Elimination and Prevention of Microbial Colonization of Central Venous Catheters Using Antibiotic Lock Technique and Non-Leachable Form of Catheter Surface Incorporated Antibiotic by Gamma Radiation

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Abstract: **Aim**: To test the efficacy of antibiotic lock technique (ALT) and catheter material modification for the eradication and prevention of biofilm formation on central venous catheters (CVCs). **Methods:** Four resistant clinical isolates, namely Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae and Candida tropicalis were used to study the effectiveness of ALT using levofloxacin (for bacteria) or fluconazole (for candida) to eliminate microbial biofilms. Moreover, with the aid of gamma radiation, gentamicin was covalently attached to the polyurethane catheter and the modified polymeric material was tested for their antimicrobial and anti-adherence properties. **Results**: The highest and lowest concentrations of levofloxacin tested (1024 and 64 mg/L) eliminated the biofilms of Pseudomonas aeruginosa and Acinetobacter baumannii after 3 and 10 days, respectively and after 1 and 7 days in case of Klebsiella pneumoniae, whereas Candida tropicalis biofilms were eliminated by fluconazole after 3 and 10 days. The modified polymeric material acquired antibacterial and anti-adherence properties. **Conclusion**: The results suggest that ALT and catheter modification could be effective for eradicating and preventing biofilm formation on CVCs.
Key words: Biofilm; Antibiotic lock technique; Central venous catheter; Glycidyl methacrylate, polymer modification.

I. Introduction

The central venous catheter (CVC) is an important tool in modern medical practices. It provides secure access to the central circulation for infusion therapy, nutritional support, hemodynamic monitoring, plasmapheresis and hemodialysis. The use of central venous catheters (CVCs) is associated with increased risk of catheter-related blood stream infections (CRBSIs). They have a higher infection risk than other indwelling vascular access lines (Patil et al 2011). The infective agents, causing such infections, colonize the catheter surface forming biofilm and resulting in increased rates of morbidity and mortality, and prolonged hospitalization periods (Rodney 2011;Cherifi et al 2013 and Kostakioti et al 2013). Aside from septic complications, failure to prevent or eradicate microbial contaminants from catheter surfaces may result in phlebitis, skin infections, and/or catheter loss and removal (Shirtliff et al 2009). However, catheter removal may not be a viable option if thrombocytopenia, coagulopathies, a lack of alternative venous access, or critical illness is a confounding issue. In addition, the clinical need for these catheters in some patients, including parenteral nutrition and intravenous drips, further complicates the decision of catheter removal. Therefore, alternative strategies are needed to prevent contamination or permit salvage of catheters in these clinical situations (Peters et al, 2013).One current approach is the use of antimicrobial catheter lock solutions, which are formulated to sterilize the catheter lumen or prevent biofilm growth and subsequent septic infection (Toltzis 2006). Several studies recommended ALT to prevent catheter-related infections (Casey and Elliott 2010 and Kulawik 2010).

Polyurethane is widely used in vascular catheters, given its favorable biocompatibility and excellent mechanical properties (Yuan et al 2004). However, it is slightly hydrophobic, a characteristic that facilitates bacterial adherence and biofilm formation (Egboh et al 1984). Modification of the biomaterial surface was regarded the most promising preventive strategy for biofilms. The covalent immobilization of the antimicrobial agent offers many privileges, including long term stability and protection against bacterial colonization, lowered toxicity and

decreased risk of emergence of antimicrobial resistant strains (Makal *et al* 2006; Humblot *et al* 2009 and Xu *et al* 2009). The aim of this study was to evaluate the efficacy of ALT and catheter material modification to eradicate and prevent microbial biofilms.

II.   Materials and Methods

1. Equipments:
Incubatory Shaker (Lab-Line Instruments Inc., Melrose, USA),   pH Meter (Jenway Ltd. UK), Vortex (Gemmy Industrial Corp., Taiwan), VCX-400 Sonicator (Sonics & Materials Inc. Danbury. CT, USA), Gamma Radiation Cell (^{60}Co- Atomic Energy of Russian$^{)}$), IR Spectroscopy (FTIR Jasco 9200 Japan) and Scanning Electron Microscopy (JEOL JSM-5400, Japan).

2. Culture media:
Nutrient agar, MacConkey agar, Sabouraud dextrose agar and trypticsoy broth. All the media were the products of Oxoid Ltd. (England). The media were autoclaved at 121° C for 20 minutes. Heat labile components were separately sterilized by filtration and aseptically added to the medium.

3. Microbial Strains:
Three clinical bacterial isolates, namely *Acinetobacterbaumannii* (*A. baumannii*), *Pseudomonas aeruginosa (P. aeruginosa) and Klebsiella pneumoniae* (*K. pneumoniae*) in addition to a *Candida tropicalis* (*C. tropicalis*) isolate. The test strains were selected among 117 isolates previously isolated from 83 CVCs collected during 2006 from ICU patients. They were selected based on their antibiotic resistance and slime productivity.

4. Antimicrobial Agents:
Levofloxacin and fluconazole were used as antibiotic lock solutions for bacteria and yeast, respectively. For modification of catheter polymeric material, gentamicin was used. These antimicrobial agents were obtained from the National Organization for Drug Control and Research (NODCAR), Egypt.

5. Catheters: Polyurethane central venous (CVPU) cathetersare a product of (AmeCath., Egypt).

6. Chemicals:
6.1 Glycidyl methacrylate (GMA); A transparent colorless liquid, contains carbon-carbon double bond and an epoxy group which make free radical reaction and ionic reaction occur. It was the product of Fluka, Biochemika, Switzerland.

6.2 Solvents used: acetone, methanol, and ethyl acetate, all analytical grade reagents (Romil ltd., GB).

7. Antibiotic- Lock Experiments:
7.1.   Biofilm formation on catheter surface
Mature biofilms on CVPU fragments were formed using the method of *Golomb and Shpigelman (*1991). Briefly, sterile segments were cut and suspended at 30°C / 5 days in microbial suspensions (10^{7} CFUs/ml) using tryptic soy broth supplemented with 0.25% sucrose in incubatory shaker, at 100 rpm.

7.2. Model of antimicrobial lock technique
The method of Lee *et al* (2006 and 2007) was applied**.** Briefly, segments with biofilms were washed three times with phosphate buffered saline (PBS), placed in fresh 10 ml tryptic soy broth supplemented with 0.25% sucrose, containing different concentrations (64-1024 mg/L) of Levofloxacin for bacteria or fluconazole for *Candida tropicalis*. At suitable time intervals of 1, 3, 5, 7 or 10 days, three segments were picked up from each tube, washed three times with PBS, vortexed and sonicated separately in 3 ml fresh PBS for 2 minutes using VCX-400 sonicator (Sonics & Materials Inc. Danbury. CT, USA) with a pulse rate of 3.5 seconds to separate and re-suspend the adherent cells, as confirmed by scanning electron microscopy (SEM). Then the sonicated microbial suspension was vortexed and serially diluted, and the viable count was determined using agar plate technique. The experiments were done in triplicate and the effect of concentration and contact time was statistically analyzed using F-test.

7.3 Scanning Electron Microscopy (SEM) Studies
Scanning electron microscopy (SEM) studies, using the method of Storti *et al* (2005), were carried out on *P. aeruginosa* or *C. tropicalis* for the lock concentrations of 64, 256 or 1024 mg/L and compared to the antibiotic free PBS.

8. Modification of Catheter Material by Gentamicin
8.1. Graft copolymerization of GMA on to CVPU (GMA-g-CVPU) (Fig 1a).
A method similar to that used by Egboh *et al* (1984) was performed to carry out radiation-induced graft copolymerization of glycidyl methacrylate (GMA) onto segments of polyurethane (PU) catheters. Graft copolymerization reaction was carried out in different solvents, including acetone, ethyl acetate or methanol. In brief, polyurethane segments were washed with acetone, dried at 50°C in a vacuum oven, weighed, and then

immersed in GMA solution in glass ampoules. The reactant solutions were de aerated by nitrogen atmosphere to remove air from grafting solution. The sealed glass ampoules were then subjected to ^{60}Co-γ rays. The grafted films were removed and washed thoroughly with methanol/DMF to extract the residual monomer and homopolymer, which may be occluded in the segments. The films were then dried in a vacuum oven at 40-50°C for 24h and weighed. The degree of grafting (DG) was determined as it follows;

$$DG (\%) = [W_g - W_o / W_o] \times 100$$

Where W_o and W_g are the weight of the ungrafted and grafted segments, respectively.

8.2. Chemical immobilization of gentamicin on (GMA-g-CVPU) (Fig 1b).

Covalent bonding of gentamicin, to the grafted segments, was performed by refluxing with super saturated alcoholic gentamicin solution, at 70ºC/4 hrs. in presence of few drops of dimethyl formamide, to enhance solubility of gentamicin. The treated catheter segments were then washed and dried. The conversion to amine structure was determined by the disappearance of the epoxy groups to secondary amine groups by FTIR spectra (Fig 6).

8.3. Fourier Transform Infrared (FTIR) Spectroscopy

A commercial FTIR (Jasco 9200 Japan) spectroscopy, at scanning range of 4000 to 200cm^{-1} wave number, was employed to investigate the chemical characteristics of virgin PU, graft copolymer (CVPU-g-GMA) and gentamicin-modified grafted films (CVPU-g-GMA-Gentamicin).

8.4. Antimicrobial Effectiveness
8.4.1. Test for antimicrobial activity of graft copolymer and modified copolymer:

The graft copolymer (CVPU-g-GMA), gentamicin-modified copolymer (CVPU-g-GMA-Gentamicin) and polyurethane (CVPU), as a control, were tested for their antimicrobial activity by placing strips of these polymers on agar plates inoculated with *P. aeruginosa, A. baumannii or K. pneumoniae*. After incubation, plates were examined for any inhibition zones around the strips or their contact inhibition zones.

8.4.2. Test for the anti-adherence capacity of gentamicin- modified copolymers:

The anti-adherence capacity of graft copolymer (CVPU-g-GMA), gentamicin-modified copolymers (CVPU-g-GMA-Gentamicin) and polyurethane (PU), as a control, was tested against highly slime producer bacterial strains used above in the lock experiment. Briefly; segments were placed separately, in microbial suspension (10^8 CFUs/ml) of these organisms, incubated at 37°C and examined for biofilm formation during 30 days, at time interval of five days. Where; three segments from each suspension were washed with PBS, sonicated to re-suspend any adherent bacterial cells (Sherertz *et al* 1990). After sonication, aliquots were well mixed using high speed vortex, 10 fold serially diluted and 0.1 ml from each dilution was inoculated on the surface of nutrient agar plates. The number of colonies was counted after 24-48 hours incubation at 37^{0}C. Results of test and control were confirmed using the scanning electron microscopy as described before.

III.   Results
Effect of levofloxacin as a lock solution on bacterial biofilms:

The results of levofloxacin on bacterial biofilms of initial counts of 4.5×10^7, 1.7×10^7 and 1.3×10^8 CFUs/segment for *P. aeruginosa, A. baumannii and K. pneumoniae*, respectively, showed that the count was decreasing by time, where a concentration of 1024 mg/L eliminated their biofilms within 3 days and a concentration of 64 mg/L within 10 days. Conversely, the elimination time was earlier for *K. pneumoniae* (7 days),and various elimination times were recorded for other concentrations tested (128-512 mg/L) (Fig 2a, b, c). The results were confirmed for *P. aeruginosa* by SEM (Fig 3).

Effect of fluconazole as antifungal lock solution:

As for bacteria, an initial biofilm count of 5.3×10^6 CFUs/segment for *C. tropicalis was* decreasing by time (Fig. 2d) and complete elimination was recorded and confirmed by SEM (Fig. 4) within 3 and 10 days for concentrations of 1024 and 64 mg/L, respectively.

Effects of radiation dose, solvent and monomer concentration (GMA) on the grafting yield:

Among radiation doses of 10, 20 and 30 kGy, different solvents (acetone, ethyl acetate and methanol), as well as GMA concentrations of 20 and 30% used ,the results showed that the degree of grafting increased with the increase in monomer concentration (up to 30 %) and radiation dose up to 30 kGy (Fig. 5). For different solvents, the grafting yield was < 20% for acetone, <10% for ethyl acetate, while the best yield (> 50%) was recorded for methanol.

Modification of graft copolymer by gentamicin:

Figure (6) shows the FTIR spectrum of CVPU-g-GMA copolymer in comparison with that of the original CVPU. CVPU spectrum (Fig 6 a) shows its characteristic peaks at 3294.8 and 1536.1 cm^{-1} assigned to NH absorption, 2880–2890 cm^{-1} attributed to C–H symmetric and asymmetric stretching absorption and 1704.0 cm^{-1}attributed to the urethane –NHCOO– carbonyl absorption. Upon grafting of GMA (Fig 6 b) a new absorption band appeared at 1724 cm^{-1} belonging to the C=O ester and at 1193 and 1159 cm^{-1} which belonged to the epoxy group of the grafted GMA (Xifei 2001). This confirmed the occurrence of the grafting process by showing the absorption peaks of grafted function groups together with that of polymer substrate. Figure (6 c) shows in addition to the absence of the absorption band belong to the epoxy ring, some broadness in the absorption peaks at 3300-3500 due to the appearance of –OH as result of epoxy ring opening. Such information shows that the epoxy group in the side chain of CVPU-g-GMA has reacted with the functional (NH2) group of gentamicin. The above observations verified the successful covalent attachment of gentamicin to the CVPU. This was also confirmed microbiologically, where only gentamicin modified polymer showed contact inhibition zones against the three test bacterial strains, on inoculated agar plates, as well as prevention of their adherence to the modified polymer surface when suspended in their broth culture, as confirmed by scanning electron microscopy (Fig 7).

IV. Discussion

Biofilm formation is the main problem in the pathogenesis of catheter-related infections. These biofilms cannot be penetrated by antibodies or phagocytes and are 1500 times less susceptible to antibiotics than are their planktonic counterparts. This led to targeted therapy aimed at eliminating the biofilms while salvaging the catheter (Costerton *et al* 1999 and Donlan, 2001). The concepts behind antibiotic lock technique (ALT) are to prolong the life of the catheter and reduce morbidity and costs of managing catheter-related infections. In addition, it may spare patients from certain toxic effects of systemic antibiotic administration, and the risk of bacterial resistance may be reduced (Capdevila *et al* 1996 and Oppenheim, 2000). ALT is especially beneficial for patients with underlying comorbidities complicating catheter removal or patients who are catheter dependent. It is also indicated for central line associated bloodstream infections involving long-term catheters with no signs of exit site or tunnel infection with the goal of catheter salvage (Estes *et al* 2013 and Peters *et al* 2013). In vitro study for prevention and/or elimination of bacterial and fungal biofilms on central venous catheters, especially long term ones, was performed. As the lock solutions are trapped inside the catheter without any systemic effect, so the use of as high as 23-512 folds of the MIC of the lock antibiotics was expected to be a highly effective and safe technique. The elimination of biofilm was concentration dependent; levofloxacin eliminated *P. aeruginosa and A. baumannii* biofilms within 3 days and up to 10 days at concentrations of about 1 mg/ml and 0.06 mg/ml, respectively. However, the elimination time was shorter for *K. pneumoniae* (24 hrs and 7 days) at the same concentrations. Similar elimination rate was reported by Lee *et al* (2007) for *P. aeruginosa* using ciprofloxacin as a lock solution.

Elimination of *C. tropicalis* biofilms, by fluconazole, showed similar response to that of *P. aeruginosa* and *A. baumannii* (within 3 and 10 days at similar concentrations). Similar observations were recorded but at longer duration (1 mg/ml at 7 days) by *Ko et al* (2010) and higher concentration (2 mg/ml at 7 days) by Andris *et al* (1998).

As shown by the SEM, lock solutions (either levofloxacin or fluconazole), eliminated both microbial cells within biofilm and the expolymeric material around them. It is an advantage for the lock technique to target both biofilm components (matrix and microbial population), so it not only eliminates biofilm but also prevents catheter re-colonization. Similar observation was reported by Totéa *et al* (2009).

Although polyurethane has excellent mechanical and anti-thrombogenic properties, nevertheless its hydrophobicity facilitates adherence and biofilm formation. Different methods were developed for synthesis of antibacterial surfaces. Covalent bonding of active antimicrobial agents to the substrate surface is a useful approach for the development of non-leaching or contact biocide surface. The antimicrobial action results from the contact of the microorganisms with the surface without release. Cationic antimicrobial agents play a key role in the development of permanent and non-leaching contact biocidal surfaces where they affect the integrity of the negatively charged bacterial cells, causing release of the intracellular components (Biering-Sorensen, 2002; Lee *et al* 2004; Makal *et al* 2006; Murata *et al* 2007 and Xu *et al* 2009). Gamma radiation is often used to sterilize

polymeric medical devices at doses exceeding 15 kGy. Commercial polyurethanes show good resistance to high-energy radiation, withstanding a single dose of up to 100 kGy (Gorna and Gogolewski 2003).

In the present study, the grafting yield of GMA onto CVPU increased with increasing GMA concentration and the radiation dose. The optimum conditions were the use of a radiation dose of 30 kGy, 30 % GMA, and methanol as a solvent. The increase in grafting yield may be attributed to the increase in free radicals concentration that arises from the increase in the monomer concentration (Charlesby, 1960) and hence increase in the epoxy groups' content on polyurethane that act as surface linkers for the nucleophilic amino group of gentamicin. Other nucleophilic groups, as linkers, including NH2, OH, SH were reported by others (Eckert *et al* 2000 and Nishiyama *et al* 2002). On the other hand, methanol facilitates the accessibility of GMA to the polymer active site; similar results were recorded by Inderjeet *et al* (1999) and El-Hag Ali *et al* (2008). In spite of being effective, the maximum radiation dose used was 30 kGy, because at a higher dose (50 kGy) the physical properties of GMA monomer were affected and yielded a powdery form. Similar observation was reported by Jansen and Ellinghorst (1979), who observed gelation of GMA.

Being a heat stable antibiotic (up to 100 C) (Wang *et al* 2004), gentamicin was covalently immobilized into graft copolymer by refluxing at 70 C°/4 hrs, as confirmed by FTIR. The immobilized antibiotic retained its antibacterial activity as confirmed by contact inhibition zone. The persistence of the antimicrobial activity of immobilized gentamicin may be explained by its flexibility due to the presence of a linker spacer (Liu and Ming, 2008 and Humblot *et al* 2009). In addition; gentamicin was reported to have a surface action on bacterial cell envelope (Kadurugamuwa *et al* 1993 a, b). In conclusion, our study suggests that both ALT and catheter polymer modification could be effective in eradicating and preventing microbial biofilms on CVCs, hence preventing catheter related infections ; however, further studies are required to verify their effectiveness in vivo.

Acknowledgment

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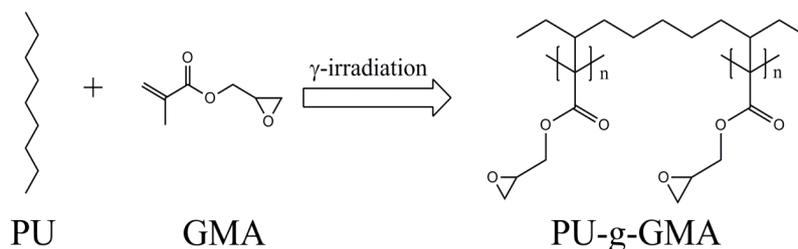
Fig 1a. Schematic representation of the radiation induced grafting of GMA onto CVPU segments
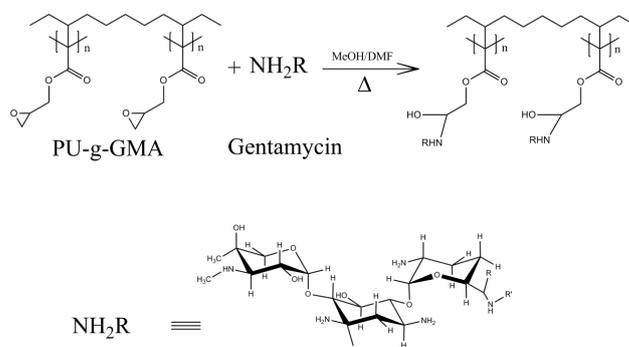

Fig 1b. Schematic representation of the chemical immobilization of gentamicin to the radiation modified CVPU catheter
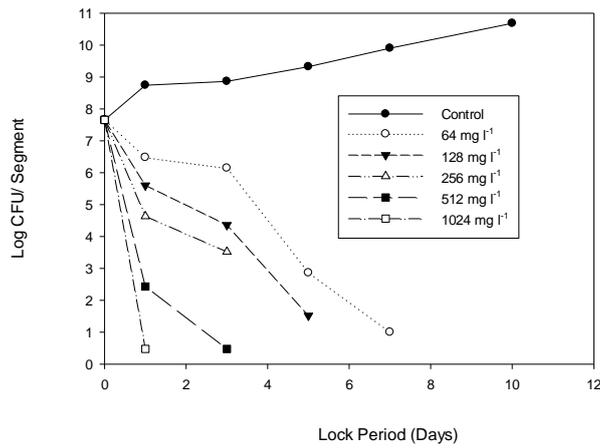
Fig 2a. Change in microbial count of *P. aeruginosa* biofilm in presence of different concentrations of levofloxacin at different lock periods.
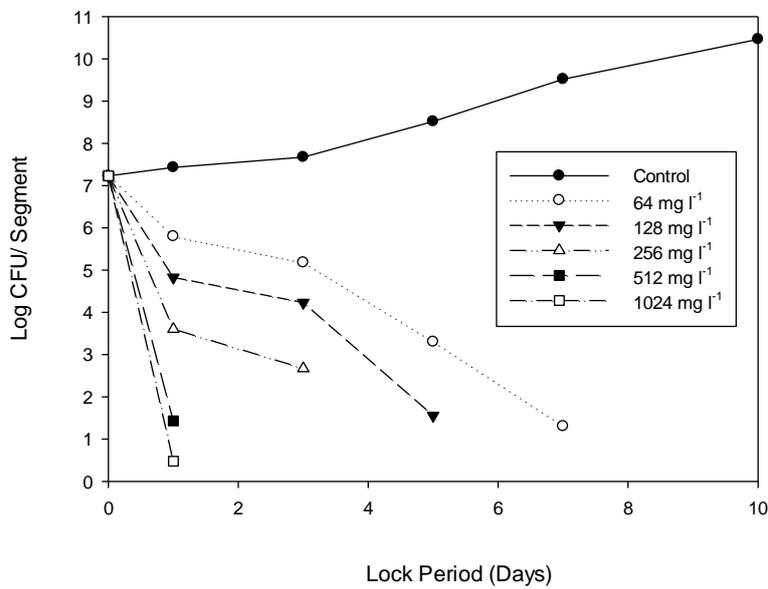
Fig 2b. Change in microbial count of *A. baumannii* biofilm in presence of different concentrations of levofloxacin at different lock periods.
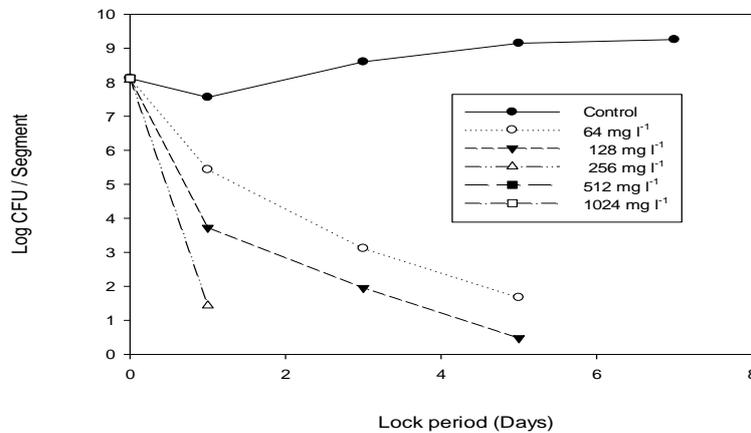
Fig 2c. Change in microbial count of *K. pneumonia*e biofilm in presence of different concentrations of levofloxacin at different lock periods.
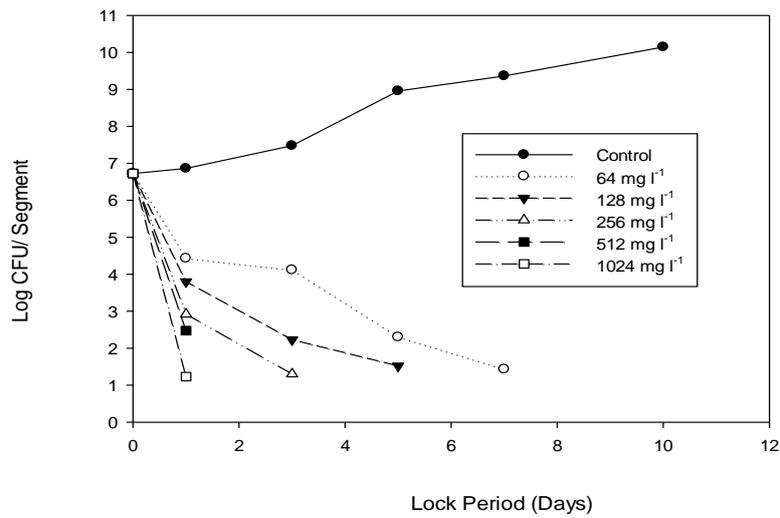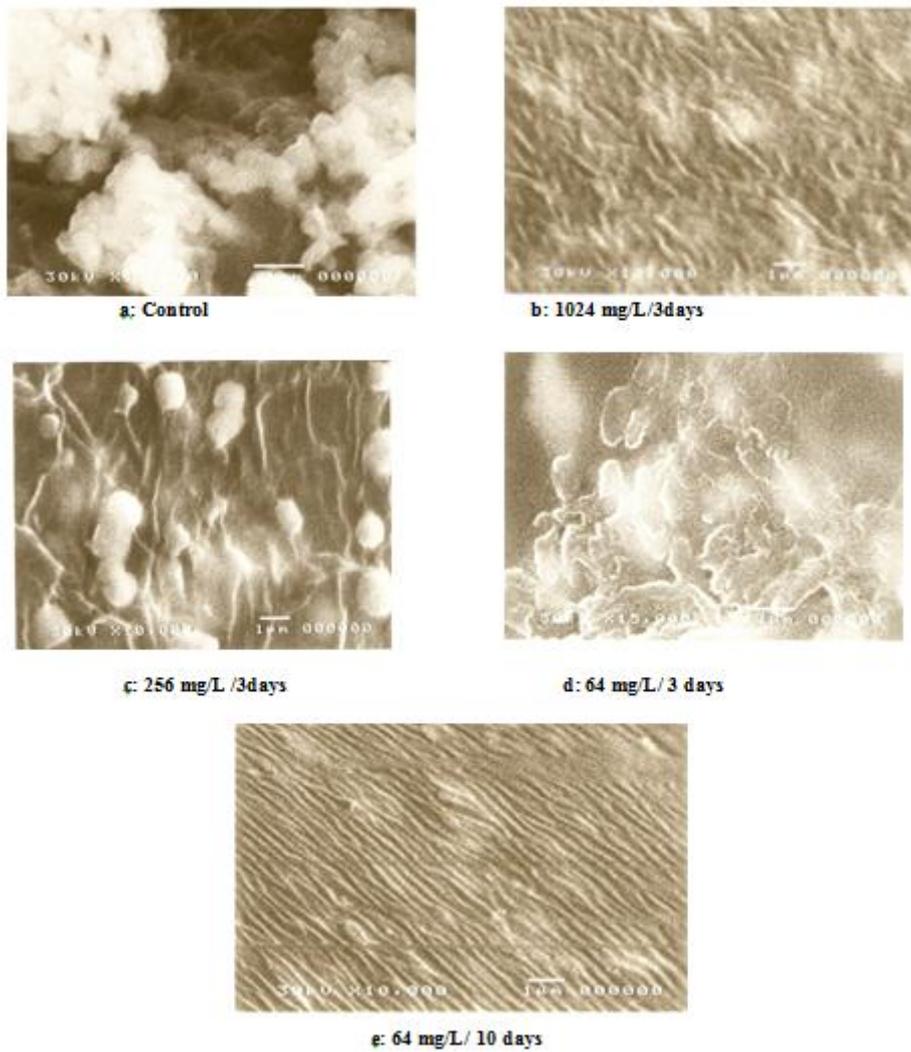
Fig 2d. Change in microbial count of *C. tropicalis* biofilm in presence of different concentrations of fluconazole at different lock periods

Fig 3. Scanning electron micrographs of *P. aeruginosa* biofilm after different lock periods
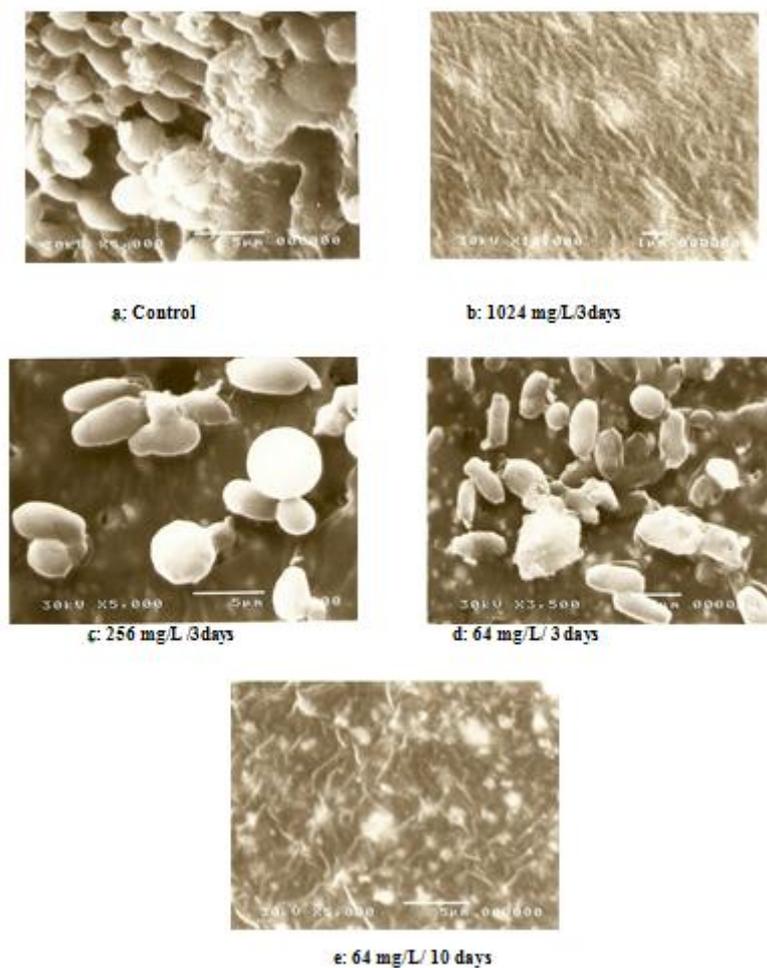
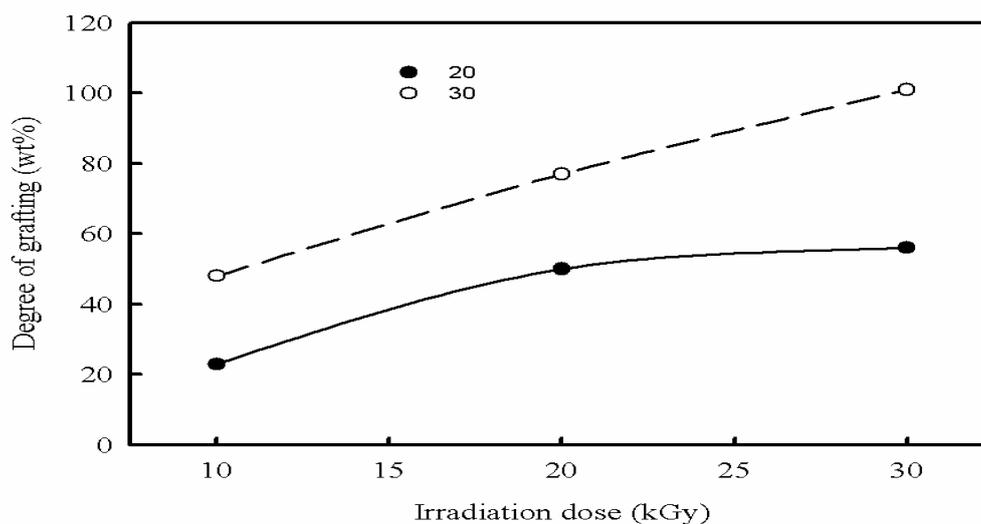**Fig 4. Scanning electron micrographs of *C. tropicalis* biofilm after different lock periods**

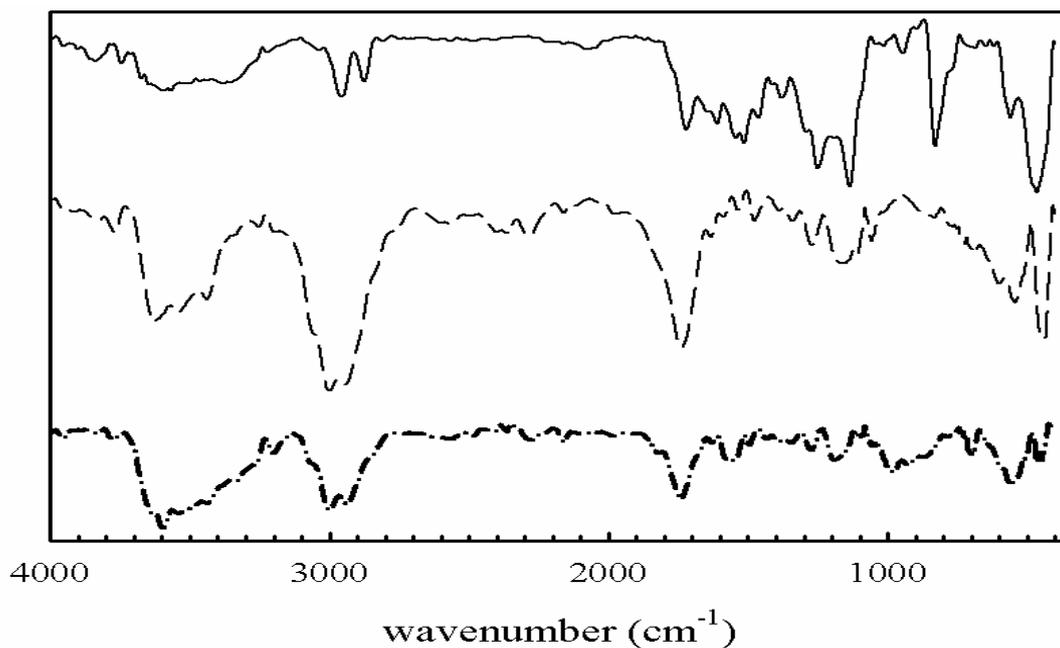**Fig 5. Effect of radiation dose and monomer concentration on grafting yield**

Fig 6. FT-IR spectra of (a) virgin (CVPU), (b) CVPU-g-GMA and (c) gentamicin modified polymer CVPU-g-GMA-Gentamicin
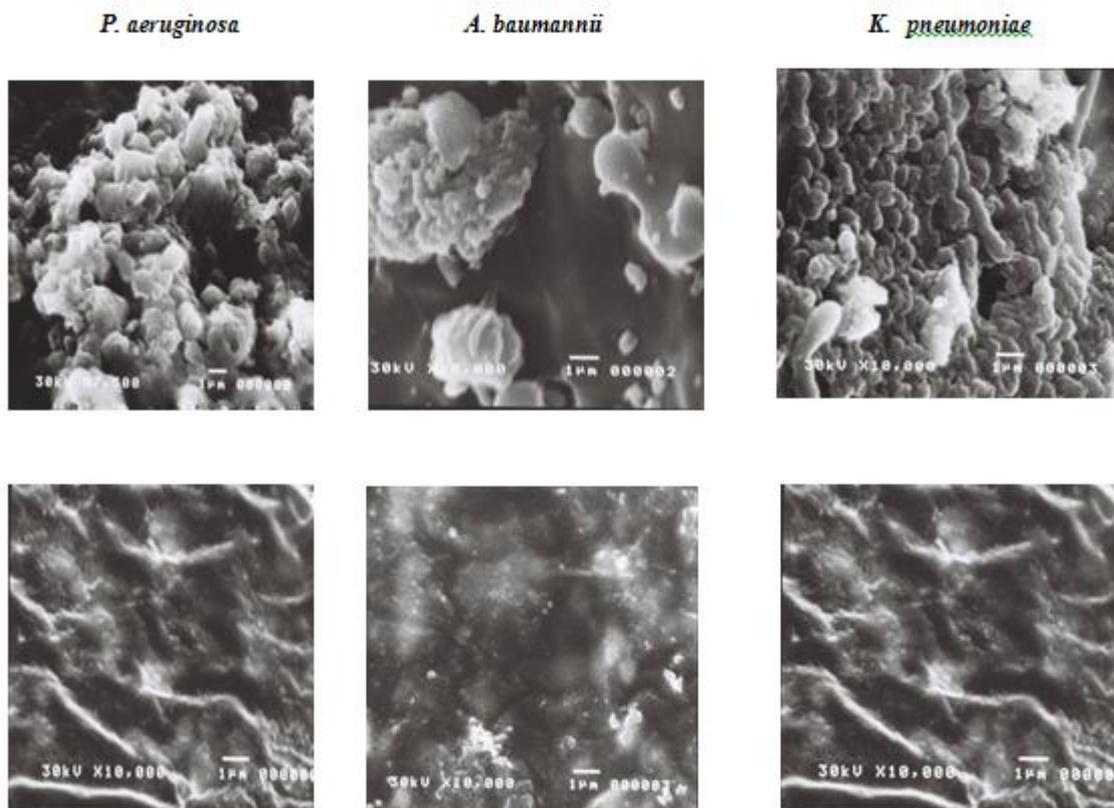

Fig 7. Scanning electron micrographs for commercial CVPU (control) in comparison with gentamicin modified CVPU (CVPU-g-GMA-Gentamicin) after immersion for 30 days in a heavy suspension of test strains