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Expression of *dnak*, *groES* and *cps* Genes in Irradiated *Klebsiella pneumoniae* Strains Isolated from UTI Egyptian Patients

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THIS INVESTIGATION presents a brief study for monitoring the correlation between the expression of heat-shock protein (HSP) genes; *dnak*, and *groES* as well as the capsular polysaccharide (*caps*) gene and antimicrobial susceptibility of extensively drug resistant (XDR) *Klebsiella pneumoniae* in response to a low dose of Gamma (γ) radiation. The XDR *K. pneumoniae* strains, that were obtained from (UTI) Egyptian patients were identified using the API 20E strip automated system. Their antimicrobial susceptibilities were monitored using standard disc diffusion method in parallel with their minimum inhibition concentrations (MICs) towards standard antibiotics using a micro-titer method, before and after exposure to γ-radiation at a dose of 24.4 Gray (Gy). The expression of *dnak*, *groES*, and *cps* genes was detected by qRT-PCR under the same conditions. The results demonstrate increasing antibiotic resistance after exposure to γ-radiation. Also, a doubling in the MIC of the tested strains towards CAZ (ceftazidime) was observed. These results were confirmed by an elevation in the expression of dnak, *groES*, and *cps* genes in response to γ-radiation. These preliminary results need more confirmaions, because they assume difficulties in curing immunocompromised patients infected with XDR *K. pneumoniae* by the currently available antibiotics.

Keywords: Pathogenic bacteria, Virulence factor, Antibiotic resistance, Ionizing radiation, Heat-shock protein genes.

Introduction

Klebsiella pneumoniae (*K. pneumoniae)* is a Gram-negative rod shaped bacterium. The whole bacterium surface is covered with capsular polysaccharides (CPS) that are regarded as one of its important components contributing to its virulence factors. The CPS facilitates the bacterial invasion of the host cell (Wu & Li, 2015). In addition, it contributes to pathogenesis by mediating resistance to phagocytosis and killing by the host immune response as well as responding to the action of antibiotics (Cescutti et al., 2016). CPS is expressed *in vivo* and promotes the biofilm formation causing a resistance to antibiotics (Diago-Navarro et al., 2014). *K. pneumoniae* is the most predominant pathogen in urinary tract infections (UTIs) (Guermazi-Toumi et al., 2018), where it is considered the most common human infection with a wide anatomic location from kidney and bladder(upper part) to prostate and urethra (lower part). The treatment of UTIs prolonged from 7days to 12 months; depending on the site of infection, whether the infection is complicated or not, and re-infection or relapse (Najar et al., 2009). An antimicrobial resistance problem will harmfully affect the outcome treatment and has been linked to the high mortality rate in patients suffering from severe infections (Harbarth et al., 2003). Moreover, *K. pneumoniae* is commonly found as normal flora of mouth, skin and intestines as well as in natural environments. Therefore, it is easy to infect the soft tissue of immunocompromised individuals, as a result of; radiotherapy, chemotherapy, surgery, stem cell and bone marrow transplantation or steroids (Khamees et al., 2015).

Gamma rays are ionizing radiations emmitted from a radioactive source,having a high energy, short wavelength and strong transmittance that has been used in many processes as medical treatment (Shathele, 2009). The timing and dose of radiotherapy depend on the type of cancer being

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treated and the aim of treatment whether cure or palliation (Lutz et al., 2007). Gamma-rays damage the DNA directly or by ionizing water molecules. In solutions, the ionizing radiation forms three different free radicals, through the radiolysis of water; OH radicals (•OH), the solvated electron (e^-_{eq}), and the H-atom (•H) (Sonntag, 1978). These free radicals can destroy proteins and reactive oxygen species (Lee et al., 2001) . Also, they induce DNA single or double stranded breaks (Sonntag, 1978). Consequently, this stimulates the bacterial cell to produce the major HSPs; Dnak, and GroES, that play a crucial role to overcome and repair these damages by preventing protein denaturation, aggregation, and to reactivate partially denatured proteins (Maleki et al., 2016). These HSPs were translated from mRNA *dnak* and *groES* genes (Cardoso et al., 2010). In agreement with Laport et al. (2006), who noted that not only changes in temperature trigger the HSPs expression in bacteria. Caillet et al. (2008) noticed a significant induction of Dnak and GroES proteins in *E. coli* in response to γ-radiation at dose 0.4-1.3kGy.

To our knowledge, limited studies have been carried out to evaluate the significance of γ-irradiation on HSPs expression genes encoding HSPs in *K. pneumoniae* strains and their virulence. Therefore, the objective of this study is conducted to monitor the effect of low dose of γ-radiation at 24.4(Gy), which is biologically equivalent to in-vitro fractionated multiple therapeutic dose of some cancer patients (Barton, 1995), on the expression of HSP genes, *dnak* and *groES* as HSP genes and *cps* as the most important virulence factor of *K. pnumoniae* strains, in parallel with reporting the change in their antibiotic resistance.

Materials and Methods

Urine collection

Early morning midstream urine samples (about 100ml) were collected from patients suffering from UTIs at the microbiology laboratory at Ain-Shams University Hospital, Egypt. The samples were collected in sterile tightly locked containers.

Isolation and identification

The collected urine samples were cultured separately on MacConkey agar (Oxoid, England). Twenty five isolates were identified as *Klebsiella sp.* on the basis of their typical colonial appearance (viscous/mucoid characteristic colonies), Gram stain to determine their microscopic shape and arrangement. The non-motile, Gram-negative, lactose fermenter, encapsulated, non-spore forming rode shape bacterium that tested catalase positive and oxidase negative underwent a further identification using API 20E strip automated system (BioMe´rieux, France), as shown in (Fig. 1). The culture was maintained at 4°C on nutrient agar slants.

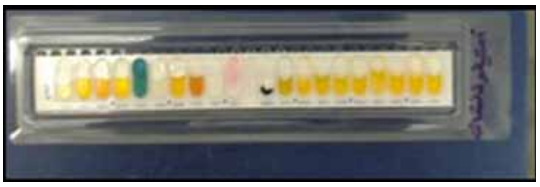

Fig. 1. Identification of *Klebsiella pneumoniae* by API20E.

Antimicrobial susceptibility

All identified *K. pneumoniae* strains were tested for their susceptibilities to antibiotics by standard disk diffusion susceptibility method (Wayne, 2017). The standard antibiotic disks (Oxoid, England) were chosen as they are commonly used in UTI treatment in local hospitals and recommended worldwide (Alabsi et al., 2014 and Arslan et al., 2005). They included the following antibiotic groups; amoxycillin-clavulanic acid (AMC, 20/10µg), amikacin (AK 30µg), gentamicin (CN, 10µg), aztreonam (ATM, 30µg), ceftazidime (CAZ, 30µg), colistin (CT, 10µg), imipenem (IMP, 10µg) and trimethoprim-sulfamethoxazole (SXT, 1.25/23.75µg). Pure colonies (3-5) were re-cultured in Luria-Bertani (LB) broth for 3h at 37°C and adjusted to 0.5 McFarland standard. Then, they swabbed on the top of the solidified Mueller-Hinton (MH) agar plate and allowed to dry for 10 min. Then, antibiotic disks were placed individually on the surface of the medium and left for 30 min. at room temperature. The plates were incubated at 32±2°C for 18-24h. Zones of inhibition were recorded in mm and compared with a standardized chart supplied by (Oxoid-England). The test was repeated in triplicate (the results not shown).

Minimum inhibitory concentration (MIC)

The MIC is defined as the lowest concentration of the tested antibiotic that inhibited 90% of bacterial growth. The XDR *K. pneumoniae* strains (three) were slected to estimate the MIC of ceftazidime (CAZ) [Fortum®1g-GlaxosmithKline, kindly provided by The National Organization for Drug Control and Research (NODCR)-Egypt]. Fortum (ceftazidime) was selected according to

CLSI-2007 guidelines because it is inexpensive and a readily available antibiotic, also, it is routinely used in Egyptian hospitals.The 96-well micro-titre assay using resazurin as an indicator of cell growth was employed (Sarker et al., 2007). Under aseptic conditions; the CAZ powder was dissolved with sterile distilled water, a volume of 100 µl of antibiotic dilution (1000µg/ml) was pipetted into the first row of the plate. To all other wells, 50µl of normal saline was added. Serial dilutions were performed. Each well had 50µl of the tested antibiotic in serially descending concentrations (from 1000 to 0.24µg/ml). To each well, 10µl of the resazurin indicator solution was added. Finally, 10µl of the selected bacterial suspension prepared as in (2.5 method) and was added to each well. The plate had a set of controls: a column with all solutions with the exception of the tested antibiotic, another column with all solutions with the exception of the bacterial suspension adding 10µl of nutrient broth instead. The plates were prepared in triplicate and placed in an incubator set at 32°C for 18–24h. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive indications. MIC was determined as the lowest antibiotic concentration at which color change occurred. The average of the three values was calculated and was the MIC for CAZ and *K. pneumoniae* isolates.

Irradiation process

Three different bottles, each one containing 100ml of tryptone soya broth (TSB) inoculated with pure colonies; K_1, K_2, and K_3 respectively, then they were incubated for 3h at 32± 2°C, the turbidity was adjusted to 0.5 McFarland. Each bottle was divided under sterile conditions, into another two sterile and autoclaved bottles. The 1st bottle was used as a control (non irradiated). While, the second one was irradiated in Gamma cell 40 irradiator occupied by Cesium 137 (^{137}Cs), Atomic Energy of Canada Limited, Commercial products located at The National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt. The radiation dose rate was 0.688 rad/sec at the time of the experiments. The 1st and 2nd bottles for each isolate were used for different investigations.

Primer design

The complete genomes of different *Klebsiella pneumoniae* strains were downloaded from the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov/nucleotide/). The design of *cps* primers was based on finding a short length of conserved sequence in multiple different *K. pneumoniae* strain′s genome. Multiple sequence alignment was performed by CLUSTAL W Multiple Sequence Alignment Program (Larkin et al., 2007). Primer design was performed using Primer 3 (version 0.4.0) and *in Silico* PCR software. 16S rRNA was used as reference gene (housekeeping gene) **(**Christensen et al., 2004 and Lim et al., 2007). All primers were purchased from (Jena Bioscience, Germany) with the sequences shown in (Table 1).

TABLE 1. Primer sequences, expected product length for amplification of 16S rRNA, *dnak*, *groES*, and *cps* genes in *Klebsiella pneumoniae* strains.

Gene	Primer sequence 5' - 3'	NCBI accession No./ Template	Amplicon loci	Amplicon size (bp)
16S rRNA*	Fd AGTCATGAATCACAAAGTGGTAAGCG Rv AGGGCTACACACGTGCTACAATG	UNIVERSAL	NA	267 bp
dnak	Fd TGGTGGTCAGACTCGTATGCCGAT Rv ATCGCCACGGCTTCATCCGGGTTA	CP025088.1 Klebsiella pneumoniae strain KP7 chromosome	548505-548603	99 bp
groES	Fd TCGTCCGTTACATGATCGTGTG Rv TGCAGAACCGGTCAGAACGA	CP025088.1 Klebsiella pneumoniae strain KP7 chromosome	148398-148482	85 bp
cps	Fd AGAGTGGAATCTGCGTGCAA Rv AGGCTCGCTTGGGTTTCTG	LT174552.1 Klebsiella pneumoniae capsular polysaccharide biosynthesis gene cluster, type: K39	12161- 12486	327 bp

bp= base pair * House keeping gene
All these primers were designed at this study

Extraction of RNA and cDNA preparation

Immediately, after irradiation, one and half ml of $1x10^9$ of each irradiated K_1, K_2, and K_3 was transferred alone to a microcentrifuge tube, centrifuged for 2min at (12,000x**g**) and 4°C. Then, each pellet was washed twice using the cooling isotonic solution for preparing RNA extraction. Then, RNA extraction was carried out using GeneJET RNA purification kit (Fermentus, Thermo Fisher Scientific Inc, UK) according to manufacturer′s instructions. A 1µl sample of purified RNA was read on the nanodrop 2000 spectrophotometer (Thermo Scientific, USA) at OD 260\280nm gave 1.8- 2 (Boom et al., 1990). Then, they were adjusted to the same concentration, and same quantities were used for synthesis of cDNA according to the instructions of high capacity RNA-to-cDNA Kit (Fermentus, Thermo Fisher Scientific Inc, UK).

Quantitative real-time PCR (qRT-PCR)

Quantitative Real-time PCR was used as a sensitive method for quantification of the gene expression. qRT-PCR amplification for *dnak*, *groES,* and *cps* cDNA in each isolates of *K. pneumoniae* before (as control) and after exposure to low dose γ-radiation (24.4Gy) was accomplished in (a Rotor-Gene 2000 real-time fluorescence thermal cycler, (Corbett Ltd., Australia). The reaction was prepared in 25µl containing 1X of GoTaq® Master Mix (Promega, USA), 10pmol of primer and 1µl of earlier reverse transcription reaction prepared cDNA product. In addition to, theDyNAmo™ Syber green fluorescent qPCR Kits (Finnzymes Oy, Finland) was used to quantify the progression of amplification. The programs for amplifications are,1 cycle 95°C for 5min for pre-denaturation. fEach one of the forty cycles consists of 95°C for 40sec, 60°C of annealing for 40sec and 72°C of extension for 20sec. The PCR conditions were optimized to produce the least non-specific signals by primer dimers, as evaluated by earlier amplification melting curve analysis. Each cDNA fragment was amplified in triplicate for each gene as well as 16S rRNA.

The specificity of the amplicons was verified by building the melting curves for the PCR products. It is very important for each gene to detect the number of cycles required for the fluorescent signals to cope with the background level; called the threshold cycle (C_t) and it was detected automatically. Then, we calculated $2^{-\Delta\Delta C}{}_t$ to obtain the change in the gene expression by normalizing them by housekeeping gene and relative to control gene, before exposure to γ-radiaton (Livak & Schmittgen, 2000).

Statistical analysis

Three replicates were performed for each experiment. Then, the data were expressed as (Mean ± Standard deviation) and paired t-test for testing antibiotics before and after irradiation using Sigma plot 10.0. Differences were considered statistically significant at ($P \leq 0.05$). $2^{-\Delta\Delta Ct}$ method was used to obtain the change in the gene expression by normalizing them by housekeeping gene and relative to control gene (Livak & Schmittgen 2000).

Results and Discussion

K. pneumoniae causes about 14-20% of severe infections leading to complications with high morbidity and mortality rates, up to 40% in some hospitals (Lepper et al., 2003). In addition, they are the second most predominant pathogen that causes UTIs with long treatment period (Najar et al., 2009), especially in immunocompromised individuals (Wu & Li, 2015).

In the current study, twenty five clinical isolates of *K. pneumoniae* were isolated from UTIs by the microbiology laboratory at Ain-Shams University Hospital, Egypt and identified by API 20E strip. The antibiotic resistance profile of all isolates were tested against eight antibiotics with different mode of actions (Table 2). Out of them, three isolates (K_1, K_2, and K_3) were selected for further studies as they showed a high resistance towards the tested antibiotics, to evaluate the effect of 24.4Gy (accumulative dose of γ-radiation that use in radiotherapy) on their antibiotic sensitivity and gene expression of *dnak*, *groES,* and *cps* .

The results in (Fig. 2) indicated that their resistance was significantly increased with P-value<0.05 after exposure to gamma radiation dose. This result was additionally confirmed by an increase in the MIC of K_2, and K_3 by double fold for ceftazidime, from 16 to ~32µg/ml for both strain (Fig. 3). This is may be due to the overexpression of proteins after expoure to γ-radiation. The results of the present study agreed with Mortazavi et al. (2017) who reported that there were significant reductions in bacterial susceptibility to antibiotics especially in *K. pneumoniae* after exposure to γ-radiation with with inhibition zone 20.3mm of and 14.7mm, respectively.

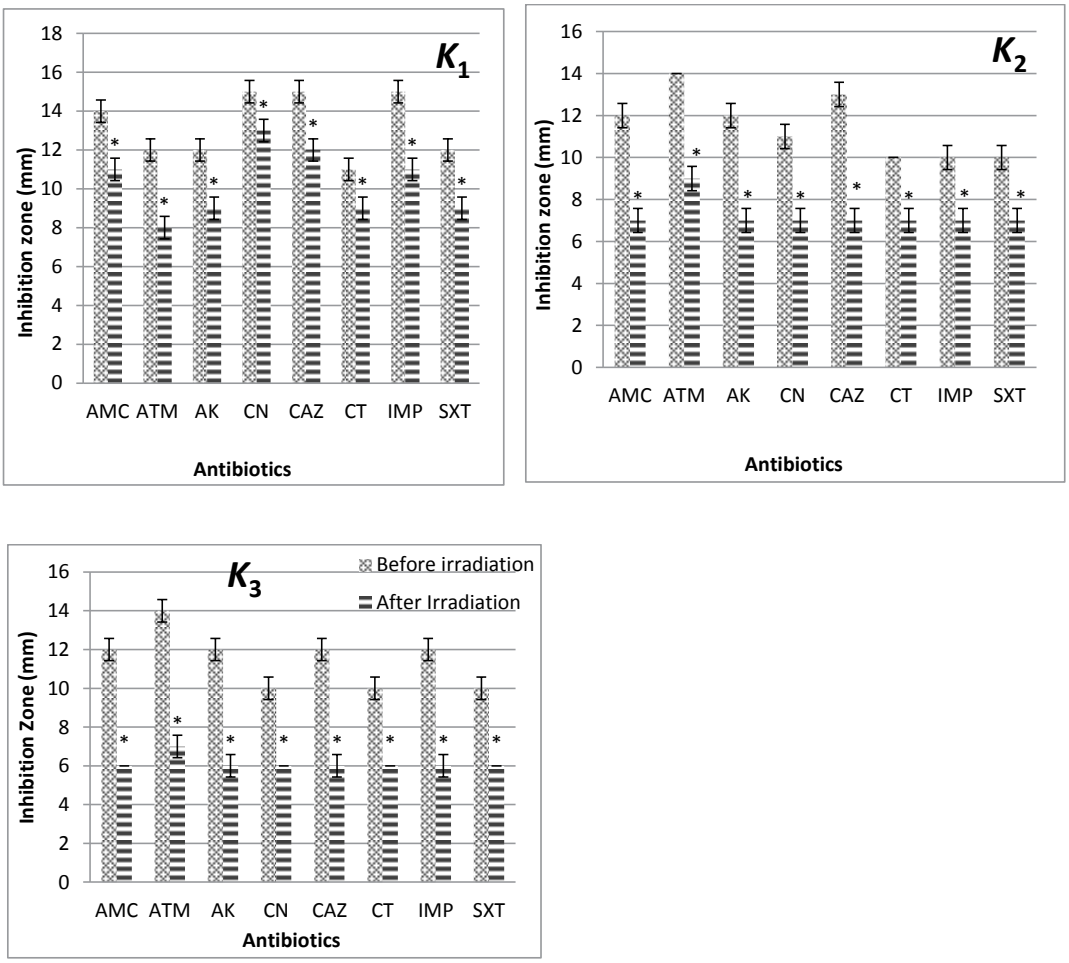

Fig. 2. Antimicrobial susceptibility of K_1, K_2, and K_3 before and after irradiation at dose 24.4Gy. AMC: Amoxycillin-Clavulanic acid (20/10µg), ATM: Aztreonam (30µg), AK: Amikacin, CN: Gentamicin (10µg), CAZ: Ceftazidime (30µg), CT: Colistin(10µg), IMP: Imipenem (10µg), SXT: Trimethoprim-Sulfamethoxazole (1.25/23.75µg) (* significant P value≤ 0.05, Error bars represent standard deviation).

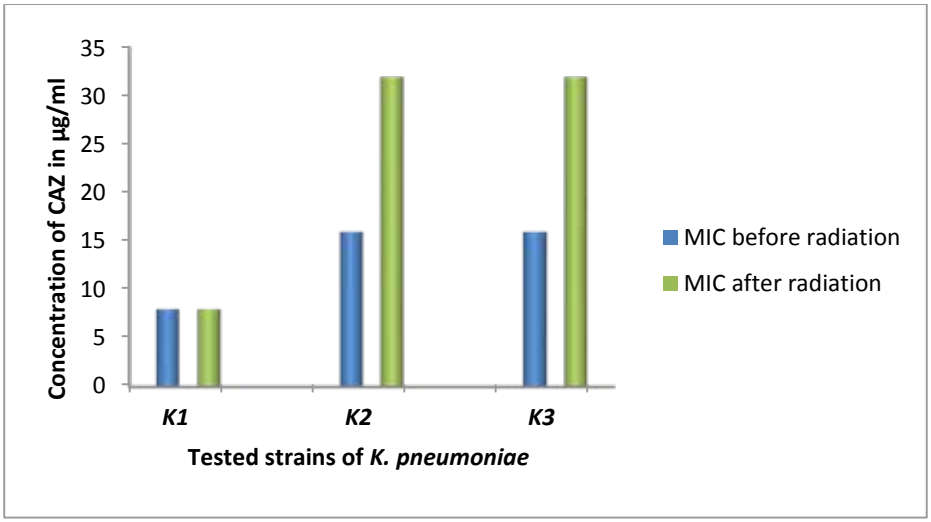

Fig. 3. Minimum inhibitory concentration of ceftazidime (CAZ) for the XRD strains of *K. pneumoniae* (K_1, K_2, and K_3) before and after exposure to 24.4Gy.

Quantitative RT-PCR was performed as a sensitive method to quantify the gene expression of *dnak* and *groES* that quickly translated to Dnak and GroES proteins as reported by Vanghele et al. (2016). The transcriptional changes in those genes were procured from the threshold cycles (C_t) which are obtained from the qRT-PCR performance as shown in (Table 3). The results of the present study revealed that the highest gene expression was shown by *dnak* in K_1, K_2, and K_3. ,this is in agreement with Calini et al. (2003), who reported that Dnak was highly induced in response to gamma radiation as a radioprotective mechanism towards the first event of DNA damage and increases long-term viability. A similar profile of *dnak*, *groES, and cps* genes expression in irradiated K_1, K_2, and K_3 (Fig. 4) suggests the presence of a common mechanism tracked by the *K. pneumoniae* to overcome and repair the protein damages in response to the external stress of γ-radiation and this perhaps trigger on or act simultaneously with the virulence and antibiotic resistance machinery to maintain the cell survival.

In contrast to the present results, Yamaguchi et al. (2003) demonstrated that the *dnak* genes have an influence on the antimicrobial activity of the fluoroquinolone in *E. coli* and mutations in them increased the bacterial susceptibility to levofloxacin. Furthermore, Singh et al. (2007) noticed, in *Staphylococcus aureus* methicillin-resistant strain, a mutation in the *dnaK* gene which increased their susceptibility to oxacillin and methicillin.

As for the *cps,* the main virulence gene in *K. pneumoniae*, the results in Table 2 revealed up-regulation by 1.5, 2.5 and ~2 fold in irradiated K_1, K_2, *and* K_3 ,respectively. Gomide et al. (2018) reported that in many pathogens, virulence factors are under control of transcriptional activators, any stress leading to the expression of virulence genes at 37 °C as reported in *L. monocytogens*. Also, the present results are in agreement with Pallen (1989) who noticed that the oxidative stress on *Vibrio cholera* was switched on its virulence genes and increases the production of Dnak and GroE proteins. However, the present results are in contrast with Lim et al. (2007) who noticed 3- to 1000-fold reduction in the expression of virulence gene levels located within the pathogenicity island on the chromosome of *Salmonella typhimuriumand Vibrio* toxin genes of some *Vibrio spp.* as food borne pathogens after exposing to 0.5 and 1kGy of γ-radiation. This conflict with the present results may be due to the diffrence in the type of organism or the γ-radiation dose.

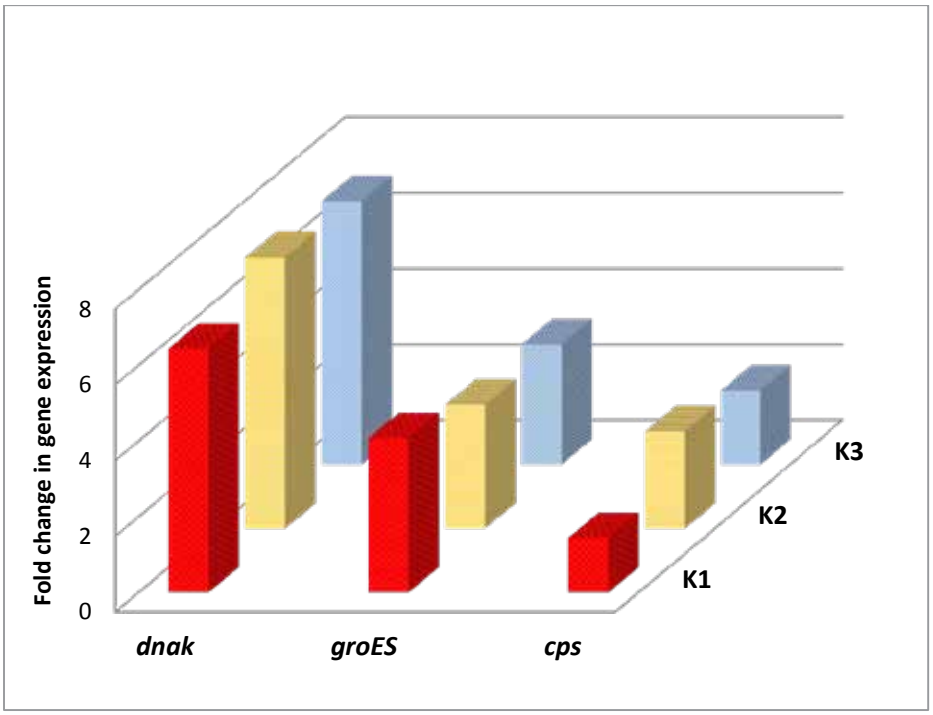

Fig. 4. Response of *dnak*, *groES*, and *cps* genes in *K. pneumoniae* to 24.4Gy dose of irradiation.

TABLE 2. Gene expression of *dnak*, *groE*, and cps mesured by RT-PCR in XRD *K. pneumoniae* after exposure to 24.4Gy dose of γ-irradiation.

Strain	Gene	Cycle threshold (C_t) *		$2^{-\Delta\Delta C_t}$ **
		Before irradiation	After irradiation	
K_1	*dnak*	30.96±1.04	28.27±1.4	6.45
	groES	32.29±1.87	30.26±0.69	4.08
	cps	31.46± 0.25	30.92±0.92	1.45
K_2	*dnak*	33.03±0.45	30.19±1.0	7.16
	groES	31.4±1.58	29.22±0.75	4.53
	cps	30.75±0.8	29.39±0.38	2.57
K_3	*dnak*	31.22±2.144	28.42±0.42	6.96
	groES	32.29±2.0	31.26.±0.36	2.04
	cps	30.36±1.34	29.39±1.18	1.96

*C_t values are the means±SD of RT-PCR analysis performed three replicates.
** Calculated by method of (Livak & Schmittgen, 2000).

Conclusion

This study is limited, just to spotlight on the fact thata low dose of γ-radiation may cause an increase in the expression of the *HSP* genes as *dnak*, and *groES* which trigger other virulence genes in *K. pneumoniae* as *cps* gene, which contribute to increasing their resistance towards the normal used antibiotics. It is possible that this may pose a therapeutic problem in immunocompromised persons, such as cancer patients receiving γ-radiation therapy. Further studies are required to confirm the expression of these genes in infected cancer patients before and after receiving their radiotherapy sessions.

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التعبيرالجيني *dnak*, *groES* and *cps* لسلالات كليبسيلا نيمونيا المشععه والمعزوله من مرضى إلتهاب المسالك البوليه المصريين

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يدرس هذا البحث بإختصار العلاقه بين جينات الصدمه الحراريه *dnak* , *groES* مع جين الكبسوله عديدة التسكر *cps* في عزلات كليبسيلا نيمونيا قبل وبعد تعرضها لجرعه إشعاعيه تكافيء الجرعه العلاجيه المجمعه التي يحصل عليها بعض مرضى السرطان عند علاجهم بالإشعاع.

حيث أن العوامل الخارجيه التي تؤثرعلى البكتيريا بالسلب تدفع جينات الصدمة الحراريه للأرتفاع وذلك لحماية الخليه من التلف وبالتالي تؤثر بالتبعيه على العديد من الجينات أما بالأرتفاع أو الأنخفاض أو حثها على التوقف. يعد جين الكبسوله عديده التسكر *cps* من أهم الجينات التي تزيد من ضراوة بكتيريا كليبسيلا نيومونيا ومقاومتها للمضادات الحيويه, وبالتالي تزيد من قدرتها على إحداث المرض وأستدامته مما يعرض حياة المريض لخطر عدم تأثير أي نوع من المضادات الحيويه المستخدمه على هذا النوع من البكتيريا الممرضه. ولهذا الغرض تم عزل البكتيريا من بول مرضى مصريين مصابين بإلتهاب المسالك البوليه وتم تعريف سلالات كليبسيلا نيومونيا باستخدام شرائط API 20E. ومن ثم تم اختيار ثلاث عزلات K_1, K_2 K_3 أظهرت مقاومة لمضادات حيويه من مجموعات مختلفه. تم تعريضهم لجرعة تشعيع جامي 24.4 جرآي و دراسة أقل جرعة تثبيط من مضاد السيفتازيديم (CAZ) والتي تعمل على قتل 90% من كليبسيلا و دراسة التعبير الجيني *dnak*, *groES*, *cps* باستخدام RT-PCR. وجد انه بتعريضها لجرعه 24.4 جراي يزداد التعبير الجيني لكلا من هذه الجينات بشكل ملحوظ بالمقارنه بالعزلات التي لم تتعرض للتشعيع وهذا أدى لزيادة مقاومتها للمضادات الحيويه وزيادة الكميه من السيفتازيديم التي تستطيع القضاء على 90% من البكتيريا بعد التشعيع. وهذا يثبت أن تعرض مريض السرطان لمثل هذه الجرعات من التشعيع الجامي وهو مصاب بعدوى بكتيرية عديدة المقاومه ستؤدي إلى زيادة ضراوة وشراسة البكتيريا الممرضه المصاب بها هذا المريض مما يصعب معه علاج مثل هذه البكتيريا بأي من المضادات الحيويه المعروفة والمتداولة.