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EFFECT OF LOW DOSE OF GAMMA RADIATION ON MULTIDRUG RESISTANT GRAM- NEGATIVE BACTERIA ISOLATED FROM BURN AND WOUND INFECTIONS

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ABSTRACT

The effect of gamma irradiation on the growth and multi drug resistance of Gram- negative bacteria isolated from burn and wound Infections were studied. These isolates included 23 isolates of *Pseudomonas aeruginosa*, 17 isolates of *Klebsiella pneumonia* and 10 isolates of *Acinetobacterbaumannii*. The susceptibility to different antimicrobial agents was evaluated and the production of extended spectrum beta lactamases (ESβLs) and metallo beta lactamases(MβLs) were studied. All isolates were multi-drug resistant, and the resistance was 100% to tetracycline while all isolates were sensitive to colistin. The results also showed that (43.47%),(26.08%) of *P.aeruginosa* had ability to produce ESβLs and MβLs respectively, (50%), (71.42%) of *K.pneumoniae* isolates had ability to produce ESβLs and MβLs respectively, while none of *A.baumannii*had ability to produce these enzymes. PCR wasused to amplify the bla_{TEM}; $bla_{CTX;}$ bla_{IMP} and bla_{VIM} genes that encoded to produce ESβLsandMβLs. Results were revealed that percentage of bla_{TEM}(90%), (90%) and (70%) in *P.aeruginosa*, *K.pneumoniae* and *A.baumannii*isolates, the presence of bla_{IMP} in (40%) of *P.aeruginosa*and*K.pneumoniae* isolates (for each) ,and (50%) of *A. baumannii* isolates. The results of irradiation bacterial isolates showed that Cesium (^{137}CS 5µci) and Sodium (^{22}Na) were effected against *P. aeruginosa* isolates, 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%). The resistance of these isolatesto the tested antibiotics were not changed after exposure to gamma irradiation, except in case of ciprofloxacin against *P.aeruginosa* and ciprofloxacin, amoxicillin-clavulanic acid, cefepime, ceftazidime and cefoxitin against *K. pneumoniae*. Also, their ability to produce ESβLs and MβLs were not changed.

KEYWORDS: Gram- negative bacteria, multi drug resistance, ESβLs, MβLs, gamma rays.

INTRODUCTION

Nosocomial infections are a significant public health concern and economic cost to health care systems (Wright *et al.*, 2014). *Pseudomonas aeruginosa* a leading cause of infection associated with healthcare especially in patients admitted to critical care units such as intensive care units and burn care centers (Bhatt *et al.*, 2015). *Acinetobacterbaumannii*isimportant opportunistic bacteria that can cause nosocomial infection in burns units. Resistance to extended spectrum beta-lactams antibiotics in *A. baumannii*is mediated by metallo-beta-lactamase and extended spectrum beta-lactamase enzymes (Owlia*et al.*, 2012). *Klebsiellapneumoniae*, is the most clinically important *Klebsiella* species and occurs in nosocomial infections (Pereira and Vanetti, 2015). The bacterial nosocomial infection remains one of the principal causes of mortality and morbidity of the burnt patient (Rafik*et al.*, 2015). Extended-spectrum beta-lactamases (ESβLs) producing G-ve bacteria have become one of the major problems in terms of nosocomial infections in human medicine (Ewers *et al* ., 2011). Carbapenem-resistant gram-negative bacteria pose a serious problem due to the genes encoding most of these carbapenemases reside on transposons or plasmids which carry additional genes encoding resistance to other classes of antibiotics (Rahamathulla*et al.*, 2016). Metallo-β-lactamases able to hydrolyze all β-lactam with the exception of monobactams (Siqueira*et al.*, 2013). Carbapenem-resistant *K. pneumoniae*(CRKP) has emerged as a major public health threat, imposing considerable clinical and epidemiological challenges. These strains are usually extensively drug resistant, have very few available treatment options with antibiotics, and those are often of uncertain effectiveness and carry high toxicity. (Amit*et al.*, 2015).

Ionizing radiation induces damage to DNA by both direct energy deposition in DNA (direct effect) and by generating reactive species from the radiolysis of water and other biomolecules surrounding the DNA (indirect effect), which subsequently react with DNA (Sahbani*et al.*, 2014).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 (Trampuz*et al*.,2006). The aims of this study were to detect the effect of gamma irradiation on multi drug resistance 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, using commercially available 6mm disks (Bioanalyse/Ankara/Turkey).The susceptibility of the isolates was determined against 13 antibiotics included: Cefotaxime (CTX), Pipracillin(PRL), Tetracycline(T), Ceftazidime(CAZ),Cefoxitin (FOX);Cefepime(CPM)Imipenem (IMP), Carbenicillin(PY), Tobramycin (TN), Gentamycin (GM), Ciprofloxacin (CIP); Amoxycillin/ clavulanic acid (AUG) and Colistin (CO),On Mueller-Hinton agar Plate(Lab M Limited TopleyHouse,United Kingdom),using overnight culture at a 0.5 McFarland standard followed by incubation at 35^{o}C for 18.

Phenotypic Detection of ESβLs production: Each isolates were showed resistant to third generation of cephalosporins were tested to investigate the production of ESβLs by disk approximation test ,the results were interpreted according to Colle*et al*.(1996) and Drieux*et al*. (2008) :Tested isolates were inoculated according to Kirby-Bauer method onto plate of Muller-Hinton agar media .Augmentin (Clavulanic acid+ Amoxicillin) disk was placed in the center of plate; Ceftazidime and Cefotaxime disks were placed at 3cm from center disk. The inhibition zones of the Ceftazidime, Cefotaxime and Clavulanic acid discs were compared after 16-18 hr. of incubation at 35C°. It demonstrates the breadth occurrence in inhibition zone between Ceftazidime, Cefotaxime and Clavulanic acid disc was considered as produced isolates of ESβLs.

Detection for Metallo-β lactamase(MβLs): Disk synergy test used to test each isolates were resistant to imipenemto investigate the production of MβLs, the test was done according to Bashir *et al*.,(2011) as follows:Tested isolates were inoculated according to Kirby-Bauer method onto plates of Mueller-Hinton agar media . Two discs of Imipenem antibiotic were placed on the plate; 5 µl of EDTA solution(final concentration is 0.5M) was added to one of them . The inhibition zones of the imipenem and imipenem -EDTA discs were compared after 16-18 hr. of incubation at 35C°. An increase in the zone size of at least 7 mm around the imipenem - EDTA disc more than other was considered as produced isolates of MBLs.

Molecular Detection of β-lactamase enzymes from producing isolates using PCR technique: All of ESβL*s* and MβLsproducersisolateswere submitted to PCR technique to detection for some genes ; bla_{TEM},$bla_{\text{CTX-M}}$ (encoded for some ESβL*s*) ;$bla_{\text{IMP-1}}$,$bla_{\text{VIM-2}}$ (encoded for some MβLs); DNA amplification was carried with a Gradient PCR System(TechNet-500 /USA). PCR was performed with a final volume of 25 µl. The primers used for PCR amplification are listed in [Table 1]. Each reaction contained 20 mMTris-HCl (pH 8.4); 50 mMKCl; 0.2 mM each deoxynucleoside triphosphate; 1.5 µl each primer ; 1.5 mM MgCl2; 1.25 U of *Taq*DNA polymerase. Template DNA (2 µl). Amplified PCR products were detected by agarose gel electrophoresis. A DNA marker was run with each gel, and the genotype was determined by the amplified product size.

Table (1): Primers selection that used in this study

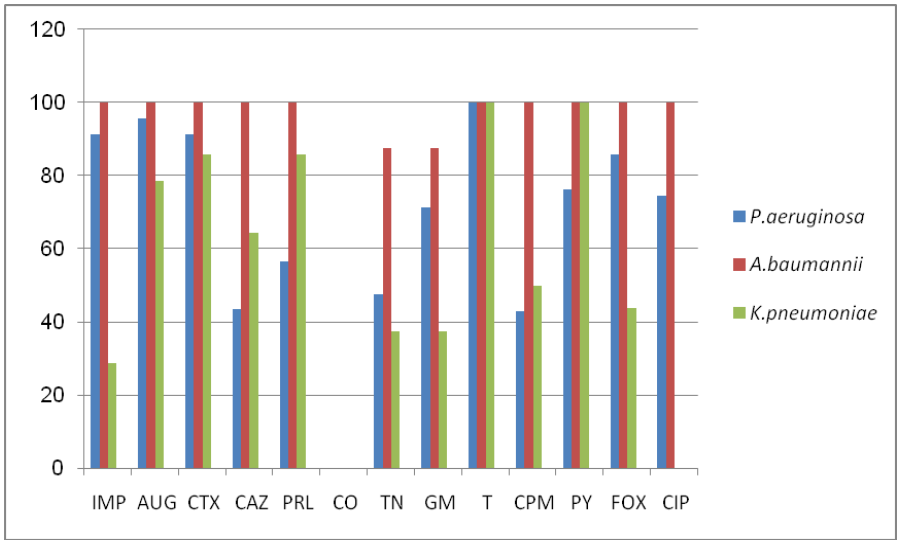
Genes	Primer name	Sequence	Product Size(bp)	Reference
bla_{TEM}	TEM -F	5'-ATGAGTATTCAACATTTCCG-3'	861	Grimm *etal*. (2004)
	TEM -R	5'-TTAATCAGTGAGGCACCTAT-3'		
bla_{CTX}	CTX - F	5'-CGCTTTGCGATGTGCAG-3'	550	Bhattacharjee *et al.*(2007)
	CTX - R	5'-ACCGCGATATCGTTGGT-3'		
bla_{IMP}	IMP -F	5'-CATGGTTTGGTGGTTCTTGT-3'	488	Sung et*al* . (2008)
	IMP -R	5'-ATAATTTGGCGGACTTTGGC-3'		
bla_{VIM}	VIM -F	5'-ATTGGTCTATTTGACCGCGTC-3'	780	Sung *et al* . (2008)
	VIM -R	5'-TGCTACTCAACGACTGAGCG-3'		

Effect of Gamma Irradiation on Growth of Bacterial cells: 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) and Sodium 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 asTrampuz*et al* .(2006)and Shokier*et al*.(2010).

Effect of Gamma Irradiation on the Multi-drug Resistant Isolates and β- lactamases Production.
Antibiotic susceptibility, MICs, detection of ESβLs and MβLS were retested after gamma irradiation.

RESULTS AND DISCUSSION
In this study, fifty isolates included Twenty three of *Pseudomonas aeruginosa,* Seventeen of *Klebsiella pneumonia*and ten of *Acinetobacterbaumannii*which initially diagnosed in hospitals from burns and wounds sources were collected. The resistance patterns of the isolates are shown in (Figure 1), all isolates were multidrug resistant, the resistance was 100% to tetracycline, but all isolates were susceptible to colistin.



(Fig 1): Resistance Percentages of Bacterial isolates in the current study.

Detection for extended spectrum β- lactamases (ESβLs) andmetallo β – lactamase (MβLs) enzymes were illustrated that (43.47%) of*P.aeruginosa* and (50%) of *K.pneumoniae* isolates were produced ESβLs, all *A.baumannii* isolates were negative for ESβLS production ,(26.08%) of *P.aeruginosa* and (71.42%) of *K. pneumoniae* isolates were positive for metallo –β-lactamases, all *A. baumannii*isolates were give negative result for metallo- β- lactamase production test .Goel*et al* .(2013) showed that 42.30% of *P.aeruginosa* isolates were positive for ESβLs ,Also, a study by Jabalameli*et al.* (2011) revealed that (42.8%) of *P.aeruginosa* isolates were positive for ESβLs ,Melake*et al.* (2015) mentioned that (50%) of *Klebsiellasp* were positive for ESβLs. Al Marjani *et al*· (2013) revealed that *P.aeruginosa* isolates were resistance 100% for Carbencillin;80 % for Cefixime, 84% for Amoxicillin/clavulanic acid .

Anvarinejad *et al.* (2014) reported that 20.37% of *P.aeruginosa* isolates were positive for metallo-β-lactamse, Peymani *et al.* (2016) illustrated that *P. aeruginosa* isolates were positive for metallo –β-lactamase with percentage (52.3%).

Out of 30 isolates that were subjected to detection of ESβLs and MβLs genes by PCR **,**The results in current study showed the presence of bla_{TEM} in (90%) of *P.aeruginosa*and*K.pneumoniae* isolates (for each) ,(70%) of *A. baumannii* isolates ;bla_{CTX} in all of *K.pneumoniae* isolates and in (50%) ,(70%) in*P.aeruginosa* and *A. baumannii* isolates respectively (Table 2).Study done by Ahmed*et al* .(2015) in Saudi Arabia about prevalence of ESβL genes of *P. aeruginosas*trains mentioned that percentage of bla_{CTX} gene was (10.7%) in *P.aeruginosa*isolates , while bla_{TEM} did not appear in any isolates of *P.aeruginosa*.

Results also showed that bla_{IMP}were found in (40%) of *P.aeruginosa*and*K.pneumoniae* isolates (for each) ,(50%) of *A. baumannii*isolates ;bla_{VIM}gene were found in (90%) of *P.aeruginosa* , (60%) of *K. pneumoniae* and in (80%) of *A. baumannii* isolates (Table 3).

Al-Ajeeli (2013) mentioned in study done in Iraq that percentage of bla_{IMP}*inA.baumannii* isolates was (23%) , while did not appear in any of *P.aeruginosa* isolates, all isolates of *A.baumannii* and *P.aeruginosa* in his study did not carrybla_{VIM}gene ,Aghamiri*et al* .(2014) reported that (9%) and (33%) of *P.aeruginosa* isolates were carrying bla_{IMP} and bla_{VIM} in Iran , Also Aghamiri*et al* .(2016) reported that (40%) and (36%) of *A. baumannii* were carrying bla_{IMP} and bla_{VIM}genes.

Table (2): Percentage of ESβLs genes in Bacterial species

Bacterial species	Genes	%
P.aeruginosa	bla_{TEM}	90
	bla_{CTX}	50
K.pneumoniae	bla_{TEM}	90
	bla_{CTX}	100
A.baumannii	bla_{TEM}	70
	bla_{CTX}	70

Table (3): Percentage of MβLs genes in Bacterial species

Bacterial species	***Genes***	***%***
Pseudomonas aeruginosa	bla_{IMP}	40
	bla_{VIM}	90
Klebsiellapneumoniae	bla_{IMP}	40
	bla_{VIM}	60
Acinetobacterbaumannii	bla_{IMP}	50
	bla_{VIM}	80

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 4).

In a study ofAtsumi*etal.* (2014), 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.*(2005) 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.Trampuz*et al.*(2006) 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*.

Table (4): 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

The resistance of those isolates to the tested antibiotics were not changed after exposure to gamma irradiation, except in case of ciprofloxacin against *P.aeruginosa* and ciprofloxacin, amoxicillin-clavulanicacid, cefepime, ceftazidime and cefoxitin against *K. pneumoniae*.

Also, their ability to prouduce ESβLs and MβLswere not changed ; MICs value of some antibiotics were increased and all isolates still resist to antibiotics after exposure to gamma, while the biofilm inhibition ratio of *P.aeruginosa* was increased up to (53.7%) after exposed to ^{22}Na and ^{137}Cs , increased to(58.3%) after exposed *A.baumannii* to ^{60}Co.

In current study, very low doses of gamma irradiation changed *K.pnemoniae* resistance to ciprofloxacin (inhibition zone 30-35mm) ,cefepime(20-33mm), cefoxitin(23mm), ceftazidime(25-30mm) and augmentin(20mm), while the previous doses had no effect on antibiotic resistance in *A.baumannii*isolates.

For ciprofloxacin the increase in inhibition zone, this effect can be explained the interaction of gamma irradiation with DNA. ciprofloxacin shares with other quinolones a common mechanism of action .Quinolones to varying degrees inhibit the bacterial enzymes DNA gyrase and topoisomerase IV, which are responsible for introducing negative supercoils into DNA in the case of gyrase and for relieving topological stress arising from the translocation of transcription and replication complexes along DNA, Formation of drug-enzyme-DNA complexes blocks DNA replication (Kim and Hopper , 2014).

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