Prevention of adhesion and surface growth of orthopedic implant microbialinfection by surface modification using antibiotics and irradiated hydroxyapatite

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Abstract

One of the major drawbacks in the use of biomedical materials is the occurrence of biomaterial-centered infections. After implantation, the host interacts with a biomaterial by forming a conditioning film on its surface and an immune response towards the foreign material. When microorganisms can reach the biomaterial surface they can adhere to it. Adhesion of microorganisms to an implant is mediated by their physico-chemical surface properties and the properties of the biomaterial surface itself. Subsequent surface growth of the microorganisms will lead to a mature biofilm and infection, which is difficult to eradicate by antibiotics.Surface treatment to modify device properties has been used to increase the bio-compatibility and decrease the susceptibility to bacterial adhesion.

In this study a total 52 clinical samples were isolated from implant infection. The microorganism were identified by API system .the susceptibility test was carried out for all isolates to detect multidrug resistant isolates and biofilm formation test was carried out to detect positive slime producing isolates . Antimicrobial activity of irradiated Hydroxyapatite (HAp) was carried out against positive slime producing isolates

The synergistic interaction between irradiated HAp and some antibiotics was carried out to evaluate the effect of irradiated HAp and antibiotics on microbial growth.

The microbial adherence on 316stainless steel chips as biomaterial was detected before and after coating with irradiated HAp and / or antimicrobial agent and it was noticed that the number of adherent strains decreased after coating with irradiated HAp and /or antimicrobial agent. HAp was exposed to gamma irradiation at doses levels 15 and 25kGy to study the effect of radiation on the antimicrobial activities of HAp. The results revealed that the doses 15 and 25kGy increased this activity but the 25KGy showed higher antimicrobial activity.

Key Words
316 Stainless Steel chips, Gamma irradiation, Microbial adherence, Biofilm formation, Hydroxyapatite and Bone infection.

1.Introduction

Biomedical devices have become essential parts of the human healthcare system. Over the past two decades, the number of artificial hip and other organ replacements has been used successfully to save lives and to restore quality of life for many people**(Griesser*et al.*, 2008**).

A wide variety of materials is used for medical purposes. These include ceramic,

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glasses, metallic and polymeric biomaterials. Metallic biomaterials that are used include stainless steel, titanium, tantalum, nickel titanium alloys. Medical grade stainless steel (316L SS) is widely used in fracture repair devices and joint replacement components due to the fact that it is a more economical alternative as compared to other metallic biomaterials**.(Saxena and Rout ,2011)**

A significant issue in implant surgery and also with short term biomedical devices is bacterial infection. The colonization of surfaces of biomedical devices and implants by bacteria can cause infections that pose a health risk to patients, often require re-operation and replacement of the infected device, and incur considerable healthcare costs. Delayed infections, occurring many weeks or months after surgery, continue to pose a serious problem. It is thought that these late-stage infections are caused not by the act of surgery but by bacterial spores circulating in the vascular system. Spores landing at an incompletely healed wound site may attach to the implant surface, multiply, and form a biofilm that eventually leads to infection**. (Griesser *et al.*, 2008).**
Accordingly, many strategies for reducing the occurrence of infections were used to prevent the initial attachment of bacteria to implant and device surfaces.
Hydroxyapatite (Ca_{10} $(PO4)_6(OH)_2$), abbreviated as HAp, is an inorganic compound, a non-toxic bioactive ceramic and its chemical composition is similar to the composition of bone **(Samar *et al.*, 2007).** HAp has antibacterial activity against most common gram-positive and gram negative bacteria. **(El-Tantawy *et al.*, 2012 and 2013).**

Gamma irradiation has been frequently used for sterilization of biomedical materials **(Weir,*et al.*,2004)** and can alter different aspects as degradation, hydrophilicity, bioactivity, and sterility **(Chu *et al.*, 2002).**
Some structural changes in bioceramics such as hydroxyapatite may occur after gamma irradiation like an increase in their enthalpy **(Kubisz *et al.*, 2003)** and decrease in contact angle with increasing in its biocompatibility**.(Sima *et al.*, 2014)**

Surface modification of stainless steel is done before its usage in the body for improving its corrosion resistance and bioactivity .Coating of the metallic implants with bioactive HAp leads to a rapid bonding between hydroxyapatite and the surrounding bone tissue. **(Saxena and Rout, 2011)**
The sol–gel dip coating process can be usefully utilized to synthesize both HAp powders and HAp films under significantly mild conditions. The versatility of the sol-gel method opens a great opportunity to form thin film coatings in a rather simple process, an alternative to thermal spraying which is currently widely used for biomedical applications(**Gupta and Kumar, 2008**).

Biomaterial associated infections are dramatic threat to human body. So, this work analyzed the influence of surface modification of 316LSS chipsby coating with γ irradiated HAp and/or antimicrobial agents on the extent of relative growth and adhesion ability of some selected strains from implant infection.

2.Materials and Methods

Samples, isolation and identification of pathogenic microorganism.

Clinical samples were taken from fifty two patients with implant infectionfrom microbiological laboratories belonging to Ain –Shams University Hospitals through January to June 2014.All pathogenicisolates were cultivated on nutrient agar medium (NA),nutrient broth (NB) (Oxoid, UK), MacConkey agar No .3(Oxoid,

UK).Identification was carried out by using the API system (API 20E strips) (The API STAPH)(BioME´RIEUX).

Gamma radiation source

Cobalt - (60Co) Gamma cell GC220, product of Canada Co.Ltd .located at the National Center for Radiation Research and Technology (NCRRT) Atomic Energy Authority was used for irradiation of HAp and the dose rate was 2.08 kGy/h at the time of experiments.

Determination of antimicrobial susceptibility patterns

All pathogenic isolates were subjected to antibiotic sensitivity test using 19different type of antibiotics agents representing different classes and different mode of actions and one antimycotic agent (NS). (Oxoid Ltd, Basingstoke, UK).

This test was done using the antimicrobial disk-agar diffusion procedure, the so-called Kirby-Bauer disk diffusion method as reported by **(Bauer *et al.*, 1996)** and described by **National Committee for Clinical Laboratory Standards (NCCLS, 2005) and (Parekh and Chanda, 2006).**Sixteen types of antibiotics (Amoxycillin /Clavulainc acid (AMC) ,Imipenem (IPM) ,Cefotaxime (CTX) and Ceftazidime (CAZ), Cefepime (FEP) , Amikacin (AK) , Gentamicin (CN), Tobramycin (TOB),Doxycycline (DO) Clarithromycin (CLR) , Azithromycin (AZM), Ciprofloxacin (CIP), Levofloxacin (LEV),Nitrofurantoin (F),Colistin Sulphate (CT),Trimethoprim/ Sulfamethoxazole (SXT)) were used in case of gram negative bacteria.

Eighteen types of antibiotics (Amoxycillin/ Clavulainc acid (AMC) ,Imipenem (IPM) ,Cefotaxime (CTX) and Ceftazidime (CAZ) , Cefepime (FEP) ,Vancomycin (VA),Amikacin (AK) , Gentamicin (CN), Tobramycin (TOB),Doxycycline (DO) ,Clindamycin (DA) , Clarithromycin (CLR) , Azithromycin (AZM),Rifampin (RD),Ciprofloxacin (CIP), Levofloxacin (LEV),Nitrofurantoin (F), Trimethoprim/ Sulfamethoxazole (SXT)) were used in case of gram positive bacteria while Nystatin (NS) was used as antimycotic agent .

The diameter of inhibition zone(IZ)was interpreted by referring to standard chart (Oxoid) and the organisms were reported as susceptible(S), intermediate (I), or resistant (R) to the agents that have been tested.

Slime production

All pathogenic isolates were subjected to qualitative assessment of slime production by the tube method (TM)and Congo Red Agar (CRA) method.

The TM was described by **(Christensen *et al.*, 1982),** the tested isolates were inoculated into a glass tube containing 5 ml of trypticase soy broth (TSB, Oxoid), with 1% glucose and incubated under static conditions at 35°C for 24h.After withdrawal of the contents, the tubes were washed twice with phosphate buffer saline (PBS) (pH 7.3) and dried, then stained with crystal violet (0.1%) and excess stain was washed with deionized water.

Slime production was judged to have occurred and adherent growth to be present if a visible continuous stained film lined the inner walls of the tube. The experiments were repeated three times.

The amount of stained biofilm was macroscopically semiquantitated as strong (+++), moderate (++), weak (+) or absent (0). The experiment was performed in triplicate and repeated three times.CRA method was described by **Freeman *et al.*, (1989)**.

CRA medium was prepared with brain heart infusion broth (BHI) (Oxoid, UK) 37 g/L, sucrose 50 g/L, agar No. 1 (Oxoid, UK) 10 g/L and Congo red indicator (Oxoid, UK) 8 g/L. First Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 min) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55°C. CRA plates were inoculated and

incubated aerobically at 37°C for 24h .Isolates that produced black colonies with dry crystalline consistency were regarded as slime positive, whereas those showing pink colonies were slime negative. The experiment was performed in triplicate and repeated three times.

Adherence assay and quantification of biofilms

Adherence assay and quantitative determination of biofilm were carried using the tissue culture plate **(Christensen *et al.*, 1985).** Aliquots (200µl) 1.5x108 Cfu/ml of cell suspension were added to the wells of sterile tissue culture plates, polystyrene, flat bottom tissue culture plate ((Sigma-Aldrich, Costar, USA)) and incubated at 37ºC for 24h.The medium and non-adherent cells were removed by washing three times in PBS (pH 7.2). Slime and adherent organisms were fixed by incubation for 1h at 60c **(Baldassarri et al., 1993)** and then stained with Hacker crystal violet (0.1%w/v) for 5min. After washing with water to remove the excess stain, the plates were dried for 30 min at 37 C.Then, the optical densities (ODs) of stained adherent biofilm were read with Micro ELISA Auto Reader at wave length 630nm .Adherence measurements were performed in triplicate and the values were then averaged. The isolates were classified based on ODs as weak, moderate or highly adherence according to **(Mathur *et al.*, 2006).**

Effect of different concentrations of irradiated HAp on some isolates.

This test was carried out according to the method of **Joshi *et al.*, 2009** on slime producer isolates using (100,150and 200mg).Twenty four hour cultures of the selected strains were inoculated in NB, incubated then swabbed over the entire surface of the agar plates. Three wells of 6mm diameter were bored in the medium for each plate with the help of sterile cork-borer and were filled with 100 µl of the suspension of un-irradiated and irradiated HAp using sterile micropipette. All plates were incubated at 37°C for 24 h, followed by measurement of the diameters of IZs. All the experiments were carried out in triplicate, the data were then averaged.

Effect of gamma irradiation on the antimicrobial activity of hydroxyapatite (HAp).

This test was carried out according to the method of **Joshi *et al.*, 2009.**A definite amount of HAp (Sigma Aldrich, USA) (100 mg) was exposed to γ - radiation doses (15 and 25 kGy) while un-irradiated HAp sample was used as negative control as previously mentioned.

Determination of minimum inhibitory concentrations (MICs) of some selected antimicrobial agents.

Determination of minimum inhibitory concentrations (MICs) of FEP, CIP, DA and CN was carried out against the selected strains according to **(Andrews 2001**) and **(Parekh and Chanda 2006)** and (MIC) of NS was carried out according to **Magaldi .*et al.*, (2001).**

In a three sterile universals, three stock solutions for each of the selected antibiotics were prepared with different concentrations of 10,000mg/l, 1000mg/l and 100 mg/l. From the sterile first tube with concentration of 10.000mg/l one ml was added to 9ml sterile distilled water to give a concentration of 1000mg/l in the second tube.

In the third tube 100µl from the initial 10.000mg/l solution were added to 9.9 ml sterile distilled water to give a concentration of 100mg/l, then serial dilutions were prepared

The inoculum used was prepared from a 24-h culture; a suspension was made in a sterile nutrient broth, The turbidity of the suspension was adjusted with a spectrophotometer to obtain a final concentration to match that of a 0.5 McFarland standard .The inoculated agarwas swabbed into the assay plate then wells were cut out of the agar, and100µl of the antimicrobial agent were placed into each well.

The plates were incubated at 35 ◦C for 24 h to determine the diameters of IZs.

Combination treatment of irradiated HAp and antimicrobial agents againstslime producer pathogens

The study of synergistic action between irradiated HAp and antibiotics or antifungal against some selected pathogenic strains was carried out according to **Bauer *et al.*,(1996).**

Bacterial or yeast cell suspension in NB or sabroud dextrose broth (SDB) was prepared and adjusted 0.5 McFarland (1.5 x 10^8 CFU/ml). The microbial suspension was applicated by using sterile cotton swab. Wells of 6mm diameter were filled with 50 µl of the suspension of the antibiotic (at its MIC) or 50 µl of (NS) (at its MIC) and 50 µl of irradiated HAp concentration using micropipette.

Plates were left for 45 min at room temperature. All plates were incubated at 37°C for 24 h, followed by the measurement of the diameters of IZs and compared with that of antibiotic, antifungal and irradiated HAp alone.

Surface modification of 316 LSS with irradiated HAp by sol-gel method

Preparation of irradiated HAp/ polyvinylpyrrolidone (PVP) Composites was carried out according to **(Ragu *et al.*, 2014).** To prepare the irradiated HAp/PVP composites, we put a suitable amount of PVP (2gm) in 20 ml double-distilled water with stirring by magnetic stirrer for about 6h and then leave the solution for 3h at the room temperature to avoid the formation of bubbles. Irradiated HAp solution was slowly added and stirred for 6h at the same temperature, a milky white coloration was observed. Finally the mixture was put in dishes in oven at 60°C.According to **Deepak and Uma (2015)**,the irradiated HAp coating on 316L SS substrates was synthesized by sol-gel method. The pre-sterilized 316L SS samples were dipped into this sol solution at a speed of 10mm/min. After dipping once the sample was withdrawn with the same speed as used for dipping to get uniform thickness. Now the coated substrate was dried by immediately transferring into an oven at 70°C for 10 minutes.

Detection of antimicrobial activity of coated 316L SS chips on selected slime producer strains.

The antibacterial effect of 316L SS chips coated with irradiated HAp and/or CN was determined by the Kirby- Bauer disk diffusion method as previously mentioned. The diameter ofIZwas measured, including the diameter of the coated stainless steel chips. The experiments was carried out in triplicate, the data were then averaged.

Detection of adherence of selected strains on the surface of 316L SS and coatedwith irradiated HAp and /antimicrobial agent.

This test was carried out according to **Parizzi. *et al.*,(2004**). Sterile clean chip with coated and irradiated HAp and /or antimicrobial agents was immersed in a flask containing 100 ml of BHI, which was previously inoculated with 24h suspensions of the selected strains. The flasks were incubated for 24h at 37c and the test was carried out as previously mentioned.

Statistical analysis

Statistical analysis was employed to detect the effect of gamma irradiation on the antimicrobial activity of hydroxyapatite (HAp) using a software program and analyzed by unpaired two tailed student's t-test **(Snedecor and Cochran, 1980).**The difference between means were considered to be statistically significant at $p<0.05$.

3. Results and Discussion

3.1 Results

Isolation and Identification of microbial isolates

Fifty two isolates,were isolated from patients(27 males (51.9%) and 25 females (48%)),were identified on the bases of their morphological and biochemical characteristics , Forty eight isolates were gram negative bacilli , two belonging to gram positive cocci and two were yeast species. The pathogenic isolates belonging to 6 genera were identified,by using API, sixspecies of pathogenic bacteria and one fungal strain were revealed (Table 1).
These species were *E.coli, K.pneumoniae, P. aeruginosa,P.fluorescens, E. cloacae, St.aureus and*C. *albicans.*

Table (1): Th e prevalence genera and species of isolated pathogens

Isolated genera and their Species	Number of isolates	Percent (%)
E.coli	18	34.6
K.pneumoniae	15	28.8
*P.*species	6	11.53
P.aeruginosa *P.fluorescens*	4 2	7.69 3.84
E.cloacae	9	17.30
St.aureus	2	3.84
C.albicans	2	3.84
Total	52	

Antimicrobial susceptibility test for all isolated pathogens against different antibiotics.

Results presented in figures (1-2) showed that some antibiotics affecting the isolated strains
It is clear that, the highest percentage of antibiotic resistance of gram negative bacterial isolates was 81% against CLR, followed by AMC andFEP (74% and 70%), respectively, while it was 68% against CTX and 62% for AZM. Regarding SXT,F,and CN the percentage of resistance was 58%, and 52%,50%,respectively. Followed by CAZ and TOB (47%), while it was 45% and 43% for DOand CIP respectively.In case of CT, the resistance was 35% followed by LEV and AK (33% and 25%), respectively,finally IPM which showed the lowest resistance 8 %.(figure 1). The highest percentage of antibiotic resistance of gram positive bacterial isolates was 100% against CLR, AZM , DO,TOB ,AK and CIP while only one isolate 50% was resistant to each FEP, CTX , CAZ,VA , IPM ,CN , DA, F, LEV and RD. Meanwhile each of the two isolates was resistant to AMC and SXT. Figure (2).

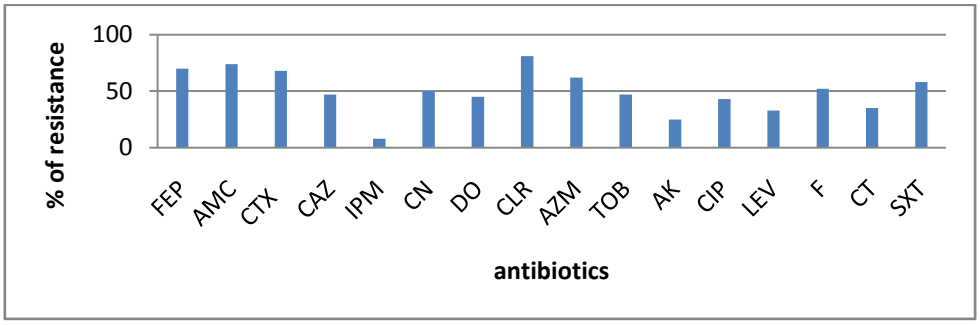

Figure (1): Antimicrobial susceptibility patterns of gram negative bacterialisolates.

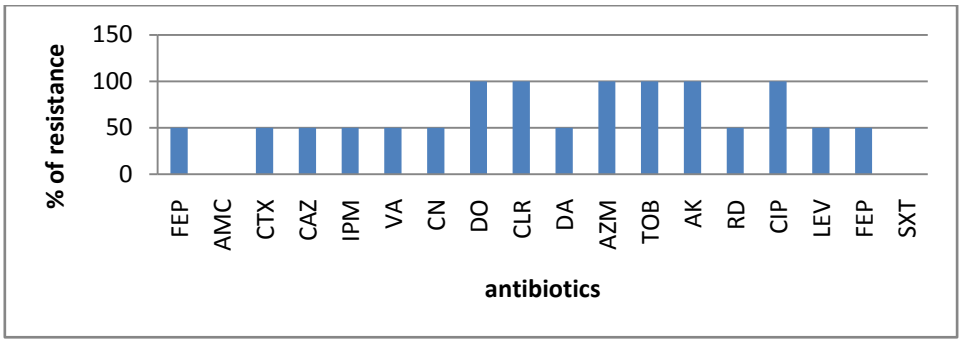

Figure (2):Antimicrobial susceptibility patterns of gram positive bacterial isolates .

Detection of slime productionand quantification of biofilms

All bacterial pathogens were subjected to a qualitative assessment of slime production by using visual methods; (TM) and (CRA).

It was found that 32(30 bacterial and 2 yeast isolates) out of 52 isolates (61.5%) were slime positive with variable degrees and 20 (38.4 %) were slime negative. Slime production score was carried out in three separate experiments, each performed in duplicate and the results were recorded with three different observers.

Quantitative assessment of slime production was carried out by the spectrophotometric method (quantitative micro method). The results in table (2) revealed that out of the tested pathogens, isolates no. 8, 22, 47 and 52 can strongly produce biofilm while 34 and 14 isolates were moderately and weakly biofilm producers respectively.

Table (2):Slime production and quantitative assessment of adherence of pathogenic isolates.

Isolate no.	Slime	Adherence (ODs)	Isolate no.	Slime	Adherence (ODs)
1	M	0.177	27	M	0.206
2	W	0.083	28	M	0.223
3	W	0.066	29	M	0.192
4	M	0.209	30	M	0.167
5	W	0.062	31	M	0.183
6	W	0.074	32	M	0.201
7	M	0.176	33	M	0.135
8	S	0.263	34	M	0.148
9	W	0.079	35	W	0.118
10	M	0.233	36	W	0.058
11	M	0.217	37	W	0.063
12	M	0.194	38	W	0.095
13	M	0.230	39	W	0.065
14	M	0.143	40	M	0.195
15	M	0.186	41	M	0.156
16	M	0.195	42	M	0.213
17	M	0.210	43	M	0.201
18	M	0.224	44	W	0.087
19	M	0.152	45	W	0.094
20	W	0.059	46	W	0.075
21	M	0.214	47	S	0.302
22	S	0.353	48	M	0.182
23	M	0.217	49	M	0.1717
24	M	0.198	50	M	0.231
25	M	0.201	51	M	0.234
26	M	0.134	52	S	0.251

W: weakly-adherent (ODs ≤ 0.120) ,M: moderate adherent (0.120 < ODs ≤ 0.240)
S: strongly adherent (ODs > 0.240).

Assessment of antimicrobial activity of different concentrations of irradiated HAp.
This test was carried out to screen the effect of different concentrations (100,150,200 mg/ml) of irradiated HAp against positive slime clinical isolates. It was found that irradiated HAp has antimicrobial activity against the tested isolates. The results presented in table (3) indicated that by increasing concentration of irradiated HAp, the diameter of IZ increased.

Table (3): Assessment of antimicrobial activity of different concentration s ofirradiated HAp.

Inhibition zone (mm)				Inhibition zone(mm)			
Isolate no.	Concentration of HAp (mg/ml)			Isolate no.	Concentration of HAp (mg/ml)		
	100	150	200		100	150	200
1	20	22	28	27	15	16	25
2	20	22	27	28	13	14	16
3	20	22	30	29	15	22	30
4	25	30	32	30	20	25	30
5	15	19	22	31	25	26	34
6	27	28	32	32	18	21	28
7	20	26	31	33	12	17	24
8	17	19	20	34	13	15	18
9	32	33	37	35	10	15	21
10	33	34	45	36	13	17	24
11	21	23	30	37	22	27	35
12	22	23	32	38	16	20	29
13	22	30	33	39	16	24	33
14	16	18	23	40	21	27	35
15	20	23	28	41	17	22	30
16	22	28	32	42	20	27	33
17	15	21	26	43	10	19	25
18	21	25	30	44	15	21	29
19	30	32	42	45	12	17	24
20	30	32	45	46	15	19	25
21	20	22	23	47	13	15	15
22	14	17	18	48	20	21	30
23	15	16	20	49	16	17	20
24	17	25	35	50	21	22	25
25	31	32	40	51	11	16	18
26	25	27	30	52	15	17	19

Effect of gamma irradiation on the antimicrobial activity of HAp against seven selected slime producer strains.

Gamma irradiation is able to affect various structural and biological properties of biomaterials and a dose of 25 kGy of gamma irradiation has been recommended for terminal sterilization of medical products Figure (3) showed the effect of two different doses of gamma irradiation

(15 and 25kGy) on the antimicrobial activity of HAp against seven selected slime producer strains .It was found that by increasing dose of γ irradiation, the antimicrobial activity of HAp increased, if compared to that of control un irradiated samples.

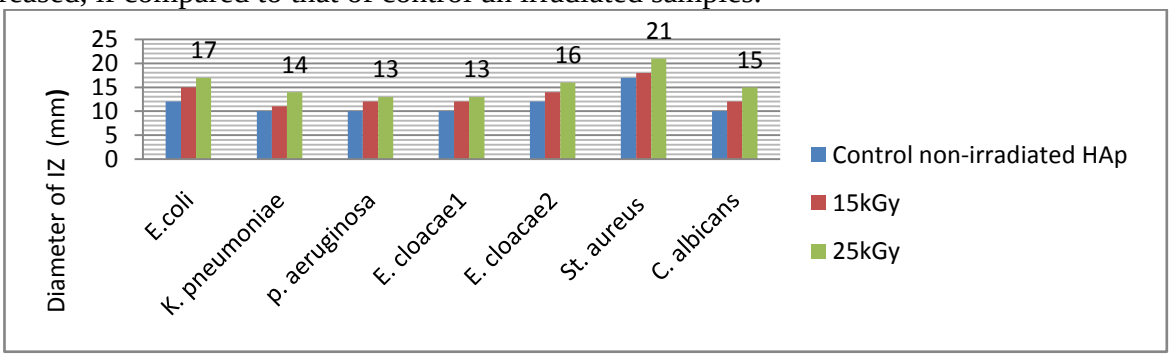

Figure (3): Effect of gamma irradiation on the antimicrobial activity of HAp against seven selected slime producer strains.

Determination of minimum inhibitory concentrations (MIC) of some selected antibiotics and antimycotic agents.

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that inhibit the visible growth of a microorganism after an overnight incubation .MICs are used as research tool to confirm resistance and to determine the *in vitro* activity of new antimicrobials. CN, FEP, CIP and DA were chosen as antibiotics that are usedcommonly in bone infection treatment.

Table (4): Minimum inhibitory concention (MIC) of common antibiotics on the selected slime producer strains.

Strain No.	Pathogenic strain	MIC of antibiotics(mg/ml)				
		FEP	CN	CIP	DA	NS
8	*E.coli*	8	1	4	ND	ND
22	*K. pneumoniae*	16	1	4	ND	ND
34	*P. aeruginosa*	128	16	1	ND	ND
47	*E. cloacae1*	2	8	2	ND	ND
49	*E. cloacae 2*	4	4	4	ND	ND
50	*St. aureus*	ND*	ND	ND	0.5	ND
52	*C.albicans*	ND	ND	ND	ND	12.5

ND*-not detected.

Combination treatment of irradiated HAp and certain antimicrobial agents against some selected slime producer strains

This study was carried out on seven identified producer strains to elucidate the effect of irradiated HAp in combination with common antimicrobial agents to identify systems which might be used to improve its the efficiency on pathogens. The results were presented in table (5).

Table (5): Combination treatment of irradiated HAp and some antimicrobial agents against seven selected slime producer strains

Strain no	Microorganism	diameter of IZ (mm)										
		FEP	CN	CIP	DA	NS	HAp (100mg)	HAp+FEP	HAp+CN	HAp+CIP	HAp+DA	HAp+NS
8	*E.coli*	14	20	21	ND*	ND	17	22	25	28	ND	ND
22	*K. pneumoniae*	14	15	15	ND	ND	14	20	24	21	ND	ND
34	*P.aeruginosa*	16	24	16	ND	ND	20	29	33	31	ND	ND
47	*E. cloacae* 1	18	12	21	ND	ND	13	22	30	30	ND	ND
49	*E. cloacae* 2	23	13	22	ND	ND	16	28	35	30	ND	ND
50	*St. aureus*	13	12	16	19	ND	21	28	33	31	33	ND
52	*C.albicans*	ND	ND	ND	ND	16	15	ND	ND	ND	ND	24

ND*: not detected.

Detection of antimicrobial activity of coated 316 L SS chips on selected slime producing strains.

Data presented in Table (6) showed the antimicrobial activity of 316LSS chips coated with irradiated HAp and CN at its MIC. It was found that after coating, the IZ of all applied strains increased if compared to that coated with irradiated HAp and/ or antimicrobial agents alone.

Table (6): Antimicrobial activity of coated 316L stainless steel chips with IrradiatedHAp and CN in respect to action of Gentamicin only on certain strains.

Strain No.	Pathogenic bacteria	MIC of CN	Irradiated HAp(100 mg/ml)	Irradiated HAp +CN at its MIC
8	*E.coli*	20	17	39
22	*K.pneumoniae*	15	14	36
34	*P. aeruginosa*	14	13	32
47	*E. cloacae1*	12	13	36
49	*E. cloacae 2*	13	16	34
50	*St. aureus*	12	21	32
52	**C.albicans*	16	15	31

*****coating with NS at its MIC

Detection of adherence of selected strains on the surface of 316L SS chips andcoated with irradiated HAp and /or antimicrobial agents.

Table (7) shows the ability of selected strains to adhere on 316 L SS andafter coating with irradiated HAp and /or antimicrobial agents. It was found that the ability of adherence of all strains decreased after coating with irradiated HAp and /or antimicrobial agents.

Table (7): Detection of adherence of selected strains on 316 L stainless steel surfacecoated with irradiated HAp and HAp with CN.

Strain No.	Pathogenic strain	Number of CFU/cm^2	Number of CFU/cm^2 after coating with irradiatedHAp	Number of CFU/cm^2 after coating with irradiatedHAp +CN
8	*E.coli*	$2.2X10^5$	$1.8X10^3$	$4.0X10^2$
22	*K. pneumoniae*	$3.6X10^5$	$2.1X10^3$	$5.5X10^2$
34	*P. aeruginosa*	$4.8X10^5$	$3.2X10^3$	$5.5X10^2$
47	*E. cloacae1*	$4.3X10^5$	$2.0X10^3$	$2.1X10^2$
49	*E. cloacae 2*	$3.8X10^5$	$2.9X10^3$	$2.7X10^2$
50	*St. aureus*	$3.1X10^5$	$2.0X10^3$	$4.4X10^2$
52	**C. albicans*	$5.3X10^5$	$4.0X10^3$	$1.3X10^2$

*the applied antimycotic agent used with *C.albicans* was NS

Statistical analysis.

Results in table (8) indicated that there was a significant effect of gamma irradiation at dose 25kGy on the antimicrobial activity of irradiated HAp against all the tested strains except *Staphylococcus aureus*, *E. cloacae 2*and *Pseudomonas aeruginosa* while gamma irradiation dose (15kGy) affects non-significantly, HAp activity if compared to control non-irradiated sample**.**

Table (8): Statistical analysis of detection of the effect of gamma irradiation on theantimicrobial activity of hydroxyapatite (HAp)

Strain No.	pathogenic strain	Diameter of IZ (mm)				
		Control non-irradiated	HAp(100mg) (15kGy)	HAp(100mg) (25kGy)	P-value	
					15 kGy	25kGY
8	*E.coli*	12	15	17	0.046	0.026
22	*K.pneumoniae*	10	11	14	0.758	0.028
34	*P.aeruginosa*	10	12	13	0.456	0.19
47	*E. cloacae1*	10	12	13	0.066	0.069
49	*E. cloacae 2*	12	14	16	0.391	0.181
50	*S. aureus*	17	18	21	0.756	0.44
52	*C.albicans*	10	12	15	0.116	0.005

3.2 Discussion

Microorganisms can reach a biomaterial implant in several ways at several time points, which determines the properties of the biomaterial surface they will meet. Airborne microorganisms, which can be present in the operating theater, can reach the surface as early as before the implantation **(Gottenbos *et al.*, 2000)** and interact with a bare substratum surface, not even covered with a conditioning film. Also during insertion of the biomaterial, microorganisms from the skin can be pushed towards the implant surface.

Furthermore, microorganisms from the skin can contaminate the operation wound and reach the implant surface through diffusion, active movement or hematogenous transport. Perioperative contamination is believed to be the most common cause of biomaterial-centered infections **((Maathuis *et al.*, 2005).**

Treatment of an established biomaterial -centered infections is difficult, as the minimum inhibitory concentration (MIC) of antimicrobial agents, necessary to kill the microorganisms, is significantly higher for microorganisms in a biofilm than for planktonic ones.

As antibiotics have little effect on biomaterial-centered infections, the standard procedure for infected orthopedic prostheses is the removal of the implant and implantation of an antibiotic releasing device at the implant site. **(Jiang *et al.*, 2010).**

In the study 52 clinical samples and specimens were collected from bone wounds, out of them 48 (92.3%) isolates were gram negative bacilli , 2 (3.8%) were gram positive cocci and 2 (3.8%) were yeast. **Khosravi *et al.*, (2009)** reported that, prevalence of isolated bacteria from orthopedic implant infections was gram positive 33.5 % and gram negative 64.5%. Other authors ,**Gomez *et al.*, (2003)** found that the incidence rate for gram positive and gram negative bacterial isolates from orthopedic implant infections was 60.6% and 33.3% respectively. This difference in results may be due to different nosocomial pathogens present in the operating rooms.

Among the isolated pathogens, *E.coli* and *K.pneumoniae* are the most common isolates followed by *E.cloacae*, P. *aeruginosa*, *P. fluorescens, St.aureus* andfinally C.albicans.

Lin *et al.*, (2013) noted that *K. pneumoniae* was estimated to be higher in long-term acute care hospitals while **Panta*et al.*, 2013**is in agreement with our results where they found that *E.coli* was the major isolates in the different tested clinical samples followed by *Klebsiella spp*.

Also a study performed by **Carvalho*et al.*, (2012) and Chihara and Segreti, (2010)** reported that, gram-negative bacteria, such as *P.s aeruginosa* and Enterobacteriaceae have grown in importance as causative agents to the increasing number of orthopedic surgeries with the use of implants. **Eid and Berbari, (2012) and Arias *et al.*, (2004)**found *thatC. albicans* may be isolated from the talus.

Antimicrobial resistance has become a major medical and public health problem. Most of the common antibiotics as Clindamycin, Cephalosporin's and Gentamicin thatare normally used for treatment of orthopedic infections were ineffective against bacteria alone. **Khosravi *et al.*, (2009)**

The obtained results in the present study was in complete harmony with the findings shown by **Keven *et al.*, (2003)** who found that, more than 70% of gram negative isolates from wound infection, pulmonary infection, and sepsis were sensitive to aminoglycosides, fourth generation Cephalosporin, Quinolones, and Imipenem.Isolates were resistant to first generation Cephalosporin and Amoxycillin\Clavulainc acid.

Bacteria having the ability to produce biofilms lead to long term persistence, promote colonization and increased rate of infections. Biofilm producing clinical isolates tend to survive on implanted medical devices and also on tissue wounds causing cystic fibrosis.**(Ercan *et al.*, 2008).**

Other authors**Rewatkar and Wadher, (2013)** showed that*P. aeruginosa*, *St. aureus*, produces strong biofilm by various methods and these microbes are highly resistant to various antibiotics **.Donlan, (2001)** found that most of gram positive and gram negative bacteria have the capacity to synthesize biofilm including *St. aureus, St. epidermidis, S. viridans, E.coli, E. faecalis, K. pneumoniae, P.mirabilis* and *P. aeruginosa.*

In this study TM and CRA methods revealed that out of 52 isolates 32 (61.5%) were biofilm producers and the remaining of the pathogens cannot produce biofilm. This finding is in a fair agreement with **Shyam*et al.*,(2015)**.

Quantitative determination of slime production was done by ELISA method. Among 52 producing biofilm isolates, four isolates were detected as strong adherent, 34 as moderate adherent and 14 as weak adherent. **Nabajit, (2014)** demonstrated that among 100 isolates

from blood samples, urine sample, others, 36 isolates were detected as strong, 47 as moderate and 17as weak/non biofilm producers.

A similar study performed by **Afreenish *et al.*, (2011)** showed among 110 isolates from the pus, intravenous and urinary catheter tips, urine and sputum the tissue culture plate detected 25 isolates as strong and 45 as moderate biofilm producers. Bacterial adherence may trigger the process of microbial growth and biofilm formation .Results, in the current study, showed that tested strains have the ability to adhere on 316L SSchips.

The use of synthetic hydroxyapatite (HAp) in biomedical applications is well warranted. It has shown to have an excellent biocompatibility in human tooth and bones. Additionally it has been documented to possess antibacterial potentials. HAp is non-toxic and did not elicit any inflammatory symptoms (**Surajit*et al.*, 2011).**The current investigation was carried out to screen the antimicrobial activity of HAp against the tested pathogens. The obtained results showed that HAp has antimicrobial activity against all the tested isolates from bone wounds, by increasing its concentration, the diameter of inhibition zone increased. Our results are in agreement with **Ragab *et al.*, (2014)** who found that hydroxyapatite is active against most common gram-positive and gram negative bacteria making it useful for clinical applications and environmental fields. Also **Tin -Oo *et al.*, (2007)** demonstrated that the antibacterial property HAp should be used to good advantage as a bioactive biomaterial in dental and maxillofacial applications.

El-Tantawy *et al.*, 2012 and 2013 reported that Gram-positive bacteria (such *as St. aureus*and *St. epidermidis*) are less susceptible to Ca ions than Gram-negative bacteria (such as *E. coli, K.pneumoniae, P. aeruginosa*,) due to differences in their membrane structure**.** Another possible explanation for the antibacterial effect is based on the abrasive surface ordering (i.e. texturing) of HAp due to surface defects and aggregates which contribute to the mechanical damage to the cell membrane of the bacteria. **(Ragab *et al*., 2014)**

Nguyen *et al.*, (2007) demonstrated that a dose of 25 kGy of gamma irradiation has been recommended for terminal sterilization of medical products**.**

The antimicrobial activity of HAp exposed to γ - irradiation doses (15 and 25) kGy was investigated. Our results revealed that the irradiated HAp has more antimicrobial activity on the tested strains if compared to non-irradiated samples.

Sima *et al.*, (2014) reported that γ – irradiation is able to affect various structural and biological properties of biomaterials. They indicated a decrease in alkaline phosphatase activity in irradiated HAp (25 and 50 kGy) which can enhance its application as biomaterial. **Thanigaiarul*et al.*, (2013)** found that low energy ion beams have also improved wettability, bioactivity, and protein absorption without major structural changes.

Concern is increasing about the emergence of antimicrobial resistance. MIC of Gentamicin for some gram negative bacilli isolates including *E. coli, K.pneumoniae, P. aeruginosa and E.cloacae* haspreviously been shown to be ranged between ≤0.06mg\l and ≤64mg\l **Ling *et al.*, (2006) , Hirakata *et al.*, (2005)**

Also **Fish *et al.*, (2002)** found that, MIC values of Cefepime against *Pseudomonas aeruginosa* were between ≤0.125- ≤8 mg/l which the results obtained in the present study.

Surface modification is considered an economical method for improving corrosion resistance and bioactivity of biomaterials. Coating approaches can effectively modify surface properties for bone implants. The sol gel dip-coating process is used mainly for the production of coatings on items This deposition method has the advantage of being capable of producing multiple coatings with a high degree of uniformity up to 1000 nm thick.**Balamurugan *et al.*, (2005) and Harle*et al.*,*(*2006)** recommended employing HAp coatings on the surface of the metallic implants. **Mehdi *et al.*, (2012)** demonstrated that Sol-

gel dip coating method is proved to be a cheap and easy coating deposition method for the metallic biomaterials. Also **Deepak and Uma, (2015***)* demonstrated that HAp coatings have been successfully deposited on 316L SS using sol–gel dip coating technique.

In the current study, we use sol–gel dip coating as a method of surface modification for preventing adherence of pathogens on the applied 316L SS. The obtained results clearly indicate that coating of chips with irradiated HAp and antimicrobial agents inhibit the ability of growth of pathogens and consequentially its ability to produce biofilm. **Thanainit et al., (2008)** found that antibiotic hydroxyapatite pellets effectively release antibiotics and can inhibit the growth of bacteria causing infection in orthopedics.

Stigtera,(2004) reported that the incorporation efficiency of an antibiotic is strongly related to the presence and number of the carboxylic groups in its chemical structure.Soantibiotics, containing carboxylic groups in their chemical structure were better incorporated than others. Hydroxyapatite contains two types of binding sites, positively charged calcium and negatively charged phosphate groups. The antibiotics interact chemically with calcium and phosphate ions in hydroxyapatite according to their chemical structure. Basic antibiotics containing amino groups might be co-precipitated with phosphate, but this mechanism is less likely. For instance, Gentamicin is basic antibiotic and lack carboxylic groups. It was poorly incorporated and quickly released from the coating. Nystatin is a polyene antibiotic obtained from *Streptomyces noursei*. The molecular formula for nystatin is $C_{47}H_{75}NO_{17}$; Nystatin is an antibiotic which is both fungistatic and fungicidal *in vitro* against a wide variety of yeasts and yeast-like fungi, including *C. albicans*. It was observed that nystatin contain carboxylic group so it interacts well with hydroxyapatite and release slowly. Our results confirmed that the synergism between irradiated HAp and the tested antibiotic or NS enhanced the effect of irradiated HAp on the growth of the selected strains.

4. Conclusion

Persistent bacterial infections associated with medical implants remain a serious and costly drawback with both temporary and permanent consequences. Clinical observations of implant-associated infections have revealed that the initial adhesion and growth of microorganisms on the surface of an implant may be the most critical event in the development of device-associated infection.

In order to overcome or reduce this problem, surface modification of the biomaterials or devices is a relatively straightforward strategy for creating the desirable surfaces which will decrease the susceptibility to bacterial adhesion .Specific coatings may be applied to a device to receive antimicrobial agents to provide device protection from infection

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الملخص باللغة العربية

منع الالتصاق والنمو السطحي للعدوي الميكروبية علي الطعم العظمي بواسطه تعديل السطح باستخدام المضادات الحيوية وهيدروكسي ابيتايت المشعع

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2-قسم الميكروبيولوجي ،كليه العلوم، جامعه حلوان –ج م ع

ان واحدة من العوائق الرئيسية في استخدام المواد الطبية الحيوية هي حدوث الالتهابات التي تتواجد على المواد الحيويه. حيث يحدث بعد عمليه الزرع ان يتفاعل المضيف مع المادة الحيويه من خلال تشكيل فيلم متكييف على سطحه واستجابة مناعية تجاه هذه المواد الغريبه و عندما تستطيع الكائنات الحية الدقيقة ان تصل إلى سطح الماده الحيويه يمكنها الالتصاق بها .انالتصاق الميكروبات بعد الزرع يرجع الي الخواص الفيزيائية لاسطح الميكروبات وخصائص سطح الماده الحيويه نفسها وحيث يؤدي النموالسطحى لهذه الكائنات الدقيقة الي تكوين فيلم متكيف (بيوفيلم ناضج) وبالتالى حدوث العدوى، وهو أمر صعب القضاء عليه من قبل المضادات الحيوية. لذا استخدمت المعالجة السطحية لتعديل خصائص الجهازوذلك لزيادة التوافق الحيوي للاجهزه الطبيه وتقليل القابلية للالتصاق البكتيري عليها .

في هذه الدراسة تم عزل 52 عينه سريريه بكتيرية وفطرية من الطعم العظمي المصاب ثم تعريفها باستخدام نظام API مع اجراء اختبار الحساسية لجميع العزلات وذلك للكشف عن العزلات المقاومة للمضادات الحيويه مع القيام بعمل اختبار لتكوين فيلم متكيف (بيوفيلم) وايضا اختبار انتاج المادة اللزجة لها .بعد ذلك تم اجراء اختبار خاصيه النشاط الميكروبي المضاد الخاص بهيدروكسي ابيتايت المشعع ضد العزلات إيجابية إنتاج الماده اللزجه. ولقد اجري اختبار تاثير التفاعل بين الهيدروكسي ابيتايت المشعع وبعض المضادات الحيوية وذلك لتقييم تأثير هم على النمو الميكروبي ثم تم الكشف عن التصاق هذه الميكروبات على 316 رقائق الفولاذ المقاومه للصدأ كماده حيويه قبل وبعد تغطيتها باستخدام الهيدروكسي ابيتايت المشعع و/ أو العوامل المضاده للميكروبات وقد لوحظ أن كميه هذه السلالات الملتصقة انخفضت بعد عمليه التغطيه بالهيدروكسي ابيتايت المشعع و / أو العوامل المضاده للميكروبات وذلك نتيجه تأثيرخاصيه المضاد الميكروبي للهيدروكسي ابيتايت المشعع و العوامل المضاده للميكروبات. وتم تعريض الهيدروكسى ابيتايت لأشعة جاما عند مستوى جرعات 15و25 كيلو جراى لدراسة تأثيرهذا الإشعاع على نشاطه الميكروبي المضاد و قد اظهرت النتائج أن جرعات 15و25 كيلو جراي ادت لزيادة هذا النشاط إلا ان 25كيلو جراي اظهرت اعلي نشاط ميكروبي مضاد.