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Research Article

Intrauterine devices associated biofilm with SEM analysis

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Abstract

A study on the isolate will help to develop sterile and biofilm adhesion prevention devices. Using structured interview, Copper-T and cervical swab specimen collection was randomly selected (Every third woman who were 20-40 years old with a mean age of 30 years visiting the hospital was included in the study), There were 105 clinical samples of used IUDs collected from patients, Antimicrobial agent at various concentrations (2µg/ml - 128µg/ml depending upon the MIC) was added to these wells incubated for 5h and then stained with crystal violet. All samples were dried to critical point gold coated and viewed under SEM. Out of 105 IUDs (obtained from women suffering from vaginitis), 97 samples had shown bacterial growth. Of these 97 bacterial isolates, 23 isolates were *Proteus mirabilis* and 74 isolates were other opportunistic pathogens. The presence of IUDs provides a solid surface for attachment and an ideal niche for the biofilm formation. SEM analysis of biofilm topography formed on those devices revealed a dense network of mono or multilayer of cells, which were similar in different species embedded within a matrix of extra cellular polymer materials. This provided an opportunity for the microorganism to accommodate each other and also to thrive under hostile conditions of pH, oxygen availability and redox potential. Dry biomass was also reported to be highest at this stage.

Keywords

Intrauterine devices (IUDs),
Copper-T and cervical swab,
Cystine lactose electrolyte
deficient agar medium
(CLED),
microtitre plate (MTP), SEM,
Proteus mirabilis.

Introduction

In developing countries Intrauterine devices (IUDs) are the most preferred means of contraception in women. The tiny IUDs that are fitted inside the uterus are now available in several shapes and sizes. The present generation of IUDs is made up of a variety of materials ranging from copper to all plastic. More innovation in design (Novo-T) and choice of materials (T-Cu 380 series, T-Cu-220 series and multiload - 375) has since been done. IUDs that steadily release hormones are also available. It is known that the insertion of IUDs stimulate inflammatory or foreign body responses, which in turn cause cellular biochemical changes in the endometrium and uterine fluid. These changes are believed to be responsible for the contraceptive effects (Gupta, 1971). Although studies have shown that IUDs effectively prevent fertilization, provide high degree of sexual; satisfaction and are cost effective (Newton, 1982), they are known to be associated with a risk of pelvic infection, heavier periods,

menstrual cramps (Lee *et al.*, 1983) and above all complications associated with colonization of microbes on these implanted devices. Most often the medical practitioner is left with little option but to remove the IUD when antibiotics cannot deal with the insult of pathogen s adequately.

Prolonged use of IUDs cause health problems like fever, pelvic pain and inflammation. Many such cases have been reported in hospitals in the study area. The users get relief only when IUDs are removed although the pathogen persists and perpetuates. Hence a study has been designed to screen the removed IUDs for any biofilm development and isolating the microbes present in the biofilm matrix. A study on the isolate will help to develop sterile and biofilm adhesion prevention devices.

Materials and Methods

Infected were IUDs removed from the patients with Reproductive Tract Infections (RTIs) were collected from Cuddalore Government hospital family planning ward in Cuddalore district (Tamilnadu and India) under aseptic conditions and brought to the laboratory for investigation. The samples were obtained over a period of two years from December 2009 to October 2011. Using structured interview, Copper-T and cervical swab specimen collection was randomly selected (Every third woman who were 20-40 years old with a mean age of 30 years visiting the hospital was included in the study), There were 105 clinical samples of used IUDs collected from patients.

Isolation of adherent microorganisms

The study population consisted of women either requesting IUDs insertion, or already using IUDs and visiting the hospital for check up. The thread that is attached to keep the device in place was found to harbor microbes, which were screened and characterized as described below. Briefly, infected thread pieces (0.5cm) from the devices were placed in a 10ml of 0.15M PBS that contained 0.1% tween-80 and sonicated in an ultrasonic cleaner water bath for 30 minutes at room temperature to detach adherent microorganisms. The microbial suspension was vortexed vigorously for 15 seconds to break up clumps. Ten fold serial dilutions of each suspension were placed on 5% CLED agar medium base using spread plate technique, incubated at 30° C for 18h and the mean number colony forming units was determined.

Identification of the isolates was done according to standard procedures. All microbial strains so obtained were maintained on slants of Cystine lactose electrolyte deficient agar medium (CLED) and sub cultured fortnightly.

Determination of biofilm dry weight

The microbes that detached from a unit size of infected IUDs after sonication were centrifuged at 10,000 rpm for 5 minutes at room temperature. The pellet so obtained was transferred to preweighed cellulose nitrate paper (0.45µm pore size; 25 mm diameter), dried at 80°C overnight and weighed. This weight was taken as measure of the dry biomass and expressed in µg/infected thread piece.

Monitoring biofilm formation

Biofilm formation was monitored by the ability of cells to adhere to the wells of microtitre plate (MTP) made of PVC. The biofilm forming microorganism recovered from IUDs were grown in CLED agar medium and samples were drawn at 12h intervals. The quantification measurement was taken for the mixed population of organisms.

One hundred microlitter (1:100 diluted CLED broth) of (bacterial isolates) samples were inoculated in MTP and incubated at 30°C for 10h. MTP wells were then rinsed thoroughly thrice with 0.15m PBS to remove free floating organisms. Crystal violet 100µl of 1% solution was then added to each well and the plates were incubated at room temperature for 15 min. Excess of strains were removed rinsing with distilled water. The strain that was taken up by biofilm forming organism was extracted twice in 200µl aliquots of 95% ethanol 100µl which was transferred to a new MTP and the absorbance was determined in a plate reader at 600nm.

Susceptibility of biofilm to antimicrobial agents

The biofilm identified on IUDs comprised of a consortium of microorganisms that included gram positive and gram negative bacteria. Therefore the susceptibility testing was carried out with repetitive anti microbial agents that were active against each of those classes of microorganism. The antimicrobial agents used were Amikacin, Ampicillin, Cefazolin, Ceftazidime, Cefepime, Cefepime- Sulbactam, Ciprofloxacin, Cefuroxime, Cefepime and briefly MTP wells were inoculated with 100µl /well of 10⁷ cells obtained after 60h as detailed earlier and allowed to form biofilm. Antimicrobial agent at various concentrations (2µg/ml - 128µg/ml depending upon the MIC) was added to these wells incubated for 5h and then stained with crystal violet. Cells detached from MTP wells were rinsed and the residual biofilm was quantified as percentage reduction in absorbance 600nm.

Scanning electron microscope (SEM) study

Microbial biofilms formed on IUDs were fixed with 2.5% (v/v) glutaraldehyde in 0.15M PBS for 1 h at room temperature. 1% (w/v) osmium tetra oxide for 1 hour, washed thrice with distilled water. They were then treated with 1 % (w/v) urinal acetate for 1h and washed again with distilled water. The samples were dehydrated in ethanol. All samples were dried to critical point gold coated and viewed under SEM.

Results and Discussion

Isolation of adherent microorganisms

Out of 105 IUDs (obtained from women suffering from vaginitis), 97 samples had given positive bacterial culture whereas eight samples showed no bacterial growth. Out of the 97 positive cultures, five were from unmarried women and 92 from married women. Of these 97 bacterial isolates, 23 isolates were belonged to *Proteus mirabilis* (Fig 1) and 74 isolates were of opportunistic pathogens (Table1).

Table: 1 Microorganisms screened and identified from infected intrauterine devices

S. No	Microorganisms	Total isolates %
1.	<i>Proteus mirabilis</i>	23 %
2.	<i>Pseudomonas aeruginosa</i>	17%
3.	<i>Escherichia coli</i>	13%
4.	<i>Staphylococcus aureus</i>	12%
5.	<i>Staphylococcus epidermidis</i>	10%
6.	<i>Candida albicans</i>	8%
7.	<i>Lactobacilli</i>	6%
8.	<i>Klebsiella pneumoniae</i>	5%
9.	<i>Neisseria gonnorrhoea</i>	3%

The vagina and surrounding regions of the reproductive tract are known to support a large number of bacteria and fungi (Lewis, 1988). The migration of those to the upper part of the female urinogenitale tract after leads to discomfort and infection. Table 1 shows the identity and % distribution of organism in 105 samples recovered from patient with IUDs infection. The microbial flora obtained from the vaginal swabs and IUDs matched to a large extent. IUDs removed from women were found to harbour *Proteus mirabilis*, *Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans*, *Lactobacilli*, *Klebsiella pneumoniae* and *Neisseria gonnorrhoea* as reported by earlier workers (Marrie and Costerton, 1983; Wolf and Kreiger, 1986). Majority of infected IUDs have been reported to be due to gram negative bacteria notably *Proteus*. Infection due to gram positive bacteria and fungi are also a matter of serious concern (O'Toole *et al.*, 2000; Donlan and Costerton, 2002). The cord threads attached to the tail of the IUDs are perhaps one of the routes of microbial migration from the vagina to the uterus. A previous study had indicated that there were fewer incidents of biofilm formations on IUDs that did not have a protruding in to the cervical region. Besides, microbial load was heaviest on the IUDs when the distal portion of the tail was directly exposed to the vaginal flora (Bank and Williamson 1983). Although the uterine secretions under normal conditions actively deal with such migrations, the presence of IUDs gives a solid surface for attachment and an ideal niche for the biofilm for and flourishes.

Dry weight of biofilm

Dry weight of bacterial mass associated with the IUDs was found to vary with time. The dry weight of bacteria indicates that the adhesion and proliferation because of a peak levels at 72h and latter a plateau state is maintained (Fig 2).

Monitoring biofilm formation

The maximum microbial adherence to the 96 wells MTP was obtained after 60 hours at 30°C. Dry biomass (1.8µg/L) was also reported to be highest at this stage. It may be clarified that this dry biomass represents a mixture of organisms listed in Table 1 in various combinations (Fig 3). Similar results were obtained during studies on biofilms and infection associated with other implanted devices by other research groups. The temperature of the uterine environment is also conducive for the formation of biofilms (Donlan, 2001). When the temperature increases the growth of biofilm increased up to 30°C, but when temperature was increased beyond, further decline in biofilm mass was noticed (Fig 4).

Antibiotic sensitivity

Data obtained on the influence of antimicrobial agents indicates that treatment with antibiotic concentration (MIC) level reduced biofilm cell counts by approximately 30-40% compared with the control sample (Fig 5). Other research groups also obtained similar results (Brown *et al.*, 1990). Our investigations strongly support the contention that infections due to biofilm formation on IUDs are difficult to resolve, as they can counter both host defence mechanism and antibiotic therapy. The study shows that release of these microbes from the IUDs would initiate a sequence of events leading to chronic infection on account of antibiotic resistance. The situation is more complicated due to the mixed nature of the flora found in biofilms. Several possibilities including phenotypic changes resulting from nutrient limitation, possible protective effect of the matrix, differential expression of drug resistance genes can be considered as the mechanisms contributing to increased resistance of antimicrobial agents to biofilm formed on infected IUDs. The strategies involving antiseptic bounded biomaterials are now in common use and future work on

such biomaterials is likely to yield improved devices. Therapeutic approaches which interfere with the expression of or activity of gene and gene products involved in microbial biofilm formation are likely to provide novel and potential beneficial alternatives to current therapies. The clinical usefulness of these strategies remains to be determined.

SEM Analysis

SEM analysis of biofilm (Fig 6) topography formed on those devices revealed a dense network of mono or

multilayer of cells from same or different species embedded within a matrix of extra cellular polymer materials such colonization was reported, (Lewandowski, 2000). This provided an opportunity for the microorganism to accommodate each other and also to thrive under hostile conditions of pH, oxygen availability and redox potential. The results are in agreement with suggestions that the heterogeneous mosaic nature of biofilm culture in response to environmental stress (Lewandowski, 2000). Besides, the complex structure could provide protection against host defense mechanism.

Figure: 1 The selective CLED agar plate showing the Transulant bluish grey colour colonies of bacteria



Figure: 2. Biomass estimation in relation to adhesion time in the biofilm forming organisms isolated from IUDs

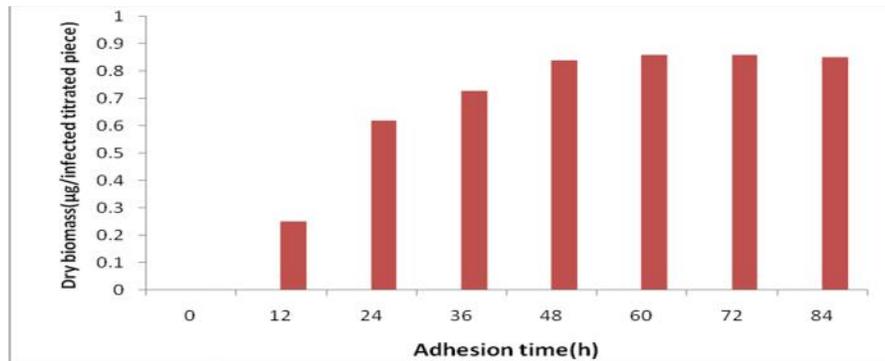


Figure: 3 Adhesion characteristics of biofilm forming organisms isolated from IUDs

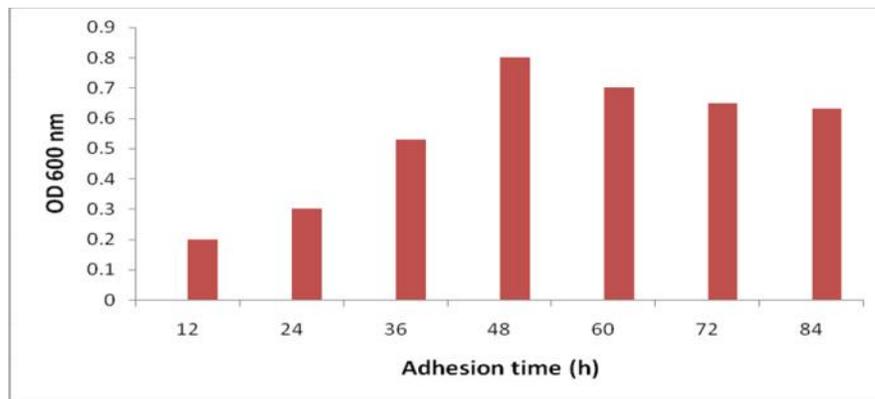


Figure: 4 Temperature characteristics of biofilm forming organism isolated from IUDs

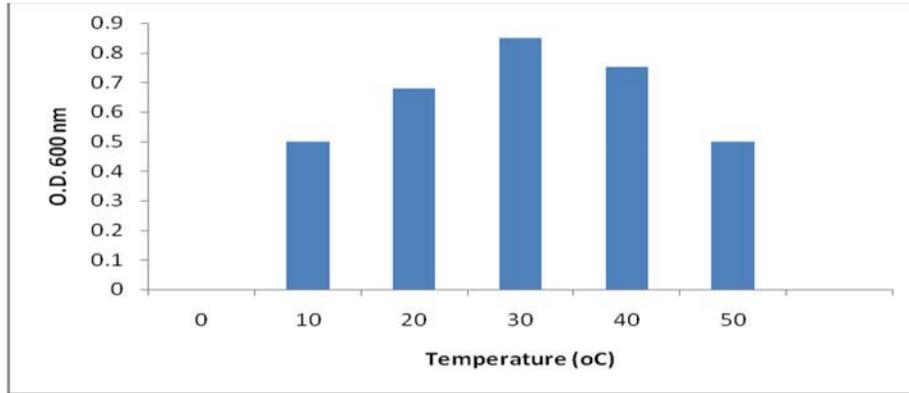


Figure: 5 The effect of different doses of antimicrobial drugs tested against total microbial isolates from IUDs biofilm

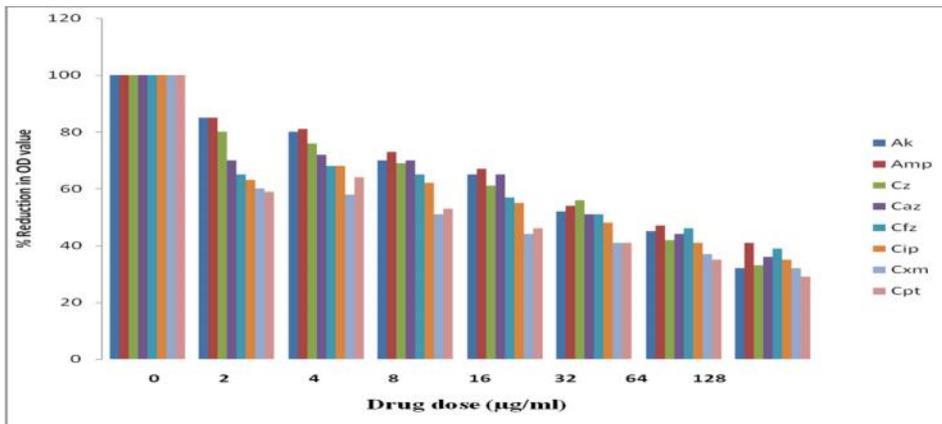
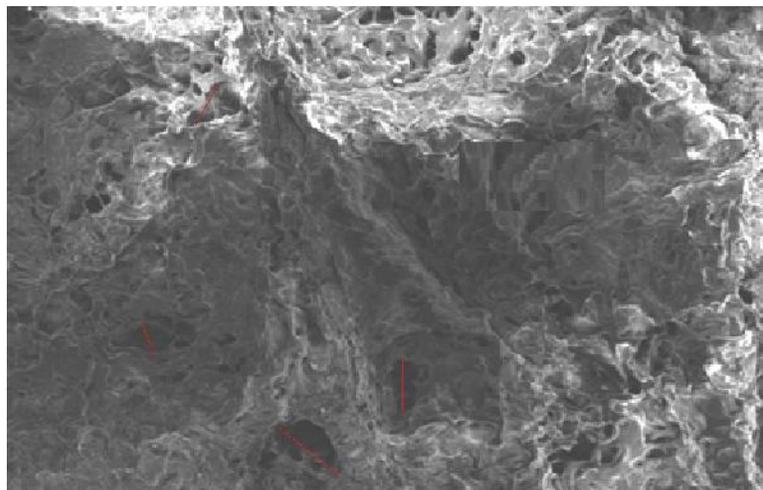


Figure: 6 SEM show the structural organization of Biofilm formed on intrauterine devices



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