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Effect of Gamma Irradiation on Biofilm Formation of Some Gram- Negative Bacteria Isolated From Burn and Wound Infections

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Abstract : Objectives: To detect the effect of gamma irradiation on Biofilm Formation of Some Gram- Negative Bacteria Isolated From Burn and Wound Infections.
Methods: A total of Fifty isolates included Twenty-three of *Pseudomonas aeruginosa*, Seventeen of *Klebsiella pneumonia* and Ten of *Acinetobacter baumannii* were isolated from burns and wound infections. The susceptibility to different antibiotics was evaluated by disk diffusion method and the effect of gamma irradiation on the growth and their ability to produce biofilm were studied.
Results: All isolates were multi-drug resistant, and the resistance was 100% to tetracycline while all isolates were sensitive to colistin.
The results of irradiation bacterial isolates showed that Cesium (^{137}CS 5µci) and Sodium (^{22}Na) were effected against *P. aeruginosa* isolate, which reduces the CFUs (95.38%) and (95.07%) respectively. Sodium (^{22}Na) was effective source against *A.baumannii* which reduced the growth (75.75%). On the other hand, results in the current study showed a reduction in the growth of *K. pneumoniae* isolate after irradiation with ^{60}Co and ^{137}Cs 5µci.The percentage of biofilm inhibition of *P.aeruginosa* was increased up to (53.7%) after exposed to ^{137}CS and ^{22}Na and increased to (54%) after exposed *A.baumannii* cells to ^{137}Cs.The results of the effect of gamma irradiation on biofilm of *P.aeruginosa* relation to different surfaces (plastic, glass, cotton, stainless steel, gauze and gloves) illustrated that the best antibiofilm effect obtained in stainless steel and plastic with inhibition rate (70.01%) and (50.24%) respectively after exposure to ^{137}Cs.
Conclusion: we report here the gamma irradiation was effectiveness against growth and biofilm formation of Some Gram- Negative bacteria isolated from burn and wound Infections.
Keywords: *Acinetobacter baumannii, Klebsiella pneumoniae*, Cobalt 60, Cesium 137, biofilm.

Introduction

Biofilm formation is one of a common strategy for bacterial survival in hard environmental conditions, bacteria can produce biofilms in water systems and on a set of abiotic surfaces commonly used in such systems as well as in natural aquatic environments[1]. Bacteria in a biofilm, as a structural community, are enclosed in a polymeric matrix constituting a protective mechanism to resist during host infection and in harsh environments[2]. These bacteria become highly resistant to antibiotics or cleaning and therefore this biofilm structure represents an important virulence factor [3].

Pseudomonas aeruginosa is a bacterium that can cause a broad range of acute opportunistic infections in patients with serious underlying medical conditions.*P.aeruginosa* high intrinsic antibiotic resistance enables it to survive in a wide range of other artificial and natural settings, including surfaces in medical facilities[4]. Biofilm formation of *P. aeruginosa* is a mechanism of resistance to antibiotic because biofilm cells are much more antibiotics resistance than planktonic cells[5].

Acinetobacter baumannii Multi drug resistance phenotype seems to play an important role in the remarkable capacity of the bacteria to persist and spread in the environment of hospital, together with its ability to colonize both abiotic and biotic surfaces and to grow as biofilm [6]. The ability of *A. baumannii* to persist in the environment may be due to its ability to biofilm formation on both biotic and abiotic surfaces.Biofilm formation is also a mechanism of pathogenesis in infections related with device and provides a source of repeated transmission by prolonging survival on inanimate objects[7].

Infections caused by microbial biofilms are a significant socio-economic burden that implicates hospitalization, lost employment, patient suffering and reduced life quality. Because the use of conventional antimicrobial compounds in many cases cannot eradicate biofilm infections [8], there is an urgent need to develop alternative measures to combat biofilms. Novel anti-biofilm strategies require detailed knowledge about the biology of biofilms, and accordingly research on biofilm infection microbiology, biofilm formation mechanisms, and biofilm-associated antimicrobial tolerance has come to the fore during the last two decades [9].

Gamma irradiation is widely used for sterilization of food preservation, medical devices and processing of tissue allograft and blood components, obviating the need for high temperatures that can be damaging to such products [10].

The aims of this study were to detect the effect of gamma irradiation on Biofilm formation of some Gram- negative bacteria isolated from burn and wound infections.

Materials and Methods

Bacterial Isolates:

Bacterial isolates were collected from patients admitted to Some Iraqi medical centers in Baghdad during a period from August to October 2015. They were obtained from wounds and burns swabs.The isolates were identified by conventional biochemical methods and vitek 2 system.

Antibiotic susceptibility testing :

The isolates were subjected to antimicrobial susceptibility testing using Kirby-Bauer disk diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines[11], using commercially available 6mm disks (Bioanalyse/Ankara/Turkey).The susceptibility of the isolates was determined against 13 antibacterial agents included: Cefotaxime (CTX), Pipracillin(PRL), Tetracycline(TE), Ceftazidime(CAZ), Tobramycin (TN) ,Gentamycin (GM), Ciprofloxacin (CIP);Cefoxitin (FOX);Cefepime(CPM) Imipenem (IMP) , Carbenicillin(PY), Amoxycillin/ clavulanic acid (AMC) and Colistin (CO),On Mueller- Hinton agar Plate(Lab M Limited Topley House,United Kingdom),using overnight culture at a 0.5 McFarland standard followed by incubation at 35 ^{o}C for 18 h.

Detection of Biofilm Formation :

In the present study, we screened the Fifty clinical isolates of *P. aeruginosa*, *A.baumannii* and *K.pneumoniae* for their ability to form biofilm by micro titer plate according to the method described by [12,13].

Twenty µl of bacterial suspension overnight culture was used to inoculate micro titer wells containing 180µl of Brain Heart infusion broth with 2% sucrose .Control wells contained 200 µl of Brain heart infusion broth with 2% sucrose. The covered micro titer plate was sealed with par film during incubation at 37C^{o} for 24 hr .Un attached bacterial cells were removed by washing the wells three times with PBS (pH 7.2) .Drying at room temperature for 15 min, then 200µl of crystal violet(0.1%) was added to the wells for 15 min. After removing the crystal violet solution wells were washed three times with PBS (pH 7.2) to remove the unbounded dye, allowed to dry at room temperature .Extracted twice with 200µl of 95% ethanol. The absorbance of each

well was measured at 630 nm using ELISA reader. The O.D value for control well was deducted from all the testO.D value.

Irradiation with Gamma Rays:

The bacterial isolates were grown in LB broth for 24 h. on a shaker (150 rpm) at 30ºC. The well grown bacterial culture was centrifuged at 8000rpm for 15 minutes. The supernatant was decanted and the pellets were suspended in sterile saline. The suspended cells were collected in a clean sterile flask to form pool. The bacterial suspension of the pool (5ml) was distributed in clean sterile screw cap test tubes and exposed to gamma source : Cobalt 60 (^{60}Co),Cesium 137(^{137}Cs)andSodium 22(^{22}Na)for different periods.(left one test tube without irradiation as a control).The non-irradiated control and the irradiated cultures were serially diluted and plated on the surface of LB agar plates and the colonies were counted and inhibition effect was evaluated and calculated percent reduction of bacterial growth using the equation described as [10,14].

Effect of Gamma Irradiation on Biofilm Formation :

The antibiofilm effect of gamma irradiation was determined after irradiated bacterial cells, two parallel wells of the same sample (control and irradiated) incubated for 24 hr, After that , un attached bacterial cells were removed by washing three times with water , then drying at room temperature for 15 min . After drying crystal violet was added for 20 min, the stained wells were rinsed three- time with D.W. allowed to dry at room temperature for 15 min and extracted twice with 95% ethanol , the rest of solution was assembled and the absorbance was measured at 630 nm using ELISA reader. The inhibition of biofilm formation percentage was calculated as equation described by[15].

Inhibition of biofilm formation on other surfaces :

Cesium 137(^{137}Cs) gamma rays was used for inhibition of biofilm formation in the glass plate , plastic plate , cotton ,gloves, stainless steel and gauze. Bacterial isolates were inoculated in 100 ml LB medium. After irradiation , the culture was divided under aseptic conditions into 1 ml aliquots into groups added to each treatment separately and remained one as a control . each control and treatment incubated for 24 hr, After that , un attached bacterial cells were removed by washing three times with water , then drying at room temperature for 15 min . After drying crystal violet was added for 20 min, the stained surfaces were rinsed three- time with D.W. allowed to dry at room temperature for 15 min and extracted twice with 95% ethanol , the rest of solution was assembled and the absorbance was measured at 630 nm using U.V. Visible Spectrophotometer .The inhibition of biofilm formation percentage was calculated as equation described by [15].

Results and Discussion

In this study, Twenty-three of *Pseudomonas aeruginosa*, Seventeen of *Klebsiella pneumonia* and Ten of *Acinetobacter baumannii* which initially diagnosed in hospitals from burns and wounds sources were collected during a period from August to October, 2015.The resistance patterns of the isolates are shown in (Table1). All isolates were multidrug resistant, the resistance was 100% to tetracycline, but they were susceptible 100 % to colistin.

A study done by[16]showed that clinical isolates of *K. pneumonia* were resistant to ceftazidime (63%), gentamycin (35.3%), tobramycin (35.7%),to cefepime(52.9%) and to cefotaxime (80%).Lari*et al.*[17]reported that *K .pneumonia* isolates showed the level of resistance to Amoxycillin/ clavulanic acid with percentage (71%).Mechanisms of resistance to carbapenems include a production of efflux pumps, β -lactamases enzymes and mutations that alter the expression and/or function of PBPs and porins. Combinations of these mechanisms can cause high levels of resistance to carbapenems in certain bacterial species, such as *P. aeruginosa*, *K. pneumonia* and *A. baumannii*[18]. At same time, all *A. baumannii* isolates have high level of resistance for most antibiotic under study include; imipenem, cefotaxime, pipracillin, carbencillin, cefepime, ceftazidime, Amoxycillin/ clavulanic acid, ciprofloxacin and tetracycline with percentage of (100%), gentamycine and tobramycin with (87.5%), and all isolates were susceptible for colistin(100%).Behzadnia*et al* .[19] revealed that all of *A.baumannii*isolates were resistance to Ceftazidime , Carbenicillin and Imipenem ,Also, study by Cen *et a* .[16]illustrated that *P.aeruginosa*isolates were resistance to cefepime with (40.4%) in 2013.

Rabirad *et al.*[20] mentioned the resistance of *P. aeruginosa* isolates to amoxi- clav(95.8%).[21]mentioned resistance to imipenem (94.7%) by*P.aeruginosa*isolates.Al Marjani*et al*.[22] revealed that *P.aeruginosa* isolates were resistance 100% for Carbencillin; 80 % for Cefixime, 84% for Amoxicillin/clavulanic acid .

Table 1: Resistance Percentages of Bacterial isolates in the current study.

Antibiotics	**Resistance percentages of *K. pneumonia* isolates (%)**	**Resistance percentages of *A.baumannii*isolates (%)**	**Resistance percentages of *P. aeruginosa* isolates(%)**
Cefotaxime	85.71	100	91.30
Pipracillin	85.71	100	56.52
Tetracycline	100	100	100
Ceftazidime	64.29	100	43.48
Tobramycin	37.5	87.5	47.62
Gentamycin	37.5	87.5	71.43
Imipenem	28.57	100	91.30
Carbenicillin	100	100	76.19
Amoxycillin/ clavulanic acid	78.57	100	95.56
Colistin	0	0	0
Cefoxitin	43.75	100	85.71
Cefepime	50	100	42.86
Ciprofloxacin	37.5	100	74.62

Results of Micro titer plate showed that (95.65 %) of *P.aeruginosa* and (100%) of *A. baumannii* and *K .pneumoniae* had the ability to produce biofilm. Biofilms have been found to be involved in a wide variety of bacterial infections in the body. [23] reported (83%), (55%) and (76%) of *P.aeruginosa*, *A.baumannii* and *K.pneumoniae* isolates respectively had the ability to produce biofilm.

The effect of gamma irradiation on the growth of isolates and their ability to produce biofilm were studied, results showed different reduction of growth percentage according to bacterial isolate, an irradiation source, and power densities.

The results of irradiation bacterial isolates showed that Cesium (^{137}CS 5µci) and Sodium (^{22}Na) were effected against *P. aeruginosa* isolate, which reduces the CFUs (95.38%) and (95.07%) respectively. Sodium (^{22}Na) was effective source against *A.baumannii* which reduced the growth (75.75%)(Table 2).

In a study of [24], they irradiated *E. coli* cells in liquid media with gamma rays from cobalt ^{60} , the swimming speeds of the bacterial cells were measured they founded that the swimming speed was un altered in cells irradiated with a lethal dose of cobalt ^{60}. Chung *et al.*[25] illustrated decrease in level of *E.coli* below 3 log with in kimbab food after irradiation with cobalt ^{60}.

It is well known that the effect of ionizing radiation on living organism is induced by DNA damage in the cell. Cell death is predominantly induced by double – strand breaks in DNA, separated by not more than a few base pairs, which can generally not be repaired by the cell [26] .Trampuz *et al.*[10]indicatedthat the viability was abrogated at 2.8 and 3.6 KGy for *S.epidermidis* and *E.coli* respectively, and the radiation dose required to reduce viable cells by one log^{10} was 0.35 KGy for *E.coli* .

In the present study, the biofilm formation of irradiated isolates was detected; the effect of gamma irradiation is given in tables (3).The percentage of biofilm inhibition of *P.aeruginosa* was increased up to (53.7%) after exposed to ^{137}CS and ^{22}Na and increased to (54%) after exposed *A.baumannii* cells to ^{137}Cs.The percentage of biofilm inhibition up to (75.97%) in *K. pneumonia* isolates (Table 3). The variation in total sugar content may be a type of protection for the bacteria against the gamma irradiation which is considered as an external stimulus for production of exopolysaccharide[27].

Table (2): Effect of Gamma Irradiation on Growth of isolates.

Bacterial isolates	Irradiation Sources			
	^{60}Co	^{137}Cs 5µci	^{137}Cs 9µci	^{22}Na
	Reduction of growth (%)	Reduction of growth (%)	Reduction of growth (%)	Reduction of growth (%)
P.aeruginosa	84	95.38	38.46	95.07
A.baumannii	66.25	55	47.5	75.75
K.pneumonia	85.86	82.75	78.96	59.31

Table (3): Effect of Gamma Irradiation on Biofilm formation of isolates.

Bacterial isolates	Irradiation Sources			
	^{60}Co	^{137}Cs 5µci	^{137}Cs 9µci	^{22}Na
	Inhibition of biofilm (%)	Inhibition of biofilm (%)	Inhibition of biofilm (%)	Inhibition of biofilm (%)
P.aeruginosa	48.5	51.1	53.7	53.7
A.baumannii	58.3	48.3	54.0	1.2
K.pneumonia	67.02	68.31	73.96	75.97

Gamma irradiation which may weaken the inter molecular interaction of theLipopolysaccharideconstituents , disorganize the structure and render it permeable to drugs by enabling them to cross the outer membrane[28], the affected cell membrane is thought to develop transient that permit the passage of a different molecules, including antibiotics [29].[30] recorded that the ability of two slime producer isolates of *Pseudomonas aeruginosa* was changed after irradiation from positive to negative or weak positive.

Effect of gamma irradiation on biofilm of *P.aeruginosa* isolate relation to different surfaces: plastic, glass, cotton, stainless steel, gauze and gloves were evaluated *In vitro* after exposure to gamma (^{137}Cs) irradiation. Table (4) illustrated results of inhibition of biofilm formation, the best antibiofilm effect obtained in stainless steel and plastic with inhibition rate (70.01%) and (50.24%) respectively , while the less inhibition obtained in gauze and glass, (-4.61%) and (-9.71%) respectively .[30] mentioned that the irradiation changes the hydrophobicity of the tested strains as well as reduce the number of cells with abnormalities in shape and size.

Table (4) : Inhibition of biofilm formation for *P.aeruginosa* by gamma irradiation.

Type of Materials	Inhibition of biofilm formation %
Plastic plate	50.24
Glass plate	-9.71
Stainless steel	70.01
Gauze	-4.61
Gloves	33.22
Cotton	9.1

The implications of these findings may be important in the pathogenesis of foreign body infections and utilization of new biomaterials to prevent bacterial adherence and colonization in immunocompromised patients.

Early adhesion of bacteria to polymer surface appears to depend mainly on hydrophobicity [31].The sterilization of the polystyrene plates with gamma radiation diminish the hydrophobicity of the polystyrene [32].Salman *et al*[33]showed anantibacterial and antiadhesive effect ofPVA and PVA- Biosufactant mixture against pathogenic bacteria in glass and plasticplates.Initial adherence is considered to depend mainly on surface properties of bacteria such as surface hydrophobicity and net surface charge , hydrophobicity of bacteria has

generally been correlated with enhanced virulence and with increased attachment to the surface of implanted devices [30].

Conclusion:

We report here the gamma irradiation was effectiveness against growth and biofilm formation of Some Gram- Negative bacteria isolated from burn and wound Infections.

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