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The Effect of Ionizing Radiation on Multi-drug Resistant *Pseudomonas aeruginosa* Isolated from Aquatic Environments in Egypt

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Authors' contributions

This research work was carried out in collaboration between all authors. Authors SME, MAAS and HMM designed the work. Authors SME, MAEB and EHAES performed the analysis in the laboratories and managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This study was conducted to determine the effect of different doses of gamma radiation on Multi-drug resistant *Pseudomonas aeruginosa* isolated from River Nile at Rosetta branch and associated drains in Egypt.
Place and Duration of Study: The study was started with samples collection in August 2010 through April 2011 in the Microbiology Dep., Central Laboratory for Environmental Quality Monitoring (CLEQM), National Water Research Center (NWRC), Cairo, Egypt and the National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA), Cairo, Egypt.
Methodology: Water samples were processed using membrane filtration, 144 strains of *P. aeruginosa* were isolated and identified and their antibiotic susceptibility was determined against 20 different antibiotics using agar disc diffusion method. Irradiation of bacterial isolates was processed using gamma irradiation unit of cobalt (Co^{60}) and the

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D_{10}-value was calculated from the survival curve.
Results: Isolates were categorized as multi-drug resistant *Pseudomonas aeruginosa* (MDRPA). 125 (86.8%) were found to be extensively drug resistant (XDR) and 19 (13.2 %) were characterized as possible pan drug resistant (PDR). The highest resistance (100%) was mostly directed to amoxycillin/clavulanic acid, ampicillin, carbenicillin, methicillin, cephalothin, kanamycin, vancomycin, tetracycline, erythromycin, clindamycin, trimethoprim/sulfamethoxazole, nitrofurantoin and chloramphenicol. More than 75% of isolates were sensitive to norfloxacin (82.6%), piperacillin (81.2%), amikacin (79.2%) and tobramycin (77.8%). 63.2%, 26.4% and 14.6% of isolates were sensitive to ofloxacin, cefotaxime and ceftriaxone, respectively. The viable counts of MDRPA decreased with increasing radiation doses of gamma rays up to the lethal dose (3 kGy). The counts of 0.5, 1.0, 1.5, 2.0, 2.5 kGy irradiated samples were respectively 7.8, 6.5, 4.7, 2.3& 1 $\log_{10}$ and the *D_{10}-value* calculated from the survival curve was 0.27 kGy.
Conclusion: Contaminated fresh water may act as reservoirs for antibiotic resistant pathogens. Regular monitoring of Multi-drug resistant pathogens in aquatic environments should be adopted constantly. Gamma radiation demonstrates a potential value for wastewater treatment and pollution control.

Keywords: Gamma radiation; multi-drug resistance; Pseudomonas aeruginosa; River Nile; wastewater treatment.

1. INTRODUCTION

The emergence of continuously evolving resistance to multiple antimicrobial agents in *Pseudomonas aeruginosa* has become a significant public health threat. There are fewer, or even sometimes no, effective antimicrobial agents available for infections caused by these bacteria [1]. *P. aeruginosa* is typically an opportunistic pathogen widespread in nature, particularly in moist environments such as water, sewage, soil, plants and animals. It can grow and multiply in a variety of water environments including river water, seawater, wastewater and bottled mineral water.

This microorganism is responsible for 10% of all hospital acquired infections, ranking second among Gram-negative pathogens [2]. According to the NNISS (National Nosocomial Infections Surveillance System) in USA and INICC (International Nosocomial Infection Control Consortium) reports, *P. aeruginosa* is the most common pathogen found in intensive care units (ICUs) [3]. Surveillance data ranked *P. aeruginosa* as the second most common cause of nosocomial pneumonia, the third most common cause of nosocomial urinary tract infections, and the seventh most common cause of nosocomial bacteraemia in the U.S.A [4].

This organism enjoys notable virulence traits including minimal nutritional requirements, tolerance towards wide variety of physical conditions and relative resistance to antimicrobial agents. This metabolic versatility contributes to a broad ecological adaptability and distribution, and reflects a genome of larger size and complexity compared with that of many other bacterial species [5,6].

Several virulence factors aid in its pathogenicity and resistance to antimicrobial agents. These include: possession of mucus-alginate capsule, presence of exoenzymes S, elastase and other proteases, phospholipase C, the ferripyochelin-binding protein, lipopolysaccharide (LPS), Exotoxin A and an active Chaperone Usher pathway [7,8]. *P. aeruginosa* has intrinsic resistance to many antibiotics due to low permeability of the outer membrane and

presence of a number of active efflux pump systems. The AmpC chromosomal β-lactamase contributes to the intrinsic resistance to penicillins and cephalosporins. Additionally, some *P. aeruginosa* strains exhibit mutations in fluoroquinolone binding sites, the loss of porin channels, and increased β-lactamase or cephalosporinase production. Acquired resistance to aminoglycosides can be due to the production of aminoglycoside-modifying enzymes encoded by horizontally acquired resistance determinants, or by mutations that reduce aminoglycoside accumulation in the bacterial cell [9,10].

Parallel to the problem of antibiotic resistance, the growing water scarcity all over the world has increased the interest in wastewater reuse and in disinfection techniques that reduce the pathological impacts of wastewater in the environment. Chlorine is the most commonly used chemical in this respect, particularly in developing countries. However, pathogens regrowth may occur after chlorination treatments as it reduce the initial count only by 2 log cycles [11]. Moreover, chlorine can react with inorganic ammonia in wastewater forming chloramines and/or organohalogen compounds such as trihalomethanes and haloacetic acids which are highly carcinogenic [12]. Several studies showed that pathogens characterized by being multiple antibiotic resistances (MAR) can find their way to different water resources in spite of chlorination [13,14,15]. Alternatives to chlorination and other traditional methods of disinfection are being studied, one such alternative is radiation processing. Nuclear techniques have recently been used to control environmental pollution. Gamma radiation has been successfully used for disinfection of harmful pathogens, degradation of toxic organic pollutants, reduction of heavy metals, biochemical oxygen demand (BOD), chemical oxygen demand (COD) and total organic carbon (TOC) all at the same time [11,12,16].

The aim of this study is to profile antibiotic resistance patterns in *P. aeruginosa* isolated from River Nile at Rosetta branch and associated drains in Egypt, as well as to address the impact of different doses of gamma radiation on these isolates and the potential value of its use as a non-conventional method for water disinfection.

2. MATERIALS AND METHODS

2.1 Study Area and Sampling

This study was started with samples collection in August 2010 through April 2011in which, the River Nile at Rosetta branch was subdivided into five reaches based on locations of known waste inputs as illustrated in Fig. 1. Totally fifteen sites were chosen, three from each reach: five at drain outfalls (El-Rahway, Sabal, El-Tahreer, Zawiet El-Bahr and Tala) and ten sites in Rosetta branch (five upstream and five downstream those drains outfalls).These are mixed drains from sewage, agricultural and industrial wastes. Water sampling was carried out according to Standard Methods for Examination of Water and Wastewater [17].

2.2 Detection, Isolation and Identification of *Pseudomonas aeruginosa*

Membrane filter technique was applied for detection and isolation of *P. aeruginosa* according to standard method No. 9213 E [17] on M-PA-C agar medium. In this procedure, water samples were filtered through sterile, white, grid-marked, 47 mm diameter membrane with pore size 0.45µmwhich retained bacteria. After filtration, the membrane was plated on M-PA-C agar medium (Difco, USA) and incubated at 41.5^{o}C/72h. Typically, *P. aeruginosa* colonies are 0.8 to 2.2 mm in diameter and flat in appearance with light outer rims and brownish to greenish black centers. These colonies were confirmed by streaking on cetrimide agar

(Difco, USA) plates, a selective medium which inhibits bacterial growth except *P. aeruginosa* and enhances fluorescein and pyocyanin 'blue green' pigment production. Confirmation was completed by microscopical and biochemical examinations (Gram staining, pigment production and oxidase test) according to Bergey's Manual of Systematic Bacteriology [18] as well as by API 20 NE assay (bioMérieux, France) according to [19].

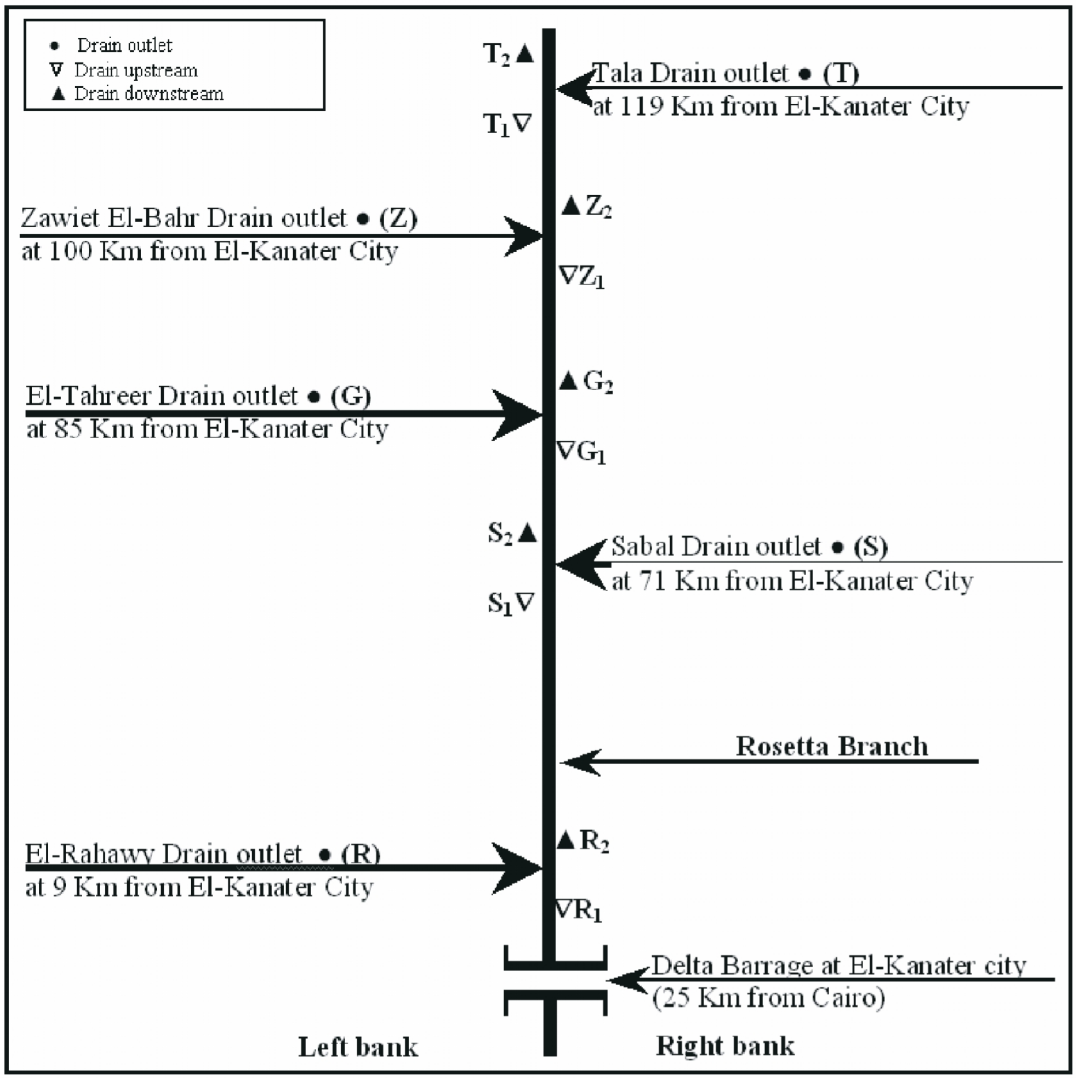

Fig. 1. Schematic diagram for water sampling locations

2.3 Antibiotic Susceptibility

The standard Kirby-Bauer disk diffusion method [20] was conducted to determine antibiotic susceptibility profiles of tested bacterial isolates for twenty antibiotics. The antibiotics tested and their concentrations were amoxycillin/clavulanic acid (30µg), ampicillin (10µg), carbenicillin (100µg), methicillin (5µg), piperacillin (75µg), cephalothin (30µg), cefotaxime (30µg), ceftriaxone (30µg), vancomycin (30µg), amikacin (30µg), tobramycin (10µg),

kanamycin (30µg), tetracycline (30µg), erythromycin (10µg), clindamycin (30µg), norfloxacin (10µg), ofloxacin (10µg), trimethoprim/sulfamethoxazole (25µg), nitrofurantoin (300µg) and chloramphenicol (30µg). All the antibiotic discs were purchased from Oxiod, UK.

Four to five similar colonies from overnight growth plate were transferred aseptically in saline solution and vigorously agitated to give a density of 0.5 McFarland turbidity standards (approximately 10^8 CFU/ml) according to [21]. Within 15 minutes, sterile cotton swab dipped into the culture suspension was used for inoculating the surface of solidified Mueller-Hinton agar (Oxoid, UK) plates. Then, antibiotic discs were placed 30 mm apart and 10 mm from the edge of the plate. Plates were incubated at 37^{o}C for 18-20h. The resulted diameters of inhibition zones around the antibiotic discs were measured to nearest whole mm and interpreted according to protocols standardized for the assay of antibiotic compounds as guided by National Committee for Clinical Laboratory Standards "NCCLS" [22].

2.4 Effect of Gamma Radiation on Multi-drug Resistant *P. aeruginosa*

The irradiation facility used was gamma (γ) irradiation unit of cobalt (Co^{60}) manufactured by Bhabba, India. The most resistant bacterial strain was grown in LB broth for 24 h. on shaker (150 rpm) at 30ºC. The well grown bacterial culture was centrifuged at 8000 rpm 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 different doses of gamma radiation (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 kGy) using three replicates for each dose. The dose rate was 1 kGy/12.5 minutes at the time of experiment. The non-irradiated control and the irradiated cultures were serially diluted and plated on the surface of LB agar plates and the viable count was determined [23].

2.5 Determination of Radiation Decimal Reduction Dose (D_{10}-*value*)

The radiation resistance of a microorganism is measured by the so-called decimal reduction dose (D_{10}-*value*), which is the radiation dose (kGy) required to reduce the number of the microorganism by 10-fold (one log cycle) or required to kill 90% of the total number. The D_{10}-*value* is the reciprocal of the slope of the exponential part of a survival curve. This value may also be obtained from the following equation [24,25].

$$D_{10}\text{-}value = D/ (\log N_0 - \log N)$$

Where, D: is radiation dose, N_0: is the initial number of viable cells and N: is the number of cells surviving the treatment D.

3. RESULTS AND DISCUSSION

In accordance with recent standard definitions [1], multi-drug resistance (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories. Extensively drug-resistance (XDR) was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and pan drug resistance (PDR) was defined as non-susceptibility to all agents in all antimicrobial categories (i.e. resistant to all approved and commercially available antimicrobials). Thus, a bacterial isolate that is characterized as XDR will also be characterized as MDR. XDR is a subset of MDR, and PDR is a subset of XDR. In case of

incomplete testing (unavailability of all approved antimicrobials), bacterial isolates can be characterized as possible PDR.

In the present investigation, a total of 144 *Pseudomonas aeruginosa* isolates were recovered from water samples collected from five drains outlets (67 isolates) and ten sites along Rosetta branch of River Nile (77 isolates). All isolates were characterized as multi-drug resistant *P. aeruginosa* (MDRPA). Out of those, 125 (86.8 %) were found to be XDR and 19 (13.2 %) were characterized as possible PDR Table 1.

Table 1. Prevalence of XDR and Possible PDR *P. aeruginosa* isolates from River Nile at Rosetta branch and drains

Water samples	No. of *P. aeruginosa* isolates	NO. (%) of isolates	
		XDR	Possible PDR
Wastewater	67 (46.5%)	58 (40.2 %)	9 (6.3 %)
Fresh water before drains discharging	31 (21.5%)	26 (18 %)	5 (3.5 %)
Fresh water after drains discharging	46 (32%)	41 (28.5 %)	5 (3.5 %)
Total	**144**	**125 (86.8 %)**	**19 (13.2 %)**

Fig. 2 demonstrates the antibiotic multi-resistance frequency of isolated strains. 144 (100%) of MDRPA isolates were resistant to 13 antibiotics, 139 (96.5%) to 14 antibiotics, 122 (84.7%) to 15 antibiotics, 79 (54.9%) to 16 antibiotics, 32 (22.2%) to 17 antibiotics, 19 (13.2%) to 18 antibiotics and 5 (3.5%) to 19 antibiotics. The highest resistance (100%) was mostly directed to amoxycillin/clavulanic acid, ampicillin, carbenicillin, methicillin, cephalothin, kanamycin, vancomycin, tetracycline, erythromycin, clindamycin, trimethoprim/sulfamethoxazole, nitrofurantoin and chloramphenicol. More than 75% of isolates were sensitive to norfloxacin (82.6%), piperacillin (81.2%), amikacin (79.2%) and tobramycin (77.8%). 63.2%, 26.4% and 14.6% of isolates were sensitive to ofloxacin, cefotaxime and ceftriaxone, respectively. Antibiotic resistance rates of all MDRPA strains isolated either from drains outlets or Rosetta branch were almost the same Table 2 and Fig. 3.

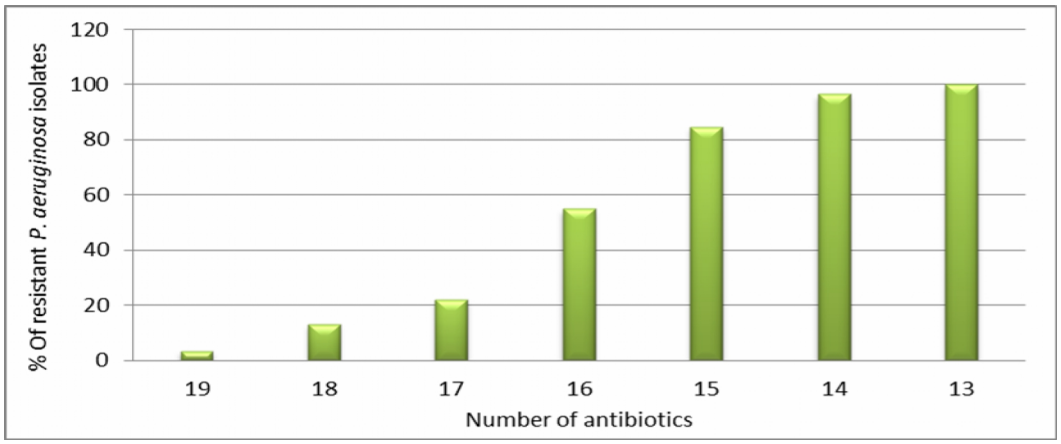

Fig. 2. Frequency of multi-drug resistant *Pseudomonas aeruginosa* (MDRPA) isolates.

The Results of gamma irradiation on MDRPA showed that the viable counts decreased with increasing radiation doses up to the lethal dose which was recorded at 3 kGy. It was found that the counts of 0.5, 1.0, 1.5, 2.0, 2.5 kGy irradiated samples were respectively 7.8, 6.5, 4.7, 2.3 and 1 $\log_{10}$ as shown in Table 3 and illustrated by Fig. 4. The *D_{10}-value* calculated from the survival curve was 0.27 kGy.

Table 2. Antimicrobial susceptibility profiles of *P. aeruginosa* isolates from River Nile at Rosetta branch and drains

Antimicrobial category	Antimicrobial agent	No. (%) of isolates		
		Susceptible	XDR	Possible PDR
Penicillins	Amoxycillin/Clavulanic acid	0	0	144 (100)
	Ampicillin	0	0	144 (100)
	Carbenicillin	0	0	144 (100)
	Methicillin	0	0	144 (100)
	Piperacillin	117 (81.2)	27 (18.8)	0
Cephalosporins	Cephalothin	0	0	144 (100)
	Cefotaxime	38 (26.4)	106 (73.6)	0
	Ceftriaxone	21 (14.6)	123 (85.4)	0
Aminoglycosides	Amikacin	114 (79.2)	30 (20.8)	0
	Tobramycin	112 (77.8)	32 (22.2)	0
	Kanamycin	0	0	144 (100)
Glycopeptides	Vancomycin	0	0	144 (100)
Tetracyclines	Tetracycline	0	0	144 (100)
Macrolides	Erythromycin	0	0	144 (100)
Lincosamides	Clindamycin	0	0	144 (100)
Fluoroquinolones	Norfloxacin	119 (82.6)	25 (17.4)	0
	Ofloxacin	91 (63.2)	53 (36.8)	0
Sulfa drugs	Trimethoprim/Sulfamethoxazole	0	0	144 (100)
Nitrofurans	Nitrofurantoin	0	0	144 (100)
Chloramphenicol	Chloramphenicol	0	0	144 (100)

Table 3. Effect of gamma radiation doses on viable count of MDRPA isolates

Irradiation doses(kGy)	Bacterial count(CFU/ml)	Log N
Control	3×10^{9}	9.7
0.5	7×10^{7}	7.8
1	3×10^{6}	6.5
1.5	5×10^{4}	4.7
2	2×10^{2}	2.3
2.5	10	1
3	0	0
3.5	0	0

Our study revealed high prevalence of a large proportion of MDRPA isolates in River Nile at Rosetta branch and associated drains along its sides. *P. aeruginosa* is inherently resistant to many antimicrobial agents, mainly due to the synergy between multi-drug efflux system or a type1 AmpC β-lactamase and low outer membrane permeability [10]. *P. aeruginosa* exhibits remarkable ability to acquire resistance by mutation or acquisition of exogenous resistance

determinants and can be mediated by several mechanisms (degrading enzymes, reduced permeability, active efflux and target modification) [8].

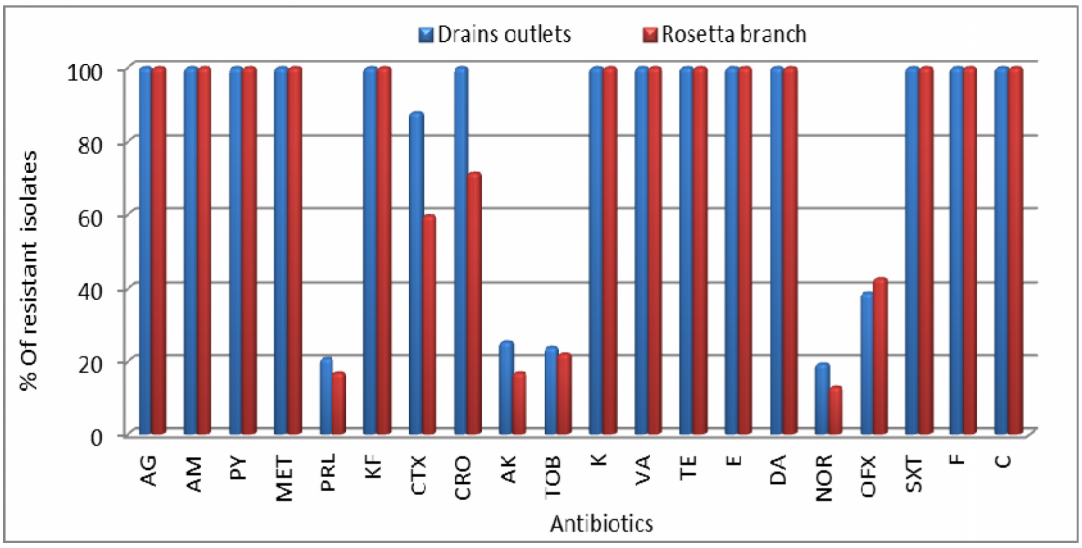

Fig. 3. Antibiotic resistance rates of MDRPA isolates from various water samples

AG, amoxycillin/clavulanic acid; AM: ampicillin; PY, carbenicillin; MET, methicillin; PRL, piperacillin; KF, cephalothin; CTX, cefotaxime; CRO, ceftriaxone; AK, amikacin; TOB, tobramycin; K, kanamycin; VA, vancomycin; TE, tetracycline; E, erythromycin; DA, clindamycin; NOR, norfloxacin; OFX, ofloxacin; SXT, trimethoprim/sulfamethoxazole; F, nitrofurantoin& C, chloramphenicol

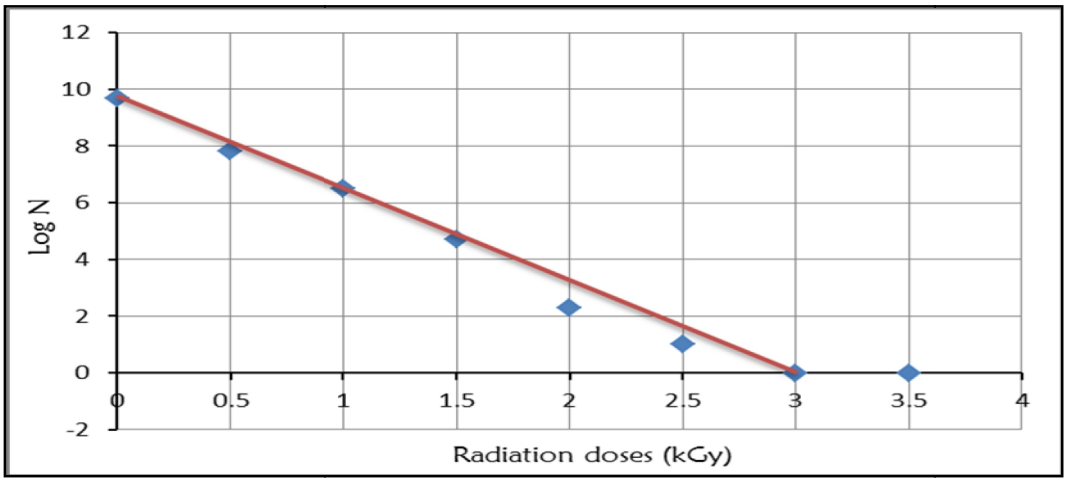

Fig. 4. Effect of gamma radiation on the viable count of MDRPA isolates

There is no standard definition of MDRPA till now [1,26]. They were defined as the isolates resistant to three agents like β-lactam, carbapenems, aminoglycosides and fluoroquinolone [27] or those isolates resistant to at least five out of seven anti-Pseudomonal categories like penicillins, cephalosporins, carbapenems, monobactams, quinolones, aminoglycosides and colistin [28]. MDRPA are defined by the CDC as resistance to one or more classes of antimicrobial agents [29,30].

The findings of this study indicated that environmental *P. aeruginosa* isolated from aquatic ecosystem have considerable levels of antibiotic resistance. Isolates demonstrated resistance to a wide range of clinically relevant antimicrobial agents, including penicillins, aminoglycosides, quinolones and glycopeptides. In Egypt, our results were in conformity with [14] who documented that all *P. aeruginosa* isolated from River Nile were 100% resistant to amoxycillin/clavulanic acid, ampicillin, carbenicillin, methicillin, cephalothin, vancomycin, tetracycline, erythromycin and clindamycin. In a similar study carried out in U.S.A, resistance of *P. aeruginosa* to ampicillin and nitrofurantoin was found to be 74% and 96%, respectively [9]. In Pakistan, [6] reported that all *P. aeruginosa* isolates from fresh water spring contaminated with domestic sewage were 94% and 73% resistant to chloramphenicol and tetracycline, respectively.

The emergence and spread of MDRPA in the aquatic environment could be attributed to the continual discharge of untreated domestic wastewater. Our data demonstrate that pollution whatever its type can create antibiotic resistant traits. Aquatic ecosystems which received wastewater, pathogenic and opportunistic bacteria, could serve as a reservoir of antibiotic resistant pathogens through transfer of resistance plasmids [6]. Thus, polluted spots in River Nile may become reservoirs for antibiotic resistant genes that can, under natural conditions, be transferred to water-borne pathogens. Discharging of wastewater into River Nile may be a source of pathogenic bacteria that originally found in patients and transfer to hospital sewage and finally to surface water. Moreover, exposing environmental bacterial communities to these wastes may results in selection of antibiotic resistance traits that was not present in unexposed communities, significant positive correlation in the level of AR with increasing concentrations of pollution and increased level of multiple drug resistance. Our results were in harmony with [15,31,32].

Appearance of MDRPA outside hospital setting is recognized as a threat to public health. While the levels of resistance among study isolates are similar to those previously reported, it is important to draw the distinction between a nosocomial setting and the non-clinical setting of the current study. In clinical scenarios, there is constant selective pressure to enhance the proliferation of multi-drug resistant strains. Given that *P. aeruginosa* has both intrinsic resistance and a dynamic ability to develop resistance during the course of infection, a high frequency of resistance is now expected in hospitals. However, in the non-clinical environment, the absence of selective pressure may reduce antimicrobial resistance levels. In addition, the presence of resistance to front line anti-pseudomonal drugs may have important clinical and prevention implications. Exposure to resistant *P. aeruginosa* results in even greater risk for immune-suppressed or other high-risk individuals [9].

With few new antibiotics and the rising incidence of MDRPA worldwide as well as growing water scarcity in parallel to increased levels of water pollution, it becomes increasingly important to utilize strategies that will minimize antibiotic resistant strains spread and mediate wastewater treatment and reuse. In this study we suggested gamma irradiation as a new, non-conventional and effective method for wastewater treatment. Lethal dose of gamma rays to MDRPA was recorded at 3 kGy. It is well known that, exposure of bacterial cells to ionizing radiation presents an additional stress to the cells which tends to disturb their organization. Nucleic acids, especially DNA, are the primary target for cell damage from ionizing radiation. Gamma radiation induced three types of damage in DNA, single strand breaks, double strand breaks and nucleotide damage which include base damage and damage in the sugar moiety [33]. The base damage is a major component of damage induced by ionizing radiation [34]. Gamma irradiation also affects protein fingerprinting and enzymes as indicated by [35,36].

Our results were in harmony with those observed by [37], who found that 3 kGy reduced completely the viable count of all Gram-negative short rods bacterium (*Pseudomonas*) isolated from soils and capable of degrading chromatic compounds. [38] Proved that Gram-negative bacilli, isolated from patients and were multi-drug resistant (MDR) strains, their viable count was completely reduced by 3.0 kGy of gamma radiation. Our results agree with those observed by [39], who found that irradiation dose of 1 kGy reduced the counts of *pseudomonas aeruginosa* by 35% of initial count.

The sensitivity of different strains towards gamma radiation varied greatly according to their structure and nature. Gram positive spore-forming bacteria are more resistant to gamma irradiation than Gram negative ones. This may be attributed to the difference in their sulfur content [40, 41]. The relative sensitivity or resistance of different microorganisms to ionizing radiation is based on their respective *D10-value*. *D10-value* is the ionizing radiation dose required to reduce the population by a 10 fold (by one log cycle, 1-$\log_{10}$) or required to kill 90% of total viable number of microorganisms [42]. Lower D_{10}*-values* indicate greater sensitivity of the organism to ionizing radiation. In this respect, the D_{10}*-value* calculated from the survival curve was 0.27 kGy. This classifies *P. aeruginosa* isolated in this study as sensitive to gamma radiation.

It seems that, irradiation could be an alternative to traditional chlorination of contaminated water, especially if reuse and/or disposal is to consider as an option. In accordance with previous opinion, [12] reported that a dose of 1 kGy from (Co^{60}) gamma source was effective to cause 99.8% reduction in total coliforms from unchlorinated effluent, while the same dose resulted in 99.3% reduction in fecal coliforms with no regrowth of both at a dose of 1.3 kGy. The D_{10}*-values* for both were 0.3 and 0.4 kGy, respectively. It is worth to mention that, there is a worldwide interest in the use of ionizing radiation as a new, non-conventional method for wastewater and sludge treatment. Irradiation for such purpose was facilitated in Germany, USA, India, Argentina, Canada, Brazil, and Korea. Egypt nowadays is stepping forward the using of radiation processing in several peaceful fields. We hope that these steps would pave the way for possible new strategy in water treatment process.

4. CONCLUSION

In conclusion, contaminated fresh water may act as reservoirs for antibiotic resistant pathogens that lead to public health concern. The emergence of MDRPA outside hospital settings is becoming a serious issue due to limited therapeutic options. Isolated *P. aeruginosa* is resistant to a wide variety of antimicrobial agents, including front line anti-pseudomonal drugs. Regular monitoring of Multi-drug resistant pathogens in aquatic environments should be adopted constantly with expanded sampling area to determine if the frequency of resistance strains remains steady over time. Additionally, optimal and controlled use policies of existing antimicrobial agents should be promoted. We suggest gamma irradiation as a non-conventional and effective method for wastewater treatment and a new strategy for pollution control. Employing an electron accelerator would improve the economics of the process and reduce any public acceptance issues associated with the use of radiation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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