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Effects of Wi-Fi Radiofrequency Radiation on Carbapenem-Resistant *Klebsiella pneumoniae*

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The hazardous consequences of electromagnetic field (EMF) exposure represent a public health concern. Common sources of EMF include smartphones and wireless fidelity (Wi-Fi). The aim of our study is to assess whether exposure to Wi-Fi radiofrequency radiation influences the pathogenic traits of carbapenem-resistant *Klebsiella pneumoniae*. The susceptibility to antibiotics was evaluated by the determination of minimum inhibitory concentrations (MIC). In this study, *K. pneumoniae* showed a non-linear response to treatments with Colistin and Gentamycin following different Wi-Fi exposure periods. Transmission electron microscopy revealed morphological changes in the bacterial cell membrane within 24 h of Wi-Fi exposure. Crystal violet quantification and quantitative real-time polymerase chain reaction showed that the ability to form biofilms was greater in Wi-Fi exposed *K. pnemoniae* when compared to control. Moreover, higher levels of *bcsA*, *mrkA*, and *luxS* messenger RNAs were observed. Our data suggest that Wi-Fi exposure can influence bacteria in a stressful way, leading to an alteration in their antibiotic susceptibility, morphological changes, and cumulative biofilm formation. Bioelectromagnetics. 2021;42:575–582.
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Keywords: **electromagnetic fields; wireless fidelity; biofilm; transmission electron microscopy; *Klebsiella pneumonia***

INTRODUCTION

Several opportunistic bacterial pathogens with multidrug resistance were identified in the area of telecommunication stations [Adebayo et al., 2015]. Research in this area seems to be of big interest and mandatory in order to improve the measures of protection assessment against the occurrence of antibiotic-resistant organisms in the environment. In compliance with the one health concept, human and animal health depend on the ecosystems where they live since they can be influenced by exposure to several stress sources and current lifestyle conditions [Destoumieux-Garzón et al., 2018]. Animals, plants, microorganisms, and mainly humans are exposed daily to polluting electromagnetic fields (EMF) such as wireless fidelity (Wi-Fi) and smartphones [Redlarski et al., 2015]. Wi-Fi radiofrequency waves, non-ionizing radiation operating at a frequency of 2.4 gigahertz (GHz), are widely used for domestic internet

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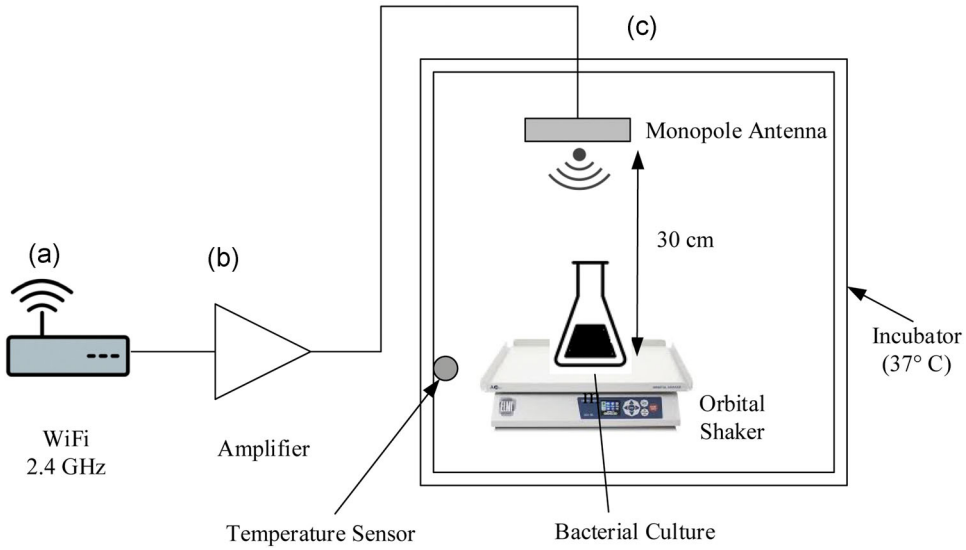


Fig. 1. Radiofrequency exposure system. (**a**) Wi-Fi router, (**b**) amplifier, (**c**) mounted incubator with patch antenna. Adapted from Said-Salman et al. [2019b].

access in homes, businesses, and at public places worldwide. These waves may influence living organisms including bacteria through non-thermal or thermal effects [Salmen, 2016]. It is important to investigate the effect that non-ionizing radiation may exert on microorganisms' virulence especially with the emergence of multidrug-resistant bacteria. The present study aimed to study the non-thermal effects of Wi-Fi radiofrequency radiation on different pathogenic characters of *Klebsiella pneumoniae*.

K. pneumonia is a rod-shaped Gram-negative opportunistic pathogenic bacterium causing nosocomial infections. *K. pneumonia* has emerged in recent years with enhanced virulence. *K. pneumoniae* carbapenemase (KPC) is the main enzymatic resistance mechanism used by *K. pneumoniae* against antibiotics [Wang et al., 2011]. Carbapenem-resistant *K. pneumoniae* carries the blaKPC gene which confers resistance to carbapenem antibiotics. Bacterial virulence factors such as antimicrobial resistance (AMR) and biofilm formation play key roles in infection settlement. The World Health Organization (WHO) stated that AMR is a "global health crisis" [WHO, 2018]. Moreover, as a response to environmental stress, bacteria can aggregate to produce biofilms through the formation of a polysaccharide matrix [Hall-Stoodley et al., 2004].

In this study, we examined the morphological changes, antibiotic susceptibility, and biofilm formation by *K. pneumoniae* after Wi-Fi exposure. Furthermore, the expression of representative genes implicated in biofilm formation and quorum sensing (*bcsA, mrkA*, and *luxS*) were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR).

MATERIALS AND METHODS

Radiofrequency Exposure System

An extended range wireless router (TL-WR524G-Tp-Link, Shenzhen, China) operating at 2.4 GHz frequency was used to generate Wi-Fi radiofrequency radiation. The system was linked to a 2.4 GHz signal booster LJ-8005 (J-Link, Shenzhen, China) and a manufactured patch antenna. The antenna was positioned in an incubator, 30 cm distant from the bacterial culture, and the temperature was maintained at 36 ± 1 °C using a temperature sensor [Said-Salman et al., 2019b] (Fig. 1). A tuned microwave absorber (lossy material) was placed within the whole cavity of the shielded incubator in order to neutralize any reflected field and to create a situation similar to the free space propagation field inside the anechoic incubator box. We measured a constant field of 6 V/m at the position of the exposed sample corresponding to a power flux density of 0.05 W/m^2. The influence of the exposure to Wi-Fi waves was investigated, and the protocol consisted of continuous radiation expositions for different periods (1, 5, 10, 20, 24, and 48 h). A control measurement was done for each experimental condition in a Faraday bag at an identical temperature to avoid any external radiation.

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Bacterial Strain

The *K. pneumoniae* carbapenem-resistant strain ATCC BAA-1705 (Microbiologics, Saint Cloud, MN) used throughout this study was generously supplied by the Faculty of Health, Lebanese University (LU), Lebanon.

Transmission Electron Microscopy (TEM)

K. pneumoniae ATCC BAA-1705 culture in Luria-Bertani (LB) broth (Fluka, Buchs Switzerland), exposed to Wi-Fi waves for 24 h under shaking at 37°C, was examined by TEM for any possible morphological changes between the control cells and exposed cells. Bacterial cells were then fixed using glutaraldehyde universal fixer for electron microscope. Electron micrographs were captured using TEM (JEM-1400 Plus, Transmission Electron Microscope; JOEL, Tokyo, Japan).

Determination of the Minimum Inhibitory Concentration (MIC)

Müller Hinton agar medium (Oxoid, Hampshire, UK) plates were inoculated with standard inocula of 0.5 McFarland of *K. pneumoniae* cultures exposed to Wi-Fi waves during different time periods (1, 5, 10, and 20 h). The MIC strip was then carefully placed and smoothed onto the agar surface. The plates were incubated at 37 °C in an inverted position for 24 h. The MIC value was determined following the manufacturer's indications. The antimicrobial MIC strips used were Colistin (CS) and Gentamycin (GN) 0.016-256 2 µg/ml (Liofilchem, Via Scozia, Italy).

Measurement of Biofilm Formation Using Crystal Violet Assay

Overnight pre-culture was subjected to 1/100 dilution in tryptic soy broth (TSB) (Oxoid) to make a 0.5 McFarland solution corresponding to an optical density (OD) of approximately 0.1 at 595 nm. Colony counting was verified to obtain nearly 10^8 CFU/ml. Standard inocula were added into flat-bottom 96-well plates and grown for 24 and 48 h in an incubator at 37 °C with or without exposure to Wi-Fi radiation. Biofilms were washed with phosphate-buffered saline (PBS) and were stained using crystal violet. The quantification of biofilm biomass was assessed as previously reported with slight modifications [Peeters et al., 2008; Kwasny and Opperman, 2010]. Stained biofilms were air-dried and the staining fixed on the adherent bacteria was solubilized with acetic acid (33%). The OD of each solubilized well was measured at 595 nm wavelength. Pure TSB medium was used as blank. The data were presented as an index of biofilm formation according to the following equation:

$$\text{Index of biofilm formation} = (\text{OD sample-OD background})/(\text{OD blank-OD background})$$

[Fijałkowski et al., 2013].

RNA Extraction

K. pneumoniae culture in LB medium was left overnight (shaking at 200 rpm) at 37 °C under Wi-Fi waves. RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) was added to bacterial culture in a ratio of 1 V/2 V where 1 ml of reagent was mixed with 2 ml of bacterial culture. Bacterial cells were then collected by centrifugation at 4000 rpm for 10 min. RNeasy MINI kit (Qiagen) was used, as described by the manufacturer, to extract the total RNA content from the control and exposed samples. On-column RNase-Free DNase kit (Qiagen) was used to remove any residual genomic DNA. The concentration of the extracted RNA was determined using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA).

qRT-PCR

Complementary DNA (cDNA) was prepared from 1 µg RNA using the FIRE Script RT cDNA synthesis kit (Solis Biodyne, Tartu, Estonia). The primers used for qRT-PCR were purchased from Macrogen (Seoul, South Korea). *K. pneumoniae* genomic DNA was used as a template to generate amplicons with estimated sizes of 100–150 nt using NCBI primer-Blast software (online software tool;

TABLE 1. Primers for the Selected Genes

Gene name	Forward primer	Reverse primer	Amplicon size	Annealing temperature (°C)
bcsA	TGCCAAAGGGGAGTTCGTTT	TGCATCATCGCCAGCTCTTT	111	60
mrkA	AGGCTGCTGATGGCACTAAA	TAGCCCTGTTGTTTGCTGGT	100	60
luxS	TGAGCCTGATTGGTACGCC	GCGAGTGCATGGTGTAAGTC	141	60
gyrA[a]	TACCGAAATCACCGCCAACA	ATTTGCCTTTACCGCCACGA	149	60

[a]Housekeeping gene.

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NCBI, Bethesda, MD) (Table 1). QRT-PCR was achieved by QIAGEN's real-time PCR cycler (Rotor-Gene; Qiagen). The reaction (10 μl) contained: QuantiTect SYBR Green PCR Master Mix (Qiagen) (5 μl), primers (0.5 μM), and cDNA 1/10 (1 μl). The performed qRT-PCR program was as follows: denaturation for 5 min at 95 °C; 45 cycles (denaturation for 10 s at 95 °C, 30 s at annealing temperature, and extension for 20 s at 72 °C). Direct PCR REDTaq ReadyMix (Sigma-Aldrich, St. Louis, MO), with temperature gradient, was used to determine the optimum annealing temperature. The studied genes were *bcsA*, *mrkA*, and *luxS*. The *gyrA* gene was used as a housekeeping gene. Three technical replicates for each condition (control and exposed) were done. In addition, a no template control (NTC) sample was used for contamination control purposes.

Data Analysis of qRT-PCR

Relative gene expression was evaluated by REST 2009 software (online software tool; Technical University Munich and Qiagen in Germany) taking into consideration the housekeeping gene standardization, and the reaction efficiency adopting the Pfaffl method [Pfaffl, 2001; Pfaffl et al., 2002]. Data standardization was done based on the reference gene (*DNA gyrase subunit A, gyrA*).

Statistical Analysis

The results of this study were displayed as means ± standard deviation from at least three replications. The statistical analysis was done using Student's *t* test taking into consideration the statistical significance ($P < 0.05$) between the exposed and control samples.

RESULTS

Effect of Wi-Fi Waves on Morphological Changes

The morphological changes of *K. pneumoniae* ATCC BAA-1705, after 24 h exposure to Wi-Fi waves, were inspected using TEM. The morphology of the negative control culture was also inspected under similar experimental conditions (Fig. 2).

The control cells appeared as rod-shaped forms with a uniform cell wall and intact cytoplasmic contents (Fig. 2a). The TEM analysis showed that, when exposed to Wi-Fi waves, *K. pneumoniae* exhibited several morphological alterations such as structural disruption of the cytoplasmic membrane and disintegration of protoplasm. Ruptured cells were still apparent, with a leak of cytoplasmic content, forming cell debris (Fig. 2b).

Effect of Wi-Fi Waves on MIC

K. pneumoniae ATCC BAA-1705 carries the *blaKPC* gene which confers resistance to carbapenem antibiotics. MIC values were used as susceptibility indicators for *K. pneumoniae ATCC BAA-1705*. The MIC of two commonly used antibiotics (Gentamycine and Colistin) were evaluated following different exposure periods to Wi-Fi waves (1, 5, 10, and 20 h) (Fig. 3). It was observed that the bacterial sensitivity to these two antibiotics was not the same in the control and the pre-exposed samples. As early as 5 h from the beginning of the Wi-Fi exposure, we noticed an increase in the sensitivity of *K. pneumoniae* to the action of Colistin and Gentamycin. The results showed an increase of *K. pneumoniae* sensitivity to Colistin and Gentamycin (at 2 and 1 μg/ml, respectively) compared to the control samples (3 and 1.5 μg/ml, respectively). Then, this bacteria regained its initial antibiotic resistance profile (3 and 1.5 μg/ml, respectively) after 10–20 h of exposure.

Effect of Wi-Fi Waves on Biofilm Formation

The effect of Wi-Fi radiation on the capacity of *K. pneumoniae* to make biofilm was performed by the crystal violet quantification assay. The measured OD values are representative of the amount of crystal violet dye fixed by the formed biofilm.

Figure 4 shows the bacterial biofilm formation index. A significant increase of the formed biofilms (1.3- and 2.1-fold change), compared to the control samples, was observed following 24 and 48 h Wi-Fi exposure, respectively.

Quantitative Real-Time PCR

Representative genes implicated in biofilm formation and quorum sensing in *K. pneumoniae* were studied in order to assess the relative gene expression profile difference between exposed and non-exposed bacteria. These genes included *bcsA, mrkA, and luxS* (Table 2).

The qRT-PCR results of *K. pneumoniae* representative genes were evaluated using REST 2009 software. G*yrA* gene was used as a reference for gene normalization. Our results showed a significant 1- to 1.8-fold upregulation ($\log_2$) of the selected genes upon exposure to Wi-Fi waves; $P < 0.05$ (Fig. 5).

DISCUSSION

Several studies reported that EMF may exert different effects on bacterial behavior. These effects depend on both physical parameters such as signal frequency characteristics and biological parameters including the organism model. Non-thermal effects of

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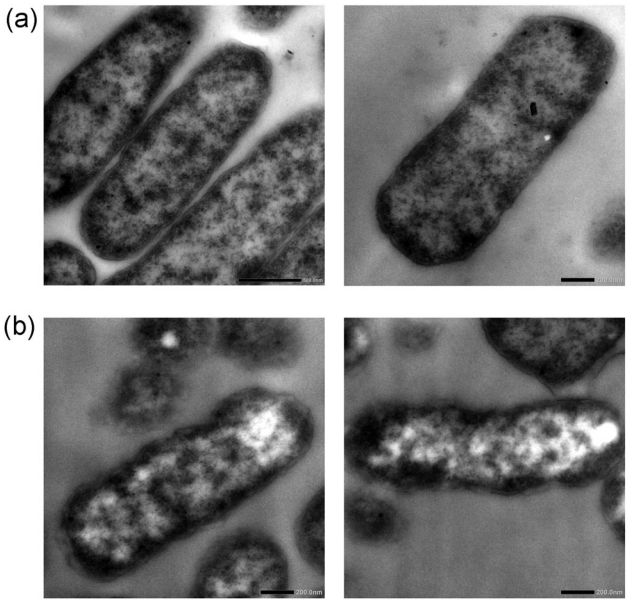


Fig. 2. Electron micrographs of control and 2.4 GHz exposed K. pneumonia. (**a**) Unexposed control bacterial cells showing preserved morphology, intact cytoplasmic membrane, and cytoplasmic contents. (**b**) Cells exposed to 24 h of Wi-Fi radiofrequency radiation showing morphological changes, irregularities in the cell wall with disintegration in cytoplasmic membrane and protoplasm.

radiofrequency have been reported to affect several bacterial strains in many ways (growth rate, antibiotic susceptibility, cell morphology, metabolic activities, and percentage of biofilm-forming bacteria) [Cranfield et al., 2003; Chang et al., 2005; Mohd-Zain and Buniyamin, 2012; Nasri et al., 2013; Taheri et al., 2015, 2017; Nakouti et al., 2017; Salmen et al., 2018; Said-Salman et al., 2019a]. Generally, the effects of EMF on a biological system had evoked some controversial

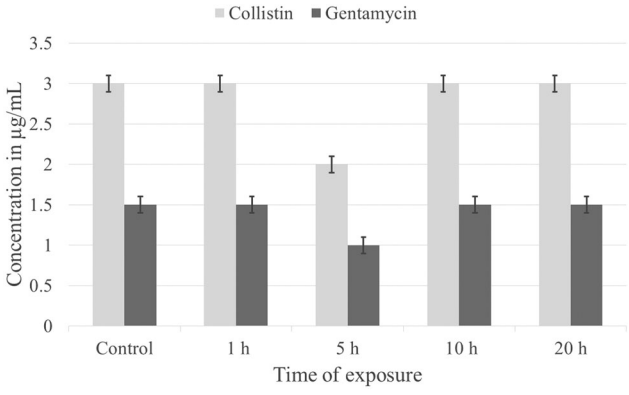


Fig. 3. Effects of Wi-Fi radiation at 2.4 GHz on MIC of *K. pneumoniae* to Gentamycin and Colistin. MIC = minimum inhibitory concentration.

results due to the difficulty of replicating published experiments and parameters.

The present study focused on the effects of Wi-Fi radiofrequency radiation on the pathogenicity of multidrug-resistant bacteria. Our findings revealed an increase in the susceptibility of *K. pneumonia ATCC BAA-1705* to two commonly used antibiotics, Colistin and Gentamycin, upon Wi-Fi radiation exposure for 5 h. This susceptibility subsequently returned to its initial profile after 10 and 20 h of exposure. The period of approximately 5 h corresponds to the mid-log phase of the bacterial growth curve. At this specific time, bacteria are in full exponential phase and have differing gene expression [Klumpp et al., 2009]. These results presented a non-linear response of *K. pneumoniae* to antibiotic treatments after Wi-Fi radiation. These findings confirm the validity of the "window theory" illustrated by Taheri et al. [2015], where the authors noticed a rise in the inhibition zone diameters in the presence of diverse antibiotics after 4.5 h of exposure to Wi-Fi waves, followed by a drop above 8 h of exposure. This data suggested that bacteria reached a certain level of damage in the exponential phase of the culture, then returned to their initial antibiotic susceptibility profile during the stationary bacterial growth phase.

Results from previous investigations proved that exposure of *Escherichia coli* to extremely low-frequency EMF (ELF-EMF) induced changes in the resistance and sensitivity profiles to some antibiotics such as erythromycin, nalidixic acid, and amoxicillin [Gaafar et al., 2006; Justo et al., 2006]. Others reported unchanged susceptibility of *E. coli* and *Pseudomonas aeruginosa* to several antibiotics when exposed to 50 Hz ELF-EMF [Segatore et al., 2012]. Exposure to mobile phone and Wi-Fi radiation showed that some bacterial strains became more resistant to antibiotics [Taheri et al., 2017; Said-Salman et al., 2019a]. Furthermore, continuous exposure of *P. aeruginosa* to mobile radiation for 24 h indicated the appearance of persister cells with increased resistance to antibiotics [Nakouti et al., 2017]. In contrast, no significant effect of radiofrequency EMF was reported on antibiotic sensitivity in exposed *Staphylococcus* [Salmen et al., 2018]. EMF could change the mechanism of the ion channels or efflux pumps, thus allowing the penetration of the particles through the bacterial cell wall [Taheri et al., 2017]. There is specific machinery for the initiation of AMR after exposure to radiofrequency radiation. Consequently, the permeability of the bacterial membrane might be altered by EMF.

The antibiotics used in the current study are Colistin and Gentamycin. The primary site of action for Colistin is the bacterial cell wall [Falagas et al., 2005], and Gentamycin penetrates through porin channels in the cell wall of gram-negative bacteria [Mohan et al., 2015].

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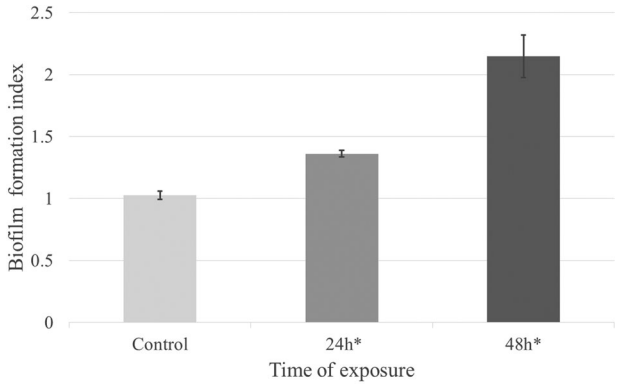


Fig. 4. Effects of Wi-Fi radiation at 2.4 GHz on biofilm formation by *Klebsiella pneumoniae*. *statisticallysignificant at $p < 0.05$.

When bound on the 30S subunit of the bacterial ribosome, Gentamycin prevents protein synthesis. Based on the findings of this study, exposure to Wi-Fi radiation might induce physical impact on the bacteria leading to the modification of its susceptibility to antibiotics. Therefore, bacterial cell membranes and substance penetration through the channels present in the membrane may be altered by exposure to radiation.

Furthermore, TEM experiments revealed that cells exposed to Wi-Fi radiofrequency radiation, for 24 h, exhibited several morphological alterations, such as structural disruption of the cytoplasmic membrane and disintegration of protoplasm as well as the presence of cell debris. The cell membrane disruptions seen may explain the influence that antibiotics exhibited in this study. The absence of an apparent capsule is due to the delicate nature of the capsule that can result in the lack of actual capsular material in up to 70% of encapsulated cells even after careful TEM processing with a cationic dye [Birkhead et al., 2017]. The observed morphological changes in Wi-Fi exposed *K. pneumoniae* seem to be comparable to those induced by ELF-EMF on several bacterial strains. Inhan-Garip et al. [2011] reported that ELF-EMF caused the disintegration of the cell wall, expulsion of the cytoplasmic contents, and retraction of the bacterial cytoplasmic membrane. Another study showed similar results induced by cationic peptides suggesting that the cationic peptides disturbed anionic target membranes in *E. coli* [Yenugu et al., 2004]. It was also reported that cell wall disintegration in *P. aeruginosa* could be induced by high voltage pulsed electric field [Fang et al., 2006]. Short electrical pulses are known to impact cell membrane by disturbing its lipid bilayer [Benz and Zimmermann, 1980]. Research in this domain showed enhanced synergic effects of nanosecond electrical pulses on bacterial susceptibility to antibiotics by affecting membrane permeabilization [Ibey et al., 2009, Martens et al., 2020]. Electrical and radiofrequency fields have been used in several medical applications such as cardiac arrhythmia and cancer treatments [Schoenbach, 2018], despite their damaging effects on the cell.

TABLE 2. Selected *K. pneumoniae* Genes for qRT-PCR Assigned to Their Function According to Uniprot

Gene name	Function according to Uniprot
bcsA	Catalytic subunit of cellulose synthase
mrkA	Fimbrial protein implicated in cell adhesion
luxS	Quorum sensing

The current investigation examined the effects of exposing *K. pneumoniae* to 2.4 GHz Wi-Fi radiation on the capability of producing biofilms. Bacterial cells of *K. pneumoniae* were continuously exposed to Wi-Fi waves for 24 and 48 h. Biofilm formation is well-known to be triggered in bacteria under stress, contributing to increased pathogenicity and attachment mechanisms [Chu et al., 2018]. Microorganisms in biofilms acquire higher protection against pH changes, osmolarity, nutrient insufficiency, and antibiotics [Fux et al., 2005]. The physical features of the exopolysaccharide layer of the biofilm provide 100 to 1,000 times more resistance to antibiotics than planktonic bacterial cells [Ito et al., 2009]. Our results showed a statistically significant rise in the ability to form biofilm by the exposed cells of *K. pneumoniae* as compared to non-exposed cells. This outcome is in line with previous reports exploring the effects of rotating magnetic field (RMF) and radiofrequency radiation on biofilm formation by bacteria [Fijałkowski et al., 2013; Said-Salman et al., 2019a]. In contrast, the ability to form biofilm by *Staphylococcus aureus* was not affected by short period exposure to mobile waves [Mohd-zain and Buniyamin, 2012]. Moreover, the exposure of *S. aureus* to mobile phone waves resulted in decreased biofilm formation ability at frequencies of 1, 2, and 3 GHz, and increased biofilm growth at frequencies of 4 and 5 GHz [Bucko et al., 2020].

The biofilm formation ability of *K. pneumoniae* is mediated by the levels of polysaccharides and fimbriae on the cell surface [Jagnow and Clegg, 2003; Schroll et al., 2010]. The regulation of cooperative activities between bacteria is controlled by a quorum-sensing process (QS), allowing them to communicate by releasing signal molecules [Li and Tian, 2012]. Quorum sensing mediates bacterial adaptation to the changing environment and has been involved in pathogenic potential such as biofilm. Thus, the expressions of the *bcsA* coding for cellulose synthase, *mrkA* coding for type 3 fimbriae, and *luxS* coding for type 2 quorum-sensing were selected for analysis by qRT-PCR. *mrkA* coding for the principal structural part of type 3 fimbriae was encoded by the *mrk*ABCD gene cluster [Ong et al., 2010]. The *bcsA,*

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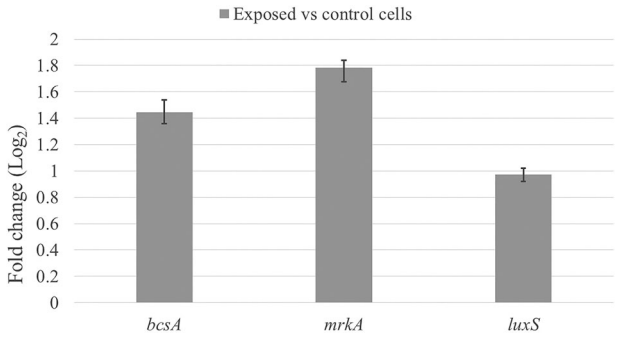


Fig. 5. Effects of Wi-Fi radiation at 2.4 GHz on relative expression (log_2 fold change) of *K. pneumoniae bcsA*, *mrkA*, and *luxS* genes.

mrkA, and *luxS* gene expressions in Wi-Fi-exposed *K. pneumoniae* were upregulated in comparison with the expression levels seen in control bacteria. Upregulation of the type 3 fimbriae encoding genes and its regulator *mrkH* was similarly observed in *K. pneumoniae* when exposed to an environmental stress factor such as simulated microgravity [Wang et al., 2016]. These outcomes confirmed our previous study, showing increased biofilm formation in different strains, and alteration of *E. coli* gene expression after Wi-Fi radiofrequency radiation exposure [Said-Salman et al., 2019a,b].

CONCLUSION

In summary, exposure of *K. pneumoniae* to 2.4 GHz Wi-Fi radiofrequency radiation affected the pathogenic traits of multidrug-resistant bacteria. There was a non-linear response of *K. pneumoniae* to Colistin and Gentamycin antibiotics after Wi-Fi pre-exposure. The 24 h exposure period to Wi-Fi radiation disrupted the cell membrane and disintegrated its protoplasm. In addition, the transcription of representative genes implicated in biofilm formation and quorum sensing in *K. pneumoniae* was upregulated due to Wi-Fi exposure.

The different bacterial responses to EMF observed among reported studies may result from the differences found in cell wall composition between Gram-positive and Gram-negative bacteria as well as the duration and type of the radiation. Low and continuous far-field exposure such as Wi-Fi may cause unexpected antibiotic resistance in the future. Under stress, bacteria tend to regulate their membrane properties through homeostasis by altering the lipid composition, thus resulting in higher permeability [Zhang and Rock, 2008]. While there might be a modification in membrane permeability by such low fields, allowing increased susceptibility to antibiotics, it is not, however, sufficient to induce cell death. Thus, bacteria are more easily affected by lower doses of antibiotics in the environment.

Further molecular experiments at gene and protein levels are required for understanding more details on the mechanisms by which these biological changes are taking place in bacteria following exposure to Wi-Fi radiation. Considering the impact of EMF on bacteria is an essential approach for controlling public health threats and may be beneficial for medical applications.

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