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Phenotypic detection of extended spectrum β-lactamase and Metallo β-lactamase production by Gram negative uropathogens after exposure to gamma radiation

Yehia Ahmed El-Zawahry[1], Lotfy Abd-El-Rehem Mousa[2], Eman Araby[2]* and Salma Abd El Monsef-Elnahass[1]

[1]Botany Department, Faculty of Science, Zagazig University, Egypt.
[2]Radiation Microbiology Research Department, National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt.

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Eighty-five uropathogen isolates were collected and differentiated on the basis of their Gram stain reaction. Among the collected isolates, 25 (29.4%) were Gram positive cocci and 60 (70.6%) were Gram negative bacilli. Antibiotic susceptibility profile towards 15 different antibiotics concluded that impenime (IMP), amikacin (AK) and Pipperacillin/tazobactam (TZP) were the most effective against Gram negative; while linezolid (LZD), and vancomycin (VA) were the most potent against Gram positive. The highly drug resistant isolates were identified as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* using 16srRNA; the two strains were subjected to different doses of gamma-radiation and the sub lethal dose of both strains was 5.0 kGy. The D_{10} values were recorded (1.2 and 1.1 kGy) for *P. aeruginosa* and *K. pneumoniae),* respectively. In addition, gamma-irradiation technique decreases the resistance of *K. pneumoniae* towards IMP and the resistance of *P. aeruginosa* towards more than one antibiotic (TZP, AZM and CIP). The results also, revealed that both of the tested strains had the ability to produce Extended Spectrum β-lactamase (ESBL) before and after gamma-radiation but only *P. aeruginosa* had the ability to produce Metallo β-lactamase (MBL), that is, ESBL and MBL co-production was detected in *P. aeruginosa.* So, these findings must be supported by other studies on the level of genes to prove the possibility for using gamma-irradiation technique to overcome microbial drug resistance problem.

Key words: Urinary tract infection, antibiotic resistance, β-lactamases, gamma radiation.

INTRODUCTION

Urinary tract infection (UTI) is one of the most common human bacterial infections both in the community and hospital setting (the health care settings) and accounts for one-third of all nosocomial infections (Dougnon et al., 2020). Infection of urinary tract marked a range of severity that spans from mild self-limiting infection to life threatening systemic disease (Lee and Kim, 2015; Klein and Hultgren, 2020). UTI causes many urinary disorders as urosepsis, renal scarring and progressive kidney damage that lead to a high health risk with high mortality, morbidity and cost a significant financial burden (Gashti et al., 2020).

Emergence of multidrug-resistance bacteria has become a major problem (Woc-Colburn and Godinez, 2020). Resistance rates vary across countries because of differences in antimicrobial agent usage and systems for the prevention of antimicrobial resistant bacteria. In addition to resistance rates, modes of resistance also differ among countries and even among cities within the same country (Kim et al., 2017).

In developing countries, the increase in resistance including third- and fourth-generation drugs like cephalosporins, penicillins and fluoroquinolones may be due to their extended use and purchase directly from the pharmacies without doctors' prescription as self-medication is a common practice (Mohammed et al., 2016).

The World Health Organization has categorized antibiotic resistance as one of the three most significant severe public health problem of the 21st century (WHO, 2014). The infections which are caused by multidrug-resistant Gram negative bacilli that produce various β-lactamase enzymes have been reported with an increasing frequency and are associated with a significant morbidity and mortality (Deshmukh et al., 2011).

Extended spectrum beta-lactamase (ESBL) producing organisms are those that hydrolyse the oxyimino beta-lactams and monobactams, but have no effect on the cephamycins and carbapenems (Ghafourian et al., 2014). Detection of ESBL producers from samples such as urine may be of highest importance because this represents an epidemiologic sign of colonization and therefore there is potential for transfer of such organisms to other patients (Aggarwal et al., 2008).

The production of hydrolytic β-lactamase enzymes is the most prevalent resistance mechanism towards β-lactam antibiotics. Metallo-β-lactamases constitute a troublesome group of enzymes, since they present a broad-spectrum profile, hydrolyse penicillins, cephalosporins and carbapenems, but not monobactams e.g.: aztreonam. Carbapenem antibiotics are currently used as the last choice for treatment of the infections caused by multidrug-resistant Gram-negative bacteria. The mortality rate associated with MBL producers is reported to be from 18 to 67% (Adam and Elhag, 2018).

Hence, this study aimed to: (1) determine the antibiotic resistance pattern of the uropathogen collected isolates (2) investigate the sensitivity of the most resistant isolates to gamma radiation. (3) Evaluate the effect of gamma radiation on the antibiotic susceptibility, and finally 4) investigate the prevalence of ESBLs and MBL among the highly drug resistant isolates by phenotypic method before and after gamma irradiation treatment.

MATERIALS AND METHODS

Bacterial isolates and cultivation media

Eighty- five isolates including 25 isolates Gram positive cocci and 60 Gram negative bacilli were obtained over 8-month period from different specialized hospitals in Great Cairo, Egypt, from UTIs samples. The tested isolates were cultivated in a standard laboratory culture media. Tryptone Glucose Yeast Extract Agar (TGY), Nutrient agar (NA), Blood agar (BA) and MacConkey agar were purchased from Difco [Difco Labs, Detroit, Michigan (USA)]. Muller Hinton Agar (MHA) were obtained from Oxoid (Oxoid. comp., Basigstoke, Hants, UK)].

Purification of bacterial isolates

The bacterial isolates were streaked for several consecutive times on nutrient agar medium until pure single colonies were obtained. The colonies were then isolated and checked by microscopic examination using Gram's stain.

Antibiotic resistance profile

Antibiotic susceptibility testing was done using the Kirby-Bauer disc diffusion method (Bauer et al., 1966) on Mueller Hinton (Oxoid) agar according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2017). The following antimicrobial discs (µg) were used: ciprofloxacin (CIP5), aztreonam (ATM10), ceftriaxone (CRO 30), carbenicillin (PY100), vancomycin (VA30), Linezolid (LZD30), amikacin (AK30), sulphamethoxazole-trimethoprim (SXT25), tobramycin (TOB10), ceftazidime (CAZ 30), gentamicin (CN10), azithromycin (AZM15), cefepime (FEP 30), piperacillin tazobactam (TZP110) and imipenem (IPM10). Bacterial colonies from a pure overnight culture were suspended in 2 ml of 0.85% NaCl in order to maintain the bacterial strains in osmotic equilibrium and the bacterial suspension was standardized to 0.5 McFarland (10^7 CFU/ml). The suspension was inoculated on Mueller Hinton agar using a sterile swab and antimicrobial agents are placed onto the surface of the agar and incubated at 37°C for 24 h. A zone of inhibition was measured and the results were interpreted as sensitive, resistant, or intermediate based on resistance data interpreted according to Clinical and Laboratory Standards Institute (Franklin et al., 2012); thereafter, the experiment was repeated in triplicate. The tested isolates which revealed 100% resistance were selected for the following studies.

Molecular identification of the most MDR bacterial isolates

The most resistant bacterial isolates (two) were biochemically identified previously, and then the identification of these selected bacteria was confirmed using 16s rRNA sequencing according to a methodology published previously (James, 2010). Briefly, the total bacterial DNA was purified from each strain using Wizard genomic DNA purification system (#A1120, Promega Corporation, USA) according to manufacturer instructions. Universal 16s primers were used for PCR amplification of 16s rRNA of the purified bacterial genomes; the forward primer is 8f (5' AGA GTT TGA TCC TGG

*Corresponding author. Email: emansk2@gmail.com. Tel: +20 01094886248.

CTC AG -3'), and the reverse primer is U1492R (5' GGT TAC CTT GTT ACG ACT T -3'). PCR procedures were carried out using the instrumentations in the centre of Virology, Cairo University, Cairo, Egypt.

Irradiation process

The irradiation process was achieved by using Cobalt 60 (^{60}Co) Gamma Cell GC 220, product of Canada Co. Ltd. located at the National Centre for Radiation Research and Technology (NCRRT) Cairo, Egypt. Irradiation process was achieved at ambient temperature. The dose rate of this source was 1.538 (kGy/h) at the time of the experiment.

Effect of different doses of gamma-radiation on viability of the selected strains (D_{10} value determination)

The two selected multidrug resistant strains*; Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were cultured and prepared in sterile normal saline solution. Five ml aliquots were distributed in sterile plug capped test tubes and exposed to gamma radiation at level doses of (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 kGy). After irradiation, appropriate dilution of 0.1 ml of the un-irradiated bacterial cells (control) and irradiated ones were serially diluted and plated onto nutrient agar plates. The plates were incubated for 24 h at 37°C. The radiation dose-response curves were graphically represented for irradiated and un–irradiated cells.

Radiation dose-response curves

Response to gamma-irradiation was expressed as the logarithm of the ratio of survivors (N/N0), where N represents the mean CFUml^{-1} of irradiated bacterial suspension and N0 the mean number of CFU ml^{-1} of un-irradiated control. D_{10} values, defined as the radiation dose (kGy) required to reduce the number of CFU ml^{-1} by one $\log_{10}$ were determined by calculating the negative reciprocal of the slope of the linear regression curve (Rajkowski et al., 2003).

Evaluation of the effect of gamma-radiation on the antibiotic susceptibility

Antibiogram test was performed to record the sensitivity or resistance of tested strains after gamma-irradiation treatment as mentioned above.

Phenotypic detection of β-lactamases

Detection of extended spectrum β- Lactamases (ESBL) production

The strains that showed resistance towards at least of the third generation cephalosporins (ceftazidime, cefotaxime and ceftriaxone) were tested for ESBL production by double disc synergy test and phenotypic confirmation test recommended by CLSI as reported by Jarlier et al. (1988).

Double disk synergy test (DDST)

Test inoculum was spread by lawn culture on Muller Hinton Agar (MHA). Antibiotic discs of amoxicillin clavulanate (20/10 µg) (augmentin) was placed at the centre and three different antibiotics including cefotaxime, ceftazidime, ceftriaxone and aztreonam were placed at different distances (10, 25 and 30 mm) (centre to centre). The plates were incubated overnight at 37°C. Distance was maintained properly in order to accurately detect the synergy. Any distortion or increase in the zone of inhibition of three antibiotic discs towards the augmentin disc was considered as positive for the ESBL production.

Phenotypic confirmatory disc diffusion test

Combined disc method

This method was designed by Jacoby and Han (1996). In this experiment, cefoperazone (CFP) (75 µg) and cefoperazone/sulbactam (SCF) (75 µg/30 µg) were used. An increase of 5 mm or more in the zone of inhibition in a disc containing sulbactam compared to the drug alone was considered as a positive test for the presence of β- lactamase enzyme.

Metallo β-lactamase production

This test was detected by Imipenem-EDTA combined disc test which was described by Behera et al. (2008). *P. aeruginosa* and *K. pneumoniae* were inoculated onto Mueller-Hinton agar as lawn culture. Two 10 µg IPM discs were placed at 20 mm centre to centre on the plate, and 10 µl of 0.5 M EDTA solution was added to one of them. After 18 h of incubation at 35°C, zone of inhibition of Imipenem + EDTA disc compared to that of Imipenem disc alone. If the increase in inhibition zone was ≥ 7 mm, then the tested organism was considered to be MBL producer.

Effect of gamma-radiation on the (ESBL) and (MBL) production

In this experiment, the tested strains which were considered as ESBL and/or MBL producers were subjected to different doses of gamma radiation, thereafter (ESBL) and (MBL) production was detected as mentioned above.

RESULTS AND DISCUSION

The infections with MDR uropathogens have become significantly challenging due to their high resistance to commonly used antibiotics. In the present study, eighty-five uropathogenic isolates were collected and identified on the basis of their morphological characters and Gram stain reaction. The results revealed that among all the examined isolates, 25 isolates (29.4%) were Gram positive cocci and 60 (70.6%) were Gram negative bacilli or short rods.

Antibiotic resistance profile of the isolated bacteria towards 15 different standard antibiotics has been shown in Table 1. The results showed that out of eighty-five isolates, 63 and 65 isolates (74.1 and 65.9%) were resistant to (CRO and FEP) and 66 isolates (77.6%) were resistant to AZM, whereas 69 and 70 isolates (81.2 and 82.3%) were resistant to CAZ and SXT. Out of 60 Gram-negative uropathogens, 59 isolates showed extreme resistance rate (98.3%) towards PY while high degree of resistance was observed towards ATM (beta-lactam

Table 1. Antibiotic resistance profile of the collected clinical isolates.

Tested antibiotics	No. of resistant isolates (from 85 isolates)	Percentage of resistance
*ATM10	45 (from 60 isolates) G-ve	75.0
CRO30	63	74.1
TOB10	55	64.7
AK 30	30	35.8
CAZ30	69	81.2
CN 10	46	54.1
*PY100	59 (from 60 isolates) G-ve	98.3
FEP 30	65	65.9
IMP 10	21	24.7
**VA 30	6.0 (from 25 isolates) G+ve	24.0
AZM 15	66	77.6
CIP 5	41	48.2
SXT 25	70	82.3
TZP 110	42	49.4
**LZD 30	7.0 (from 25 isolates) G+ve	28.0

ATM10, Aztreonam; CRO 30, Ceftriaxone; TOB10, Tobramycin; AK30, Amikacin; CAZ 30, Ceftazidime; CN10, Gentamicin; PY100, Carbenicillin; FEP 30, Cefepime; IPM10, Imipenem; VA30, Vancomycin; AZM15, Azithromycin; CIP5, Ciprofloxacin; SXT25, Sulphamethoxazole-trimethoprim; TZP110, Piperacillin tazobactam; and LZD30, Linezolid. *ATM & PY, used only with G-ve isolates; **VA& LZD, used only with G+ve isolates.

agents) with resistance rate of 75%. Among Gram positive uropathogens (25 isolates), the isolates showed low resistance rate towards VA and LZD (24 and 28%).

In general, IMP, AK, CIP and TZP were the most effective drugs towards Gram negative isolates while, VA and LZD were most effective against Gram positive isolates.

The results in this study showed that the isolated uropathogens were mostly resistant to 3rd-generation cephalosporins including CAZ, CRO and FEP (which considered extended β-lactam agents) than to non-β-lactam agents such as CIP and CN. This result is in agreement with that of Bhatt et al. (2017) who reported that β-lactams, and fluoroquinolones antibiotics have limited value for the treatment of UTI infected by Gram negative bacteria and there was extreme resistance towards CAZ, FEP and CRO higher than CN.

Talbot (2013) stated that the resistance of bacteria against β-lactam agents may be a result of numerous mechanisms comprising modification of penicillin-binding proteins, loss of porins, overexpression of efflux pumps or production of β-lactamases.

The results in this study were in accordance with those of Salleh et al. (2017) who reported that, all the isolated uropathogens (Gram negative isolates) showed high sensitivity to AK, IMP and TZP.

The data of antimicrobial resistance profile of this study are also consistent with many previously reported studies (Joly-Guillou et al., 2010; Abujnah et al., 2015) who declared that AK, IMP were highly effective against Gram negative bacteria which are highly resistant to cephalosporins (first, second and somewhat third generations) and penicillins.

Regarding the tested Gram positive isolates, the data revealed that VA and LZD were the strongest agents and this agreed with the results of Bhatt et al. (2017) who reported that VA and LZD were found to be the most effective against Gram-positive isolates.

The antibiotic susceptibility profile of the eighty five isolates indicated that two isolates were 100% resistant against all the tested antibiotics (data not shown).

Molecular identification of the selected isolates using 16s rRNA:

Neighbour-joining phylogeny trees of the output result of BLAST indicated that the submitted gene corresponding to rRNA sequence is identical by 100% to *P. aeruginosa* strain 127 16s ribosomal RNA gene, partial sequence (Figure 1) and by 99% to *K. pneumoniae* strain MLST-15 16s ribosomal RNA gene, partial sequence (Figure 2).

Effect of gamma radiation on the viability of the tested strains

The relative sensitivity or resistance of different microorganisms to ionizing radiation is based on their respective D_{10}-value. D_{10}-value is the ionizing radiation dose required to kill 90% of the total viable number of microorganisms (Niemira et al., 2006; Aquino, 2012). Lower D_{10}-values indicate greater sensitivity of the organism to ionizing radiation.

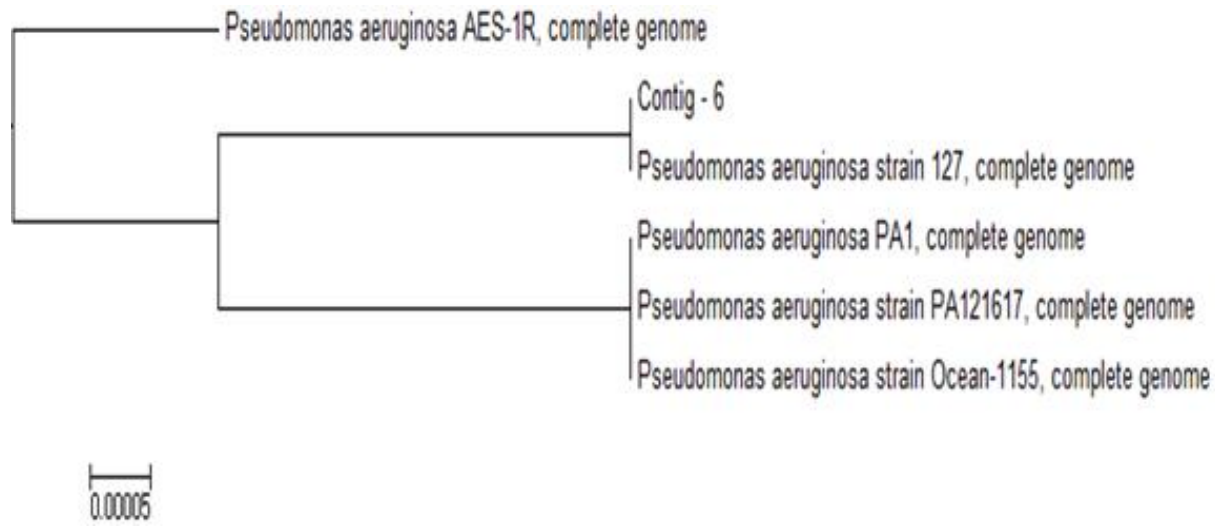

Figure 1. The phylogeny tree of. *P. aeruginosa* PCR molecular identification.

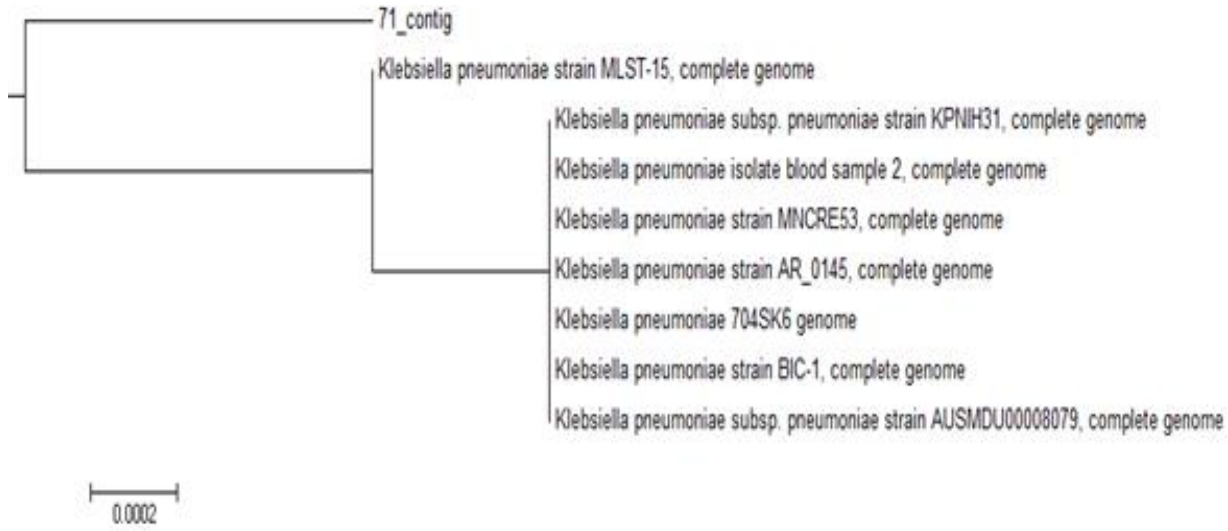

Figure 2. The phylogeny tree of *K. pneumoniae* PCR molecular identification.

In this experiment, the effects of gamma radiation at different exposure doses (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 kGy) on the viability of *P. aeruginosa* and *K. pneumoniae* was tested; thereafter the dose response curves were graphically represented as shown in Figures 3 and 4.

The survival number of irradiated cells at each irradiated dose after incubation for 18 h. at 35± 2°C was calculated. Curve was plotted between log survival numbers against different doses. The results for both strains showed that, by increasing the radiation doses, the total bacterial counts gradually decreased when compared with un-irradiated one (control). The lethal dose at which there was no growth of the tested strains was 6.0 kGy for both strains. The calculated D_{10} values were recorded (1.1 and 1.2 kGy) for *P. aeruginosa* and *K. pneuomoniae*, respectively.

Generally, the decrease in population of *P. aeruginosa* and *K. pneumoniae* in this study was probably due to the effect of energy produced from increasing doses of irradiation, which might have broken the bonds in the DNA molecules, leading to inability of microorganisms to replicate and reproduce resulting in bacterial death (Gillard et al., 2007).

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

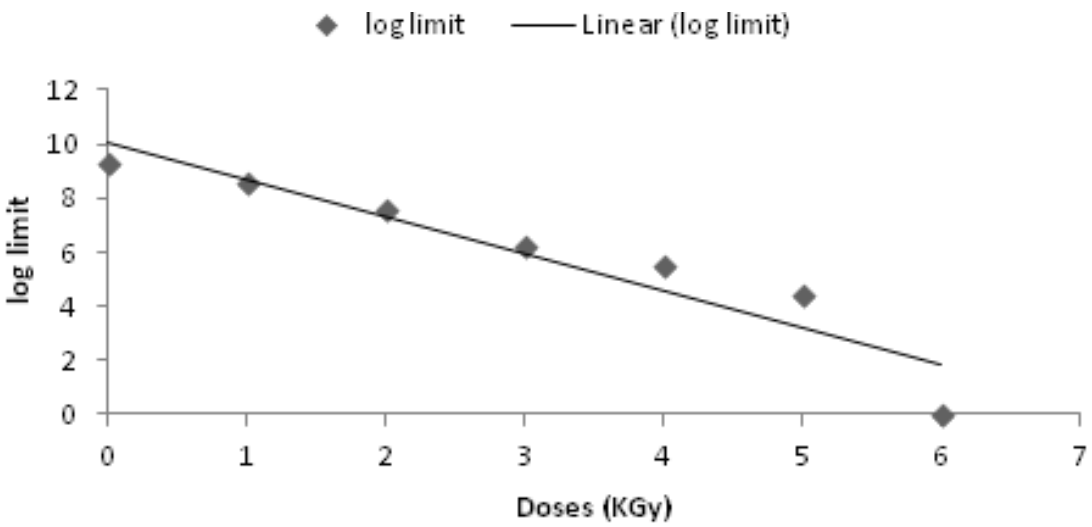

Figure 3. Effect of different doses of gamma-radiation on the viability of *P. aeruginosa*.

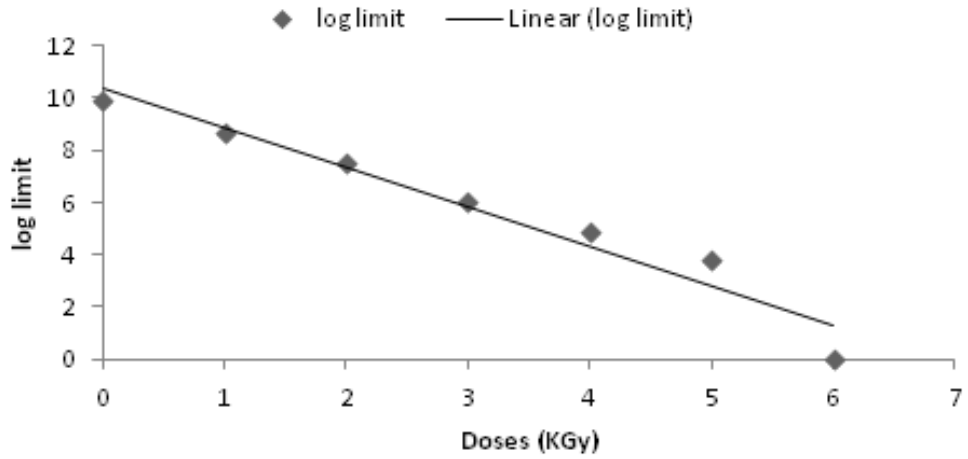

Figure 4. Effect of different doses of gamma-radiation on the viability of *K. pneumonia.*

and other biomolecules surrounding the DNA (indirect effect), which subsequently react with DNA (Kuefner et al., 2015; Sage and Shikazono, 2017). The lethal effect of the ionizing radiation was associated with other bacteria to an oxidizing stress due to the presence of reactive oxygen species (Pouget et al., 2002).

Regarding *K. pneumoniae*, it was observed that from Figure 4 which showed resistance towards gamma-radiation, this may be due to the wall composition of its capsule which consists mainly of a heavily packed accumulation of fine fibers, and represented a polymer of capsular polysaccharide with approximate layer thickness of 160 nm (Amako et al., 1988; Sridhar-Rao et al., 2008). Meanwhile, Atique et al. (2013) showed that, lower D_{10} (0.60 to 0.74 kGy) values were recorded for Gram negative *Pseudomonas* spp (associated with human amniotic membrane), their sublethal dose for *P. aeruginosa* was from 4-5 kGy and all Gram negative isolates were found to be killed after 5 kGy.

Effect of gamma-radiation on the antibiotic sensitivity

In order to determine the biological effects of gamma-radiation on the antibiotic susceptibility to the selected strains, a standardized bioassay using the disc diffusion method was applied. Bacterial cells in liquid culture were exposed to 1.0, 2.0, 3.0, 4.0 and 5.0 kGy of gamma-radiation. Antibiograms were performed and the diameter of inhibition zones of the exposed cells and un-irradiated

Table 2. Antibiotic susceptibility pattern for *P. aeruginosa* before and after treatment with different doses of gamma- radiation.

Dose (kGy)	ATM	CRO	TOB	AK	CAZ	CN	PY	FEP	IMP	AZM	CIP	SXT	TZP
C	R	R	R	R	R	R	R	R	R	R	R	R	R
1.0	R	R	R	R	R	R	R	R	R	R	R	R	30/S
2.0	R	R	R	R	R	R	R	R	R	R	21/S	R	S
3.0	R	R	R	R	R	R	R	R	R	30/S	S	R	S
4.0	R	R	R	R	R	R	R	R	R	S	S	R	S
5.0	R	R	R	R	R	R	R	R	R	S	S	R	S

C- Control (un-irradiated), R- resistant, I- intermediate, S- sensitive.

Table 3. Antibiotic susceptibility pattern for *K. pneumoniae* before and after treatment with different doses of gamma-radiation.

Dose (kGy)	ATM	CRO	TOB	AK	CAZ	CN	PY	FEP	IMP	AZM	CIP	SXT	TZP
C	R	R	R	R	R	R	R	R	R	R	R	R	R
1.0	R	R	R	R	R	R	R	R	26/S	15/I	R	12/I	R
2.0	R	R	R	R	R	R	R	R	34/S	I	R	I	R
3.0	R	R	R	R	R	R	R	R	S	I	R	I	R
4.0	R	R	R	R	R	R	R	R	S	I	R	I	R
5.0	R	R	R	R	R	R	R	R	S	I	R	I	R

C- Control (un-irradiated), R- resistant, I- intermediate, S- sensitive.

ones (control) for each dose was determined. The sensitivity test was carried out twice for each strain.

From Table 2, it is obvious that the sensitivity of the tested strains towards antibiotics depends on gamma-radiation dose and the type of antibiotic. The results also revealed that exposure of *P. aeruginosa* to gamma-radiation changed its sensitivity towards CIP, AZM and TZP from resistant to sensitive with inhibition zone of 21, 30 and 30 mm in diameter at dose levels of 2.0, 3.0 and 1.0 kGy respectively, whereas its resistance to all the remaining antibiotics was still constant without change after exposure to gamma-radiation.

Regarding *K. pneumoniae,* its resistance towards IMP changed to be sensitive at 1.0 kGy. Also, its susceptibility towards AZM and SXT changed to moderate at 1.0 kGy (Table 3), while, its resistance to all the remaining antibiotics was still constant without change after exposure to gamma-radiation. These data suggested a possible stress response to gamma-radiation. The variability of the radiation effect on susceptibility towards the different antibiotics could be explained by the nature, penetration mode inside the cell or by the mode of action of antibiotics.

The increase in sensitivity of irradiated *P. aeruginosa* and *K. pneumoniae* cells can be explained on the action of gamma irradiation on one or more mechanisms. Pouget et al. (2002) said that the increase of sensitivity may be related to membrane composition modifications following the irradiation. The main theories that try to explain the biological effects of oxidative stress are based on the possible effects on the permeability of ionic channels in the membrane (Berrier et al., 1993; Galvanoskis et al., 1999).

Another study conducted by Potron et al. (2015) documented that resistance of *P. aeruginosa* to antibiotics is the result of the production of enzymes that inactivate and degrade antibiotics, reducing the membrane permeability and the efflux system.

Dreier and Ruggerone (2015) obtained that MexAB-OprM is usually stated in wild type (WT) *P. aeruginosa* and participate in the passage of various antimicrobials such as fluoroquinolones, -lactams, macrolides and trimethoprim, sulfamides. Indeed, Nebras et al. (2016) confirmed that gamma irradiation make inhibition to gene expression of MexAB-OprM and MexXY efflux pumps of *P. aeruginosa*. As a result, it can be suggested that the tested strains showed alterations in the cell wall/cell membrane/porins which lead to change in the resistance of *K. pneumoniae* towards IMP and *P. aeruginosa* towards TZP.

Effect of gamma-radiation on extended spectrum β-lactamases and Metallo β-lactamases production

Seid and Asrat (2005) and Kumar et al. (2014) mentioned that inappropriate and incorrect use of third-generation cephalosporins in empirical therapies and lack of suitable

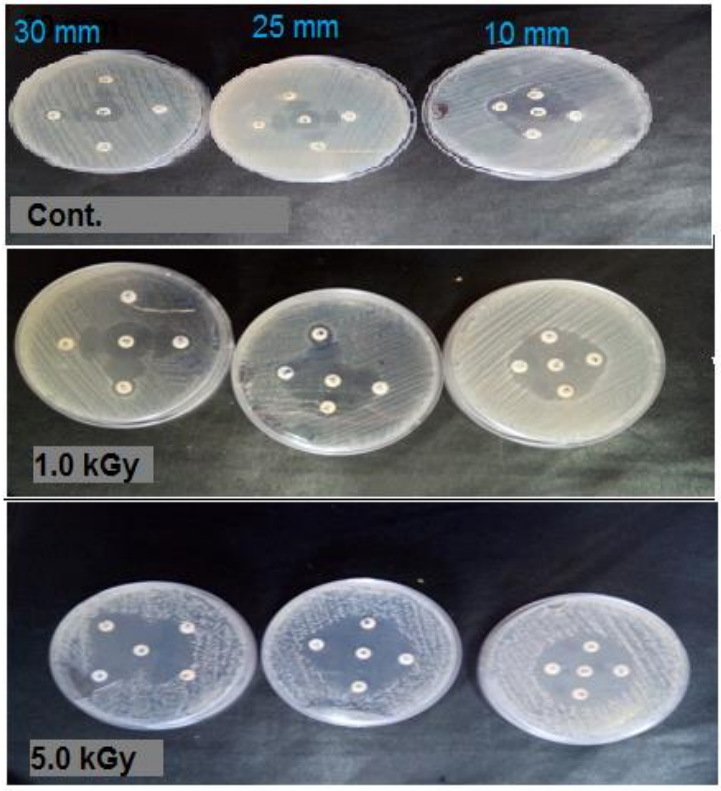

Figure 5. Expansion in the inhibition zone of CAZ, CRO, CTX and ATM towards (AMC) disc by *K. pneumoniae* before and after gamma-irradiation at different distances.

infection-control policies can be considered as the most important factors in the emergence of ESBL-producing strains.

According to double-disc diffusion synergy test (DDST), the ability of the choiced strains to produce ESBLs was tested. The results revealed that the two tested strains (*P. aeruginosa* and *K. pneumoniae*) had the ability to produce ESBLs; these results are in agreement with those of Vandana and Honnavar (2009) who reported that ESBLs are found in certain genera of the Enterobacteriaceae family including *P. aeruginosa*.

Figure 5 showed the ability of the tested *K. pneumoniae* to produce ESBLs before and after gamma-radiation at different distances. The results revealed that there was a gradual expansion in the inhibition zone of the disc containing CAZ, CRO, CTX and ATM towards AMC disc from control (un-irradiated sample) till sublethal dose. This indicated that ESBL production was not affected by gamma irradiation process and is confirmed by combined disc method which showed an increase in the diameter of inhibition zone of the disc containing Sulbactam (SCF) in comparing to the drug alone (CFP) by 5 mm which is considered as positive results for the production of (ESBLs) (data not shown).

From the previous data in this study, it was concluded that un-irradiated *K. pneumoniae* was resistant to IMP, but after exposure to gamma-radiation, its resistance was changed to be susceptible although its ability to produce ESBL was still found. These results were in agreement with those of Dutta et al. (2014) and Shaikh et al. (2015) who observed that all the ESBLs producing isolates were sensitive to IMP. So, it can be concluded that the presence of ESBL is not the reason for microbial resistance towards IMP in case of the control strain but may be due to the incidental presence of various other mechanisms of resistance and counter resistance in a given bacterium as loss of porins. In addition, increased use of carbapenems to treat ESBL-producing organisms has been involved in the emergence of carbapenem-resistant organisms.

Bhattacharjee et al. (2008) and Livermore (2009) reported that carbapenems are advocated for use in treatment of infections caused by ESBLs producing Enterobacteriacea, particularly *Escherichia coli* and *K. pneumonia,* and Giriyapur et al. (2011) stated that carbapenems are stable in the presence of hydrolytic effects of ESBLs, which may explain the consistent finding that 98% of ESBL-producing organisms retain

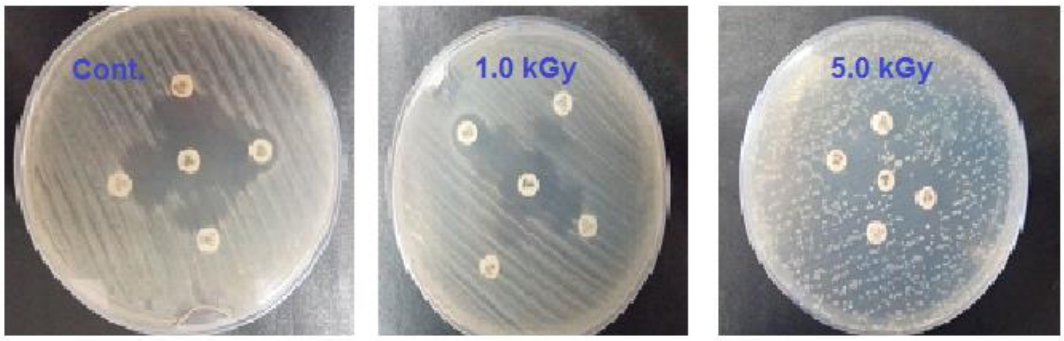

Figure 6. Expansion in the inhibition zone of CAZ, CRO, CTX and ATM towards (AMC) disc by *P. aeruginosa* before and after gamma- radiation at fixed distance.

susceptibility to either imipenem or meropenem.

Also, the results in this study revealed that irradiated and un-irradiated *K. pneumoniae* showed resistance towards all the tested third and fourth generations of cephalosporins antibiotics as well as aztreonam and this is owing to its ability to produce ESBL in both cases. Livermore (1995) discussed that ESBL producing strains show variable resistance against fourth generation cephalosporins. Giriyapur et al. (2011) documented that ESBLs are enzymes that offer resistance against wide spectrum third generation cephalosporins antibiotics along with monobactams (aztreonam).

As a result of the resistance of the tested *K. pneumoniae* towards IMP, its ability to produce Metallo β-lactamase (MBL) by combined disc test (IMP/EDTA) was screened, with the results showing its inability to produce MBL. This could be explained by the presence of other resistance mechanisms involved, such as permeability mutations via the loss of porins or the up-regulation of efflux systems (production of efflux pumps, β–lactamases enzymes and mutations) that alter the expression and/or function of PBPs and porins.

Regarding *P. aeruginosa*, by using (DDST) at a distance 25 cm, there was ghost zone of CAZ, CRO, CTX and ATM towards AMC disc (an evidence of the ability of *P. aeurginosa* to produce ESBL) before gamma radiation and there was no change in the synergy of third cephalosporine towards AMC after exposure to gamma-radiation (Figure 6).

Additionally, this result was confirmed by applying (SCF) test which showed an inhibition zone of 18 mm and 16 mm for un-irradiated and irradiated strain till sublethal dose, respectively towards Sulbactam (SCF) in comparing to the drug alone (CFP) (data not shown).

Arora and Bal (2005) reported that the number of infections caused by extended spectrum beta lactamases producing *P. aeruginosa* is on the rise and poses a threat to patients due to therapeutic failure if they remain undetected.

Mettalo Beta-Lactamases producing *P. aeruginosa* which exhibited multiple resistances to β-lactam antibiotics and multi drug-resistant has been reported to be responsible for severe problem of the management of nosocomial infections worldwide (Potron et al., 2015). The results in this study also revealed that, *P. aeruginosa* was resistant to IMP. Using combined disc test showed resistance towards IMP alone and appearance of inhibition zone in the presence of EDTA plus IMP before and after gamma-radiation at different doses. This result confirmed that *P. aeruginosa* can be considered as MBL producer (Figure 7).

Gupta (2008) concluded that MBL-producing *P. aeruginosa* have been reported to be responsible for serious nosocomial infections worldwide. In the present study, ESBL and MBL co-production was detected in *P. aeruginosa* which was in concordance with other studies (Salimi and Efterkhar, 2013) which found that among the 128 IMP resistant isolates, there were four isolates considered as co-producers of ESBL and MBL.

Thomson and Bonomo (2005) reported that IMP represents one of the last alternatives for the treatment of nosocomial infections caused by multidrug-resistant Gram-negative bacteria, particularly *P. aeruginosa,* but the use of IMP as the first choice of treatment for MDR *P. aeruginosa* in this unit provides a possible explanation for the presence of increasing imipenem-resistance (Nakamura et al., 2013). In addition, Ozyurt et al. (2008) and Hocquet et al. (2010) concluded that one of the most important ways for resistance towards IMP is the generation of MBL enzymes.

Results of this study also indicate that *P. aeruginosa* was resistant towards ATM before and after exposure to gamma- radiation at different doses. This is in accordance with Meini et al. (2014) who discussed that MBL enzymes hydrolyze all β-lactams except ATM. Also, Shahcheraghi et al. (2010) reported that all their metallo β-lactamase producing isolates were resistant to ATM. Lucena et al. (2014) concluded that MBL producing strains of *P. aeuroginosa* (clinical isolates) were 49% resistant for ATM, while Abd El-Baky et al. (2013) showed

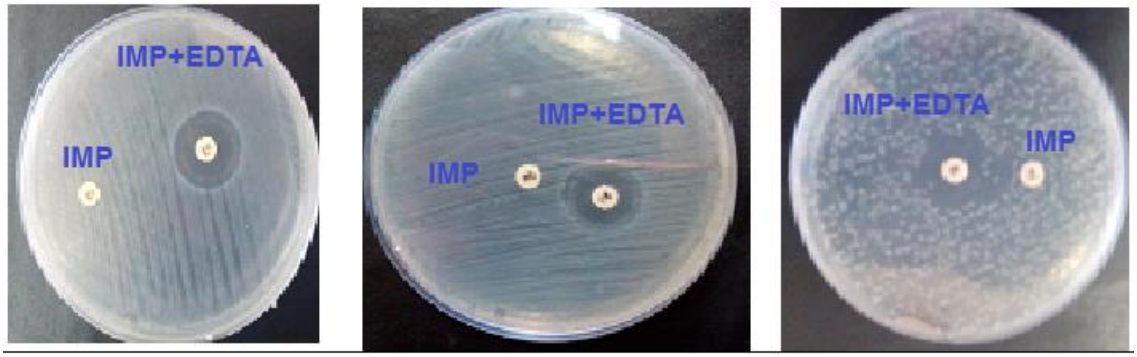

Figure 7. Combined disc test (CDT) for *P. aeruginosa* before and after gamma- radiation.

that, MBL-producing *P. aeurginosa* of clinical isolates were 90.3% resistant for ATM. Sedighi et al. (2015) showed that MBL-producing strains of *P. aeuroginosa* isolated from hospitalized patients were 100% resistant against cefepime and this is accordance with the obtained data in this study. Lucena et al. (2014) showed that 93 MBL-producing clinical isolates of *P. aeuroginosa* were 100% resistant to ciprofloxacin and gentamicin.

Salimi and Eftekhar (2013) discussed that TZP was considered the most effective antimicrobial agent against MBL producers, but in this study, *P. aeruginosa* was resistant towards TZP, a resistance that was changed into susceptible after exposure to gamma-radiation.

From the previous discussion, this study suggests that use of cephalosporins and carbapenems should be restricted in treatment of uropathogens to minimize the development and spread of multidrug resistant problem treatment.

Conclusion

From the previously mentioned results, it can be concluded that the most resistant isolates (100% resistance) were identified as *P. aeruginosa* and *K. pneumoniae* using 16srRNA. The D_{10} values were recorded as (1.1 and 1.2 kGy for *P. aeruginosa* and *K. pneumoniae)* respectively, and both of the tested strains had the ability to produce Extended Spectrum β-lactamase (ESBL) before and after gamma-radiation. ESBL and MBL co-production was detected in *P. aeuroginosa,* finally, gamma- irradiation technique decreased the resistance of *K. pneumoniae* towards IMP and the resistance of *P. aeruginosa* towards more than one antibiotic (TZP, AZM and CIP). This therefore enables us to use gamma-ray in sterilization of implemented devices in hospitals to prevent emergence of these extremely hazardous strains before causing the infection. These findings must be supported by other studies on the level of genes to prove the possibility for using gamma- irradiation technique to overcome microbial drug resistance problem.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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