Effect of low dose of gamma radiation on multidrug resistant *Mycobacterium tuberculosis*

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Abstract: Multidrug resistance (MDR), defined as resistance to at least isoniazid (INH) and rifampin (RMP) which have the most effective bactericidal activity to *M. tuberculosis*, is now common throughout the world, with average rates of resistance of 15% in high-burden countries. Exposure of bacterial cells to low doses of ionizing radiation induces mutations that lead to genetic and subsequent phenotypic effects. Our aim was to study the effect of a low dose of gamma radiation, which is commonly used for treatment of most cancer patients, in altering the resistance of *M. tuberculosis* and analyze their effect on repairing gene mutations in the *rpoB* and *KatG* involving *M. tuberculosis* resistance to RMP and INH. *M. tuberculosis* strains resistant to RMP and INH were isolated, identified and exposed to 24.41Gy *in-vitro* gamma irradiation. The susceptibility tests to RMP and INH after irradiation were performed. Mutations in the *rpoB and katG* gene were analyzed by DNA sequencing of *M. tuberculosis* MDR clinical isolates before and after exposure to gamma radiation. The results revealed that after exposure 61.1% were sensitive to RMP and INH, 22.2% sensitive to RMP and resistant to INH while 16.7% remained MDR. As a result of comparing mutations of *rpoB* and *katG* gene before and after irradiation with respect to their mutations conferring RMP and INH resistance, a marked difference in the distribution of mutations was observed. Knowledge of the effect of radiation on *M. tuberculosis* would help to improve the treatment of tuberculosis. [Journal of American Science 2010;6(10):774-780]. (ISSN: 1545-1003).

Key words: Multidrug-resistant tuberculosis (MDR-TB); isoniazid (INH); rifampicin (RMP); *katG* gene; *rpoB* gene; mutations; *in- vitro* gamma irradiation.

Introduction

Tuberculosis (TB) is a common and deadly infectious disease caused by mycobacterium, mainly *Mycobacterium tuberculosis* (*M. tuberculosis*). One-third of the world's population has been exposed to the TB bacterium. It has fatality rate of approximately 20%, even with intensive treatment (**Bartzatt *et al.*, 2008**). Major anti-TB drugs include isoniazid (INH), rifampin (RMP), and ethambutol (EMB). Drug-resistant *M. tuberculosis* strains are rapidly becoming the next global health emergency **(Klingeren *et al.*, 2007, Richter *et al.*, 2009 and Sangare *et al.*, 2010)**. MDR strains are difficult to cure and are more likely to remain sources of infection for long period of time than drug susceptible strains (Zumia and Grange, 2001). Multidrug-resistant MDRTB, defined as simultaneous resistance to at least isoniazid (INH) and rifampin (RMP), is a serious problem **(Bifani *et al.*, 2002, and Nalini *et al.*, 2010).** Strains of MDRTB appear to result from the stepwise acquisition of mutations in the genes encoding drug targets or drug-converting enzymes **(Gillespie, 2002 and Parrish *et al.*, 2007)**. Resistance to RMP in *M.tuberculosis* strains is usually caused by the point mutations in an 81bp region, (named core *rpoB*, or RMP-resistance determining region PRDR), which is part of the *rpoB* gene encoding the beta subunit of the DNA polymerase. Because synonymous changes are very uncommon in this region, the detection of any nucleotidic substitution within this genetic region is a marker for resistance. Although a single point mutation is sufficient for developing RMP resistance, a number of articles described multiple *rpoB* mutations for *M.tuberculosis* **(Pozzi *et al.*, 1999; Mani *et al.*, 2001 and Hristea *et al.*, 2010)**. For INH resistance mutations have been reported to map in at least four different genetic loci; *katG*, *inhA*, *ahpC*, and *oxyR* (**Banerjee *et al.*, 1994, Kelly *et al.*, 1997**). Despite this, one codon, *katG* 315 is most frequently associated with resistance to INH (often at high level), and therefore, the detection of resistance in many cases is simplified **(Wada, *et al.*, 2004 and Hillemann, *et al.*, 2005)**. Several molecular methods to detect drug-resistant in *M. tuberculosis* have been reported **(Garcia de Viedma, 2003)**. These methods are fundamentally based on the detection of point mutations. PCR-DNA sequencing is a straightforward technology to detect mutation (**Kapur, 1994 and Parrish *et al.*, 2007)**. The importance of TB as a major public health problem has been dramatically reinforced due to the human immunodeficiency diseases and the emergence of multi-drug-resistant *M. tuberculosis* (MDRTB) strains (**Allix-Béguec *et al.*,**

2008). The association of tuberculosis and cancer has been described and explained by many authors in many diverse ways **(Kaplan *et al.*, 1974, Pandey *et al.*, 2003 and Hofmeyr *et al.*, 2007).** Radiation therapy is one of the most important methods of cancer treatment; at least 50 % of all cancer patients will receive radiotherapy at some stage during the course of their illness. (**Tobias, 1992 and Spira and Ettinger, 2004**). It is well known that, exposure of microbial 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. Breaks in the DNA chain disrupt function of the molecule in several ways **(Scala, 1995).** 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. The base damage is a major component of damage induced by ionizing radiation **(Pouget *et al.*, 2002)**. After irradiation, some bacterial cells contain abnormal sets of chromosomes or transmit their chromosomes abnormally, while others exhibit heritable changes **(Scala, 1995)**.

To this end, as radiotherapy of cancer patients exerts many effects on microbial cells, and in order to treat patients with MDR-TB now and in the future, new approaches to anti-tuberculosis treatment are needed urgently as no new drugs specifically evaluated for the treatment of TB has been introduced since rifampicin entered treatment regimens in the late 1960s and early 1970s, the present work aimed to study the antibiotic adjuvant activity of low dose of gamma radiation commonly used in the treatment of lung cancer patients against MDR-TB with respect to their resistance to RMP and INH in an attempt to successfully combat this global problem.

Materials and methods

Strains and resistance testing: A set of Eighteen Rifampin and Isoniazid resistant *M. tuberculosis* strains obtained from sputum specimens of patients referred to Abbasia chest hospital, Cairo, Egypt were included in the study. The sputum specimens were digested and decontaminated by N-acetyl L-cystiene (NALC) and sodium hydroxide (NaOH) as described previously **(Kent and kubica, 1985)**. All specimen sediment was finally re-suspended in 2.2 ml of 0.067 M phosphate buffer (pH 6.8). For all specimens, half of the sediment was inoculated onto the culture medium and used for acid-fast staining. All manipulations of specimens were done in the certified level II biosafety cabinet to contain the aerosols that potentially generated by adding reagents.

Microscopy. Smears were stained by Ziehl-Neelsen staining **(Heifets and Good, 1994).**

Culture. Aliquots of the processed sediment were inoculated onto Lowenstein-Jensen medium for isolation of the acid fast bacilli (BBL). Slants were incubated at 37°C for 8 weeks in a 5% CO2 atmosphere. Identification of the isolates was done according to colony morphology, growth rate, niacin accumulation and nitrate reduction **(Kent and Kubica, 1985)**.

From culture strains: Few colonies of previously well-characterized *M. tuberculosis* clinical isolates that were grown on Lowenstein-Jensen slants were used. A one McFarland suspension was made and was divided to 3 parts. The first part was used for susceptibility test and DNA was extracted from the second one. The third part was exposed to gamma radiation then the susceptibility test and DNA extraction were done on the irradiated suspension.

The susceptibility tests to RMP and INH (before and after radiation) were determined using Mycobacterium growth indicator tube (MGIT). MGIT culture tube contains 7ml of modified Middlebrook 7H9 broth base, to which 0.8 ml was added an enrichment supplement containing oleic acid, albumin, dextrose, and catalase (BBL MGIT OADC) and 0.1ml antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BBL MGIT PANTA).

Antimycobacterial drugs: Lyophilized drugs (Becton Dickinson) were dissolved according to the manufacturer's instructions. The final drugs concentrations used were 0.1 µg/ml for INH and 1.0 µg/ml for RMP **(Rüsch-Gerdes, *et al.*, 1999).** Finally 0.5 ml of the test inoculums (1 McFarland) was added. For each isolate, a growth control tube with Growth Supplement and without drug was included. If the relative growth in the drug-containing tube was equal to or exceeded that of the growth control tube, the isolate was considered drug resistant; otherwise the isolate was considered drug susceptible.

DNA extraction: Bacteria were harvested from the Lowenstein Jensen slopes, heat-killed. DNA was extracted using Genomic DNA isolation kit (Fermentas life Science, EU) as per manufacture protocol. The extracted DNAs were stored at -20°C.

DNA target sequences DNA target sequences were the 363-bp *M. tuberculosis rpoB* gene and the 206-bp *M. tuberculosis* katG gene.

PCR Specific for rpoB gene and katG gene: A 363-bp segment of *rpoB* species was amplified using (5'ACACCGCAGACGTTGATCA3') and (5'CTAGTGATGGCGGTCAGGT AC3') oligonucleotide primers **(Wada, *et al.*, 2004)**. A 206-bp segment of *katG* species was amplified using (5′GAAACAGCGGCGCTGGATCGT'3) and (5′

GTTGTCCATTTCGTCG GGG'3) oligonucleotide primers **(Kim, *et al.*, 2006)**. The primers were synthesized from the Biotechnology Facility at the University of Connecticut, USA. PCR reaction for both was performed in a 50 ul volume with master mix contained final concentration of 1.25U Taq DNA polymerase, 50 mM KCl, 30mM Tris HCl, 1.5mM Mg^{+2}, 0.1% lgepal CA630 and 200uM of each dNTP (Eppendorf, NY, USA) with 100 pmol of primers with 100 ng of DNA template. The PCR amplification was carried out with an initial denaturation at 94ºC for 10 min. followed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 60 ºC for 30s, and extension at 72ºC for 30 s. The PCR sample heated at 72ºC for 30 min for a final extension for rpoB gene. While, the PCR cycle for katG was consisted of initial denaturation at 94ºC for 10 min. followed by 30 cycles of denaturation at 94ºC for 45 s, annealing at 63 ºC for 30s, and extension at 72ºC for 30s. For final extension the samples were heated at 72ºC for 5 min.

Detection of amplified PCR Products: Agarose gel electrophoresis was used to detect PCR products. Ten micro liter volumes of PCR products separated through a 1.5% agarose horizontal gel by electrophoresis at 84 volts. Gels were stained with ethidium bromide (0.5 ug/ml), and visualized by ultraviolet light and photographed.

Purification of PCR products and DNA sequencing: The PCR products of *rpoB* and *katG* segments were purified by Gene Jex PCR purification kit as manufacture instructions (Fermentas Life sciences, EU). The purified DNA was submitted for sequencing. The same primers used for PCR were used for the sequencing of forward and reverse fragments.

Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM, BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template selected primer. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). The Blast sequences computer program was used for DNA sequence comparisons (http:// www. ncbi. nlm. nih.gov/BLAST/).

Irradiation source: ^{137}C Gamma cell 40, Atomic Energy of Canada Limited, Commercial Products located at National Center for Radiation Research and Technology (Cairo, Egypt) was the irradiation source used. The dose rate was 1.30 Rad/Sec at the time of experiments. *In- vitro* 24.41 Gy gamma irradiation was given. This total single dose is biologically equivalent to the fractionated multiple therapeutic doses of 70 Gy/35 fractions used in the treatment of some cancer patients and was calculated by using the linear quadratic (LQ) formula described by **Barton, (1995)**.

Results and Discussion

A major point of concern is that patients infected with MDR-TB strains are more difficult to treat and are more likely to remain as sources of infection for longer periods of time than patients with drug-susceptible strains **(Zumia & Grange 2001).** In this study eighteen *M. tuberculosis* isolates identified according to colony morphology, growth rate, niacin production and nitrate reduction. Clinical isolates were selected according to multidrug resistance characteristic of strains (resistance to RMP and INH).

Drug sensitivity after *in- vitro* gamma irradiation: The eighteen *M. tuberculosis* isolates were resistant to RMP and INH before exposure to gamma radiation by MGIT. The *susceptibility* test of MDR resistant *M. tuberculosis* clinical isolates after irradiation revealed the following changes 61.1% were sensitive to RMP and INH which have previous been identified as MDR before radiation. While 22.2% were sensitive to RMP and resistance to INH and 16.7% remained MDR. 'spectrum' of mutations produced by γ-radiation which is significantly different from that occurring spontaneously leading to multi-drug resistance could be attributed to the random effects of radiation, whether such effects are direct or indirect. When ionizing radiation is incident on biologic systems (microbial cells) energy is transferred into the system according to fundamental physical principles. The effect is often not so predictable (Scala,1995). To date, little reports were cover such area.

Molecular analysis: All isolates before and after radiation contained *rpoB* and *katG* based upon PCR amplification of a 363 bp and 206 bp respectively as shown in figure 1 and 2. No complete deletions of the *katG* gene were found among the strains. Genomic DNA of three isolates was analyzed for mutations in the 363-pb fragment of the *rpoB* gene and 206-bp for *katG* gene by sequencing. These three strains picked according to their change from MDR to sensitive strains after gamma irradiation.

Mutations in the 81-bp core region of *rpoB* gene were identified in the three RMP resistance isolates of *M. tuberculosis* included in the study. Genetic studies have demonstrated that more than 95% of RMP resistance is associated with a mutation in the 81-bp core region of the *rpoB* gene **(Telenti, *et al.*, 1993 and Ramaswamy and Musser, 1998)**.

Before exposure to gamma radiation, mutations affecting codons 531or 526 were present in the RMP-resistant *M. tuberculosis* strains Fig.3. In our study there were substitution of TCG to TTG in codon 531 in two strains and substitution of CAC to TAC in codon 526 were present in one strain. Mutations at Codon 531 of *rpoB* gene followed by Codon 526 was reported as the location of the most frequent mutations associated with RMP resistance **(Chaves, *et al.*, 2000 and Venkatesh, *et al.*, 2003)**. Mutations in the *katG* gene were identified in the three INH resistance isolates at codon 315 the missense mutation AGC — ACC was found. Studies investigating strains with high level INH resistance have documented that 50 to 100% have mutations located in codon 315 of the *katG* gene **(Musser, *et al.*, 1996 and Hillemann, *et al.*, 2005).** After irradiation, as a result of comparing mutations of *rpoB* and *katG* genes before and after irradiation with respect to their mutations conferring RMP and INH resistance, a marked difference in the distribution of mutations was observed as illustrated in figure 3 and 4. DNA sequence analysis of *rpoB* segments showed that two had more than one mutation, and one had single mutation in the 81-bp RRDR of the *rpoB* gene. One isolate has contained five mutations at codons 510, 522 (had two base pair mutations), 529 and 531. The second one had contained 11 mutations at codons 508, 511, 514, 515, 517, 518,519, 522, 525, 527 and 528 some of these codons had more than one mutation. The third one had one mutation at codon 533. DNA sequence analysis of *katG* gene after radiation revealed that in one isolate there were mutations at codons 311, 320 and 326. The second one there was mutations in codons 319, 322 and 325, While, the third one had double mutations at codons 310 and 321. There is considerable evidence suggesting that nucleic acids are the primary target for cell damage by ionizing radiation. Gamma radiation induced three types of damage in DNA. The base damage is a major component of damage induced by ionizing radiation in prokaryotic systems. Thus irradiation produces damage which can cause mutations leading to change of some or all cell activities **(Scala, 1995 and Pouget *et al.*, 1999)**. The biological effects of radiation occur as a result of discrete changes in the nucleus and molecular structure of the irradiated cells. Chromosomal alterations, resulting from the absorption of ionizing radiations, play a key role in the development of initial molecular lesions to their eventual expression either in the reproductive death of cells and subsequent tissue damage, or permanent hereditary changes in surviving cells that may lead to genetic damage affecting succeeding generations **(Hall *et al.*, 1988)**. Figs (3) &(4) show that, there is evidence of stable DNA lesions produced in cells in response to ionizing radiation which include modifications to thymine, adenine, guanine and cytosine (substitutions), such effect could be attributed to the formation of clustered lesions or multiply damaged sites (MDSs) produced from the gamma ray track near the DNA and which are defined as ≥2 lesions within a distance of 10–15 base pairs (bp) and which may be situated on the same strand or in opposite strands **(Sutherland *et al.*,2000; Nikjoo *et al.*, 2001 and Malyarchuk *et al.*,,2003).** Some authors, **Joiner *et al.*, (1996)** reported that there is no doubt of the existence of stress responses that are upregulated in response to exposure of bacterial cells to small doses of ionizing radiation and other DNA-damaging agents. Changed expression of some genes, only in response to low and not high doses, may occur within a few hours of irradiation. When cells are irradiated, lethal damage can occur, or postirradiation conditions are modified to allow repair. Repair saturation invokes the production by irradiation of a class of DNA damage that is accurately and efficiently repaired at low radiation doses, but as the dose increases the repair process becomes saturated and "damage fixation" begins to complete. Damage fixation is the progression of the damage to a state in which it can no longer be repaired. Misrepair could occur either by error-prone repair system or by repair occurring on a template from which base sequence information has been lost **(Tobias,1985 and Hellman, 2001)**. The evidence of new mutations in this study indicates that mutations could be altered by gamma radiation. The RMP and INH resistance alteration in these isolates may be due either to the presence of a mutation in this gene or to some other mechanism caused by radiation.

Conclusions

With the significant worldwide reservoir of *M. tuberculosis* infection and the threat of MDR-TB, there is a need for novel approaches to this age-old disease. Exploration of the role of low doses of gamma radiation on MDR-TB revealed substantial induced genetic and subsequent phenotypic variability and shifts in antimicrobial resistance towards susceptibility. Mutagenic treatment by ionizing gamma radiation tumor-treating doses in the arsenal of anti-TB therapy require further *in- vivo* investigations.

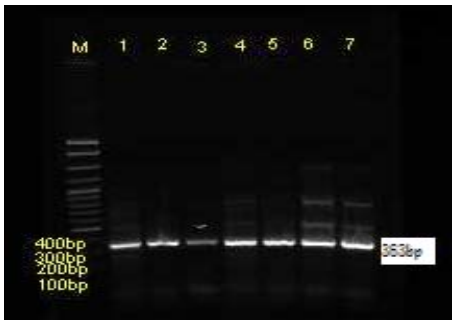

Figure (1): Agarose gel electrophoresis of positive 363 bp PCR products of *rpoB.* M, 1 kbp ladder gene, M, 1

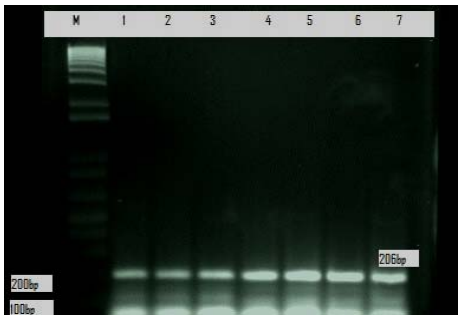

Figure (2): Agarose gel electrophoresis of positive 206bp PCR products of *katG* gene. M, 1 kbp ladder

	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533
1	ACC	AGC	CAG	CTG	AGC	CAA	TTC	ATG	GAC	CAG	AAC	AAC	CCG	CTG	TCG	GGG	TTG	ACC	CAC	AAG	CGC	CGA	CTG	TCG	GCG	CTG
2																			TAC							
3	NCC			GTG			TTT	TTT		CAT	TTT	TAC			TCN			ACT	TAC	TTT	TTT					
4																								TTG		
5			NAG												NCN							CNA		TTN		
6																								TTG		CCG

Figure(3): Location & type of mutations of the *rpoB* gene gene in region of 508-533 before and after radiation. N = not detected
Lane 1: Wild strain *M.tuberculosis H37Rv*, Lane 2 showed the mutation associated with RMP resistant strain at codon 526 before radiation, line 3 showed the mutation of the same strain after radiation, Lane 4 showed the mutation associated with two RMP resistant strain at codon 531 before radiation, Lane 5and 6 showed the mutation of the same strain after radiation. N = not detected

	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326
1	AAG	GAC	GCG	ATC	ACC	AGC	AGC	ATC	GAG	GTC	GTA	TGG	ACG	AAC	ACC	CCG	ACG	AAA
2							ACC											
3			GCN				ACC					TGN					NAA	
4							ACC				GTT		AAN			ATT		
5		GAN					ACC					ANG						

Figure (4): Locations and types of mutations in *katG* gene in region of 309-326 before and after radiation. Lane 1: Wild strain *M.tuberculosis H37Rv*, Lane 2 showed the mutation of the 3 INH resistant strains at codon 315before radiation, Lane 3, 4 and 5 Sites of mutations after radiation. N = not detected

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8/16/2010