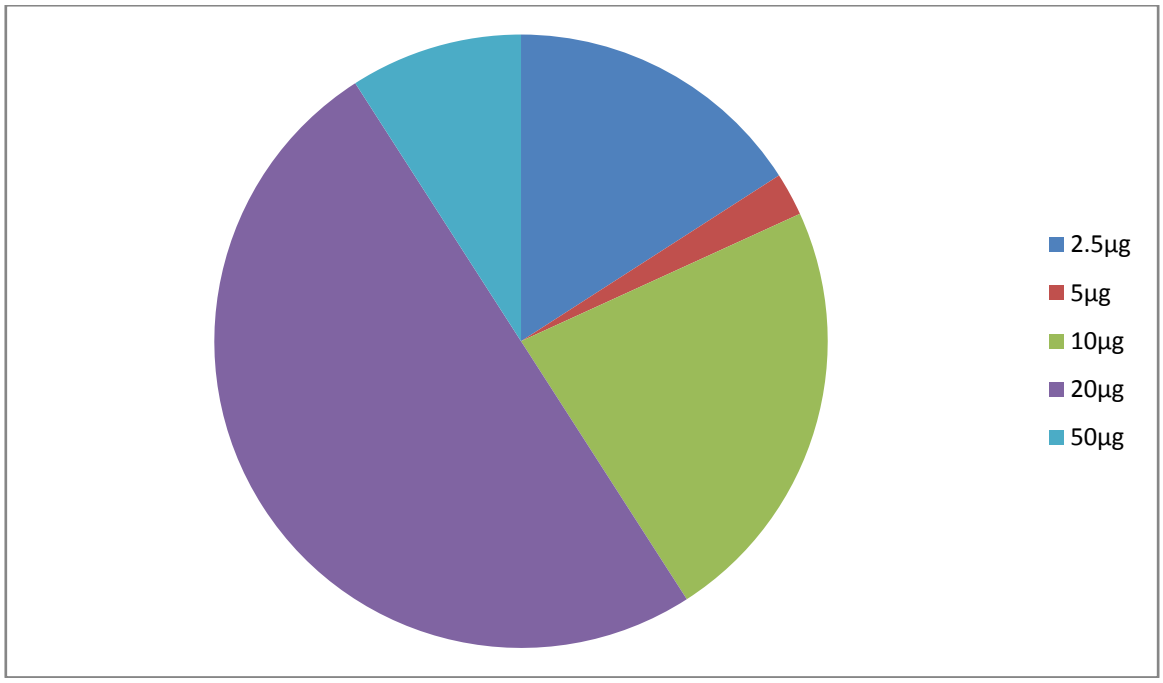

Fig: Pie chart representing the % of biofilm isolates(planktonic forms) showing MIC at various concentrations (µg/ml) of Nitrofurantoin (agar dilution method)

MIC 50: These values are defined as the lowest concentration of the antibiotic at which 50% of the isolates are inhibited [14].From the study it has been found that 44% (around 50)biofilm isolated are inhibited at 20µg/ml of concentration of the antibiotic.

V. Discussion:

An useful survival strategy for prokaryotes is to enter into consortia,arrangements in which the physiologic characteristics of different organisms contribute to survival of the group as a whole.A biofilm is a consortium of bacteria within an extensive exopolysaccharide exopolymer;which protect bacteria from host's immune mechanisms.

Inthis study,urinary catheter(>1 month in-situ) has been choosen as study material to demonstrate the behaviour of biofilm mode of growth in an in-situ device. Catheterisation often invites ascending infections by the commensals present in urethra,local part of skin or following catheter contamination by the hand of service provider.In bladder environment some organism can multiply rapidly if urine is not drained continuously.Such UTI are most commonly caused by planktonic form of single bacteria amenable to treatment with susceptible drugs.But in a long term(>30 days)catheterisation with continuous drainage,the iatrogenic or ascending infections may not be revealed as significant bacteriuria; rather they colonize on urine contact surface of the device in the form of biofilm & serve as persistent source of treatment refractor infections.

Frequent removal of urinary Foley catheter is technically not possible;so arrest of biofilm formation by timely administration of antibiotic is recommended. An experiment regarding MBEC & MIC was carried out by Farshad Sepandj[141] and his collegues. The patients having peritoneal dialysis-associated peritonitis were selected & peritoneal dialysis(PD) catheters were collected as the clinical sample. Standard MIC testing using National Committee on Clinical Laboratory Standards (NCCLS) protocol was carried out on the isolates obtained from PD-catheter. The MBEC assay was based on a 96-well ELISA platform & allowed the simultaneous determination of MBEC values for 8 different antibiotics. The assay involved the formation of 96 identical biofilms on plastic pegs on the lid of MBEC device.These biofilms were then exposed to test antibiotics for a defined time period, then placed in fresh bacteriologic medium in a second 96-well plate and incubated overnight. The MBEC value was the lowest dilution that prevents regrowth of bacteria from the treated biofilm. Eight samples of PD effluents were tested for MBEC analysis for each of E.coli & P.aeruginosa during the study period. MIC sensitivities of E.coli were favourable for all antibiotics tested except ampicillin & cephazolin (4/8 & 5/8 sensitive).The MBEC results demonstrated significant problems with ampicillin & cephazolin(2/8 sensitive each). The MIC results of P.aeruginosa were relatively favourable for all antibiotics tested except for aztreonam & piperacillin. The MBEC results revealed uniformly poor sensitivity of pseudomonal biofilms to

antibiotics. So in peritonitis caused by E.coli & P.aeruginosa, there is substantial discrepancy between MIC and MBEC results.

In my study a modified method has been carried out on pre-formed biofilm in a uro-catheter.MIC & MBEC assay of catheter isolated stains was done by nitrofurantoin (broth dilution & agar dilution method.The value obtained,denotes that MBEC are always >MIC.

The clinical significance of such experiment is that,the bacteria which survive antibiotic challenge at MIC levels can serve as nidus for recurrent chronic catheter-associated infections.MIC assay does not reflect bacterial behaviour in biofilms in terms of antimicrobial interaction.On the other hand; the MBEC asay provides an alternative method for determination of effective antibiotic outcome against bacteria within biofilm.

VI. Conclusion:

The biofilms on uro-catheters pose special problems in their management due to their high treatment refractoriness & persistant source of infections.Orgamisms within biofilm are usually multidrug resistance due to high probability of conjugation between closely aggregated microorganisms of different strains.In this study it has been shown that sessile forms have higher resistance to antimicrobial drugs(i.e,Minimum biofilm eliminating concentrations or MBEC) than that of respective planktonic forms(MIC).So any treatment based on in-vitro drug sensitivity test for planktonic form of isolates may not be effective for eliminating same organisms residing within biofilm & may require several fold higher concentration of drug.Even after removal of a biofilm-colonised catheter,persister organisms may colonise on new set of catheter.So whenever prolonged catheterisation with several changes is indicated it is better to take some effective measures to prevent biofilm formation than to eliminate organisms from biofilm.Elimination of endogenous source of infections ,reduction of iatrogenic infections ,use of biofilm-proof devices,frequent monitoring for insignificant bacteriuria & flushing of bladder with sterile normal saline through a new catheter are recommended for this purpose.

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