Control of the Activity of *Pseudomonas Aeruginosa* by Positive Electric Impulses at Resonance Frequency

Fadel M. Ali[1], A. M. Elkhatib[2], W. M. Aboutalib[3], A. M. Abdelbacki[4], A. M. Khalil[5], and N. Serag[6]

[1]Biophysics Dept, Faculty of Science, Cairo University, Egypt.
[2]Physics Dept, Faculty of Science, Alexandria University, Egypt.
[3]Physics& Chemistry Dept, Faculty of Education, Alexandria University, Egypt.
[4]Plant Pathology Dept, Faculty of Agriculture, Cairo University, Egypt.
[5]Basic Sciences Dept. Faculty of Engineering, Pharos University in Alex. (PUA), Egypt.
[6]Physics Dept. Faculty of Engineering German University in Cairo (GUC), Egypt.
Alaa.khalil@pua.edu.eg

Abstract: *P. aeruginosa* considered as a notoriously difficult organism to be controlled by antibiotics or disinfectants. The potential use of alternative means as an aid to avoid the wide use of antibiotics against bacteria pathogen has been recently arisen remarkably. The surge of interest has been marked by increasing efforts in research to explore the possibility of controlling the activity of bacteria and its sensitivity toward antibiotics by using extremely low frequency electromagnetic fields. Therefore, the aim of the present work is to study the effect of positive square pulsed electric fields at different frequencies in the range 0.1-50 Hz, exposure periods on the growth characteristics of *P. aeruginosa*. Furthermore, to study the effect of exposure on bacterial antibiotic susceptibility and molecular and morphological cellular structure. Results indicated that exposure to PEF can inhibit bacterial growth at particular resonance frequencies 0.7 Hz and 0.5 Hz and significant increase in antibiotic susceptibility to protein and cell wall inhibitors. Also, results of DNA, dielectric relaxation and TEM indicated molecular and morphological changes. Therefore, it concluded that treatment of *P. aeruginosa* cells by pulsed electric fields at for mentioned frequencies acts on its cellular activity and structure and it is a promising methodology for further in vivo applications.

[Fadel M. Ali, A. M. Elkhatib, W. M. Aboutalib, A. M. Abdelbacki, A. M. Khalil, and N. Serag. **Control of the Activity of *Pseudomonas Aeruginosa* by Positive Electric Impulses at Resonance Frequency.** *J Am Sci* 2013;9(10):120-130]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 15

Key Words: Pulsed Electric Fields, Resonance Frequency, Pseudomonas Aeruginosa, DNA and TEM

1. Introduction

P. aeruginosa, is an important Gram-negative opportunistic, dangerous and dreaded pathogen, as well as the principal cause of morbidity and mortality in cystic fibrosis patients (Jonathan and Jiyoung 2011). It is difficult organism to be controlled by antibiotics, because of its cell wall low permeability, its genetic capacity to express a wide repertoire of resistance mechanisms, the mutation on its chromosomal genes which regulate resistance genes and also it can acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages (Lambert, 2002). The possibility of a synergistic and/or antagonistic effect evoked by the combination of the appropriately patterned electromagnetic fields EMF and specific antibiotics deserves special attention in light of the risk that antimicrobial resistance poses to public health (Segatore *et al.,* 2012).

Several studies have been performed to verify direct effects exerted by extremely low frequency (ELF) electromagnetic fields (EMFs) on cell functions. Different variety of cell responses have been observed involving proliferation and differentiation (Lisi, *et al*., 2006, Vianale *et al.*, 2008), gene expression (de Mattei *et al.,*2005, Goodman, *et al.,* 2009), modulation of the membrane receptors functionality (Bersani *et al.*, 1997, Ke *et al.,* 2008), alteration in ion homeostasis (Grassi *et al.,* 2004, Piacentini *et al.*, 2008), and free radicals generation (Morabito *et al*., 2010, Wolf *et al.*, 2005). Additionally, bacteria have also been used in the studies to evaluate the effects of ELFEMF on some of its functional parameters as cell growth, antibiotic sensitivity, and cell morphology (Cellini *et al.,* 2008, Belyaev, 2011). In particular, it has been demonstrated that bacterial exposure to ELF EMF can alter its viability and growth rate with frequency and amplitude dependency (Moshe *et al.,* 2008), antibiotic susceptibility (Segatore *et al.,* 2012), and ultra-structural shape (Inhan *et al.,* 2011).

Of note, higher frequency fields are truly electromagnetic in that the electric and magnetic fields propagate together, whereas at low frequencies the fields can be effectively separated as alternating electric or magnetic fields. The consistently low thresholds at which EMF stimulates biological processes indicate that they require little energy and its frequency response is most effective at certain values that coincide with the natural rhythms of the processes affected (Martin and Reba, 2004). Biological systems, *in vivo*, generate electric currents and fields that

associated with magnetic fields, as a result of the running physiological mechanisms. In these mechanisms ionic motions are involved which are responsible for all bioelectric signals generated. The flow rate, period and direction of flow of the ions will generate an electric impulse with a specific frequency, shape and amplitude (Fadel *et al.,* 2009).

Based on metabolic biomagnetic resonance model (MBMR) to control physiological functions in biological systems, it is necessary to apply electric fields of frequency similar to that of bioelectric impulses generated during metabolic activities. This interference will affect the ion motion in the biological system, which may be enhanced or inhibition depending on the mode of interference. The bioelectric signals generated during metabolic activities of cells are known to be in the extremely low frequency range, therefore, to interfere with these signals; the applied electromagnetic wave should have the same frequency of the bioelectric signal.

The aim of the present study is to study the effect of pulsed electric field (PEF) of different frequencies at different exposure periods on the viability, antibiotic susceptibility and the molecular and cellular changes of *P. aeruginosa* ATCC 27853.

2.Materials and Methods:

Reference strain of *P. aeruginosa* (Ref 0353P, ATCC 27853, LOT 353604, OXOID) is used for all the present comparative experiments. The microorganism is plated on MacConkey agar and incubated at 37 ^{0}C 24 hrs. Thereafter, several colonies plated again on a fresh agar plate and also incubated for 24 hrs at 37 ^{0}C. For maintaining a fresh strain, this procedure is repeated weekly before running the experiment.

Determination of Bacterial Replication:

The bacterial growth of *P. aeruginosa* is determined by measuring the optical density (OD) at 700 nm (the best wave length for absorbance of bacterial suspension that selected automatically) using spectrophotometer type (JENWAY 6405 UV/Visible-U.K.). A calibration curve between the OD and cell count was established by preparing a bacterial suspension which was produced by suspending three colonies from fresh 24 hrs incubated plated agar of *P. aeruginosa* in 5 ml of MacConkey broth media and then incubated for 12 hrs at 37 ^{0}C (the experimental time range). Every 60 min intervals within the time range the OD measured and different dilutions of each sample are plated on MacConkey agar. After the incubation for 24 hrs at 37 ^{0}C of the plates, colony forming units CFU are counted and then correlated to the OD and its values plotted versus CFU/ml values and a best fit line is performed. Then the growth characteristic curve for each sample could be plotted between the CFU/ml and time directly (Obermeier *et al.,* 2009).

It is worthy to mention that the absolute reference values of CFU were being differed from experiment to another due to the various amounts of bacterial suspension used to inoculate the agar plates. However, within the one experimental setup the CFU were identical for the exposed and reference samples. Moreover, the check of the concentrations was applied by keeping the absorbance almost the same for all samples before running the experiment.

Positive Square Electric Impulses Source:

Direct current power supply (9v-DC) through an electronic switching device is maintained to produce a square pulsed current with different frequencies. The square pulses then directed into DC/DC voltage converter that produces the voltage field intensity (400 ± 25) v through squared copper plates 5X5 cm^2 and 1 cm apart at which the samples suspensions are placed and the pulse shape was displayed using oscilloscope GOS-620. The system fabricated and manufactured locally in the physics lab of German University in Cairo-Egypt as shown in figure-1.

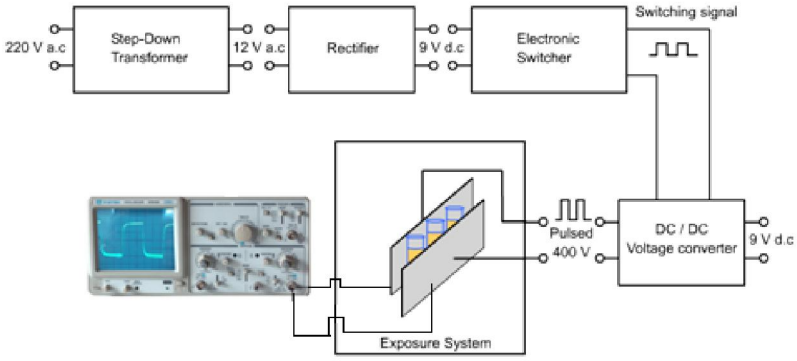


Figure-1: Schematic illustration of the experimental design used for exposure.

Inhibition Frequency Determination:

Samples of *P. aeruginosa* exposed to different frequencies of each exposure facility to determine the resonance frequency at which the peak of the inhibition is occurring.

A fresh bacterial suspension of it was prepared as mentioned before. The concentration of the suspension has been adjusted to approximately 107 CFU/ml by using the OD as mentioned before. The *P. aeruginosa* suspension then divided into two groups (three samples per each), one group kept as a control (unexposed) group and the other group exposed to PEF source where the selected frequencies from 0.1 Hz to 1.1 Hz varies in a step of 0.1 Hz. It is worth to mention here that there were several trials to select the most effective frequency range from 1.1 Hz till 50 Hz on cellular activity but without significant effects. At the end of the exposure period, the bacterial growths for both the control and exposed groups were determined in parallel by measuring the absorbance at wavelength 700 nm. Then, the sample groups are incubated at 37 0C and every 1 h the incubation are interrupted for absorbance measurements and different dilutions of each sample is plated on MacConkey agar in order to determine its cellular counts to detect the resonance frequency that cause maximum growth inhibition. The inhibition percentage difference in 10th hour incubation for each sample is calculated with respect to its control one according to equation-1 and the average value is taken. Then the average inhibition percentage difference values are plotted versus frequency in the range of 0.1Hz to 1.1 Hz to represent the resonance curve for the analysis purpose.

%D= [(Average OD (control) – Average OD (exposed)) / Average OD (control)] x 100. Eq-1

Most Effective Time Determination:

In order to determine the optimum exposure time after obtaining the resonance frequency of growth inhibition, two groups of *P. aeruginosa* suspensions are prepared; one kept as control and the other group exposed to PEF source at the resonance frequencies for periods of 30, 60, 90, 120 and 150 min. After the end of the exposure time, all groups are incubated and then absorbance measurements were done every 1 hr. The average percentage inhibition value (as compared to control) at 10th hr post incubation for each group was calculated to specify the most effective time.

Antibiotic Susceptibility Test:

The bacterial isolate of *P. aeruginosa* was subjected to susceptibility testing using five different antimicrobial agents namely (Imipenem (IPM), Ceftriaxone (CRO), Amikacin (AK), Tobramycin (TOB), and Pefloxacin (PF)). The susceptibility breakpoints for all antimicrobial agents used in the antibacterial susceptibility test, discs as well as zone reading chart were supplied by CLSI, M100-S17 (Performance Standards for Antimicrobial Susceptibility Testing; 2007). Antimicrobial susceptibility test was carried out and performed by the procedure outlined by the *National Committee for Clinical Laboratory Standards (NCCLS), 1985*.

P. aeruginosa suspension was divided into two groups (three samples per each); control and exposed to PEF at resonance frequencies for the most effective time that for mentioned previously. At the end of the exposure period, samples of control and exposed groups were used to be inoculated in MacConkey agar plates. After the inoculums had dried (3-5 minutes.) the appropriate antibiotic discs were placed on the agar surface with a sterile forceps, gentle pressure was applied over the surface of each disc to ensure contact. The inoculated plates then incubated at 37 °C for 48 hrs. Then the diameter of each inhibition zone was measured and compared with the zone reading chart.

Transmission Electron Microscope (TEM) Examination:

The morphological changes of control and exposed group to PEF at resonance frequencies for the most effective time that for mentioned previously have been determined using TEM. In order to prepare the bacterial sample to be examined by TEM, the sample should undergo some processing according to (Demicheli *et al.,* 2007). The processing of bacterial cells (control and exposed) began after 1 h post exposure, where the bacterial cells were collected and washed three times in Phosphate buffered saline (PBS). The pellet was suspended in 2% glutaraldehyde in phosphate buffer, pH 7.2, and fixed overnight in the refrigerator. After fixation, the pellet rinsed three times in phosphate buffer, pH 7.2. The resulting pellet containing the cell was fixed in 1% osmium-tetroxide in phosphate, pH 7.2, for 3 h at 4 °C and dehydrated with increasing concentrations of ethanol. After the 100% ethanol washes, cells were washed with 100% acetone and infiltrated with resin,

Acetone: resin (no accelerator) 2:1	1 h
Acetone: resin (no accelerator) 1:1	1 h
Acetone: resin (no accelerator) 1:2	1 h
Pure resin (no accelerator)	1 h
Pure resin (no accelerator)	overnight
Pure resin + accelerator	1-2 h
Embed samples into molds	60 C° oven

Semi thin sections were prepared on glass slides through cutting at 1 µm using the ultramicrotome. Sections were stained with Toludine blue for 5 minutes examined by light microscope model M-200M. Ultra-thin sections were cut using ultramicrotome Leica model EM-UC6 at thickness 90 nm, mounted on copper grids (400 mish). Sections

were stained with double stain (Uranyl acetate 2% 10 minutes followed by Lead citrate for 5 minutes and examined by transmission electron microscope JEOL (JEM-1400) at the candidate magnification. Images were captured by CCD camera model AMT, optronics camera with 1632 x 1632 pixel format as side mount configuration. This camera uses a 1394 firewire board for acquision.

DNA Study:

DNA was extracted from 50 mg of fresh *P. aeruginosa* suspension (either control or exposed) according to the method developed by (Dellaporta *et al.,* 1983). The quantity of extracted DNA was measured by means of agarose gel electrophoresis and confirmed by spectrophotometer (Hoisington *et al.,* 1994). The extracted DNA subjected to amplification reaction via the polymerase chain reaction (PCR) manufactured by Thermocycler T1, Biometra, Germany. The PCR mixture consists of a PCR beads tablet (manufactured by Amessham Pharmacia Biotech). The specific primer (PA-SS-F= GGGGGATCTTCGGACCTCA; PA-SS-R TCCTTAGAGTGCCCACCCG) used in the amplified polymorphic DNA reactions. The amplified DNA of all groups was electrophoresed using electrophoresis unit (wide mini-sub-cell GT Bio-RAD) on 2% agarose containing 0.5 µg/ml of ethedium bromide, at a constant 75 volt and 60 mA, and visualized with UV trans-illuminator. Then DNA gel was scanned for band, using gel documentation system (AAB Advanced American Biotechnology 1166 E. Valencia DP. Unit 6 C, Fullerton, CA 92631). The different molecular weights of bands were determined against a DNA standard (Mid Range DNA Ladder Jena Bioscience) with molecular weights 100, 150, 200, 300, 400, **500**, 600, 800, **1000**, 1500, 2000, 3000 bp. The similarity level was determined by un-weighted pair group method based on the arithmetic mean (UPGMA).

Dielectric Relaxation Studies:

The dielectric measurements have been done for control and group exposed to PEF at resonance frequencies for the most effective time that for mentioned previously. The samples of bacterial suspension of each group undergo to centrifugation at 14,000 RPM and 4 °C for 15 minutes under sterilized conditions, the pellets were then harvested, washed with sterile deionized water twice more and finally re-suspended in sterile deionized water. After that the concentration of bacterial cells of different samples was adjusted to approximately 10^7 CFU/ml.

The dielectric measurements were carried out for the samples in the frequency range 0.1-10 MHz using a Loss Factor Meter (type HIOKI 3532 LCR Hi TESTER, version 1.02,1999, Japan), with a sample cell type PW 9510/60, manufactured by Philips. The sample cell has two squared platinum black electrodes of 0.64 cm^2 area and separated by 1 cm apart. During the measurements, the sample between the electrodes was kept at a constant temperature of 24 ± 0.1 ^oC.

The capacitance of the samples was measured at each frequency and the resistance was recorded. Each run was taken three times and the average was considered. The relative permittivity (έ), loss tangent "tan δ", dielectric loss ε″, conductivity σ and relaxation time (τ) of the samples were calculated for each frequency using the following relations.

$$\acute{\varepsilon} = C\ d/\varepsilon_o\ A \quad \text{Eq-2}$$

$$(\delta)=1/2\pi f RC \quad \text{Eq-3}$$

$$\varepsilon'' = \acute{\varepsilon}\ (\delta) \quad \text{Eq-4} \qquad \sigma = 2\pi f\ \varepsilon''\ \varepsilon_o \quad \text{Eq-5}$$

$$\tau=12\pi f_c \quad \text{Eq-6}$$

Where f_c is the critical frequency which corresponding to the mid-point of dispersion curves.

Statistical Analysis:

The statistical analysis was performed using the student's *t*-test with a minimal confidence level of 0.05 for statistical significance. Each experiment was performed at least three times with a minimum of three samples per termination point, resulting in a total number of a six samples for each experiment.

3.Results:

A- Growth Characteristics:

The variation of sample optical density (OD) with *P. aeruginosa* cell count in MacConkey broth medium measured at 700 nm indicated a linear dependence of the absorbance on the microorganism count according to the equation (CFU) = $5.18x10^8$ (A) with correlation factor 0.96.

The growth curve is measured for different samples after the exposure to PEF for a period of 60 min at different frequencies. This experiment started with a pilot study where the frequency of the applied field was changed to cover the frequency ranges from (1.0 to 50) Hz in steps of 5 Hz. The results indicated no change in the growth curves characteristics as compared with control in this range. The study was then directed to investigate the effects of frequencies lower than 1.0 Hz. The frequency of the field was varied in steps of 0.1 Hz. Figure-2 shows the variation of decrease in absorbance of the exposed samples

measured at 10th hr post incubation (as reference to its control samples) as a function of the applied field frequency. The results indicated two inhibition resonance peaks for cellular growth at frequencies (0.5Hz and 0.7 Hz). The curves represented in figure-3 show that all types of exposure started to make growth difference compared to its control in the exponential growth period at 4th hr post incubation and that difference continued till the stationery phase after 9th hr. In this respect, the number of CFU/ml for the control samples and exposed ones at those resonance frequencies post 10th hr incubation are tabulated in table-1. It is clear from the data that the population intensity of samples exposed to PEF has been reduced by 9.2% and 18.1% at frequencies 0.5 and 0.7 Hz respectively with very highly significant inhibition (P<0.001).

B- Most Effective Exposure Time:

Figures 4 and 5 show histogram for the growth inhibition percentage differences at 10th hr post incubation after exposure of microbial cells to PEF at resonance frequencies 0.5 and 0.7 Hz for several exposure times. It is clear from the figures that 60 min exposure gives the maximum growth inhibition at the resonance frequencies for all demonstrated exposure types.

C- Antibiotic Susceptibility Test:

In table-2 the antibiotic susceptibility test results for control and exposed to 0.5 and 0.7 Hz PEF samples are given. It is clear from the table that exposure to PEF at 0.5 Hz gave significant increase in susceptibility to protein synthesis inhibitors (AK and TOB) and cell wall synthesis inhibitor (IPM). Also, the exposure to PEF at 0.7 Hz gave significant increase in susceptibility to protein synthesis inhibitor (AK) and cell wall synthesis inhibitor (IPM). No remarkable changes in the DNA synthesis inhibitor for all exposed samples.

D- TEM Examination:

The figure-6 of TEM for control *P. aeruginosa* cells illuminated the majority of the cells have a well preserved cell envelope that consists of a smooth, evenly stained plasma membrane, a semi translucent periplasmic gel and a symmetrically stained, taut outer membrane. Additionally, through the binary fission the cells look remarkably robust since its cytoplasm's is filled with ribosomes and their chromosome is spread throughout the entire cytoplasm; (it is worthy to mention that image contrast is based on the mass distribution of the biomaterial and not on the staining reagent) these are structural traits suggesting active metabolic processes and protein synthesis. On the other hand, the cells that exposed to 0.5 and 0.7 PEF showed a preserved cell wall and outer membrane but with disorganized and localized intracellular organelles and significant biomass loss as shown in figures 7 & 8.

E- DNA Analysis:

Electrophoretic amplified DNA pattern of P. aeruginosa DNA extracted from control and exposed to 0.5 Hz and 0.7 Hz PEF (E) for 1 hr are shown in figure-9. The figure indicates the appearance of a new band at 350bp for exposed sample.

F- Dielectric Relaxation Results:

Figures10-12 show the relaxation curves of *P. aeruginosa* bacterial suspensions control and exposed to 0.5 Hz and 0.7 Hz PEF respectively. Each figure represents the variation of the relative of permittivity (ε′) plotted on the left y-axis and the electric conductivity (σ) on the right y-axis as a function of frequency. It is clear from the figures the (ε′) dependence on frequency a mirror image for (σ) dependence on frequency. All measured samples (control and exposed) have dispersion phenomena at frequency range demonstrated. The relaxation time (τ), dielectric increment (Δε′), and conductivity (σ) were calculated from the curves in the figures for all samples demonstrated. The average values of each are given in table-3. The data obtained indicate pronounced decrease in the dielectric properties for the samples exposed to 0.7 Hz-PEF compared with its control ones.

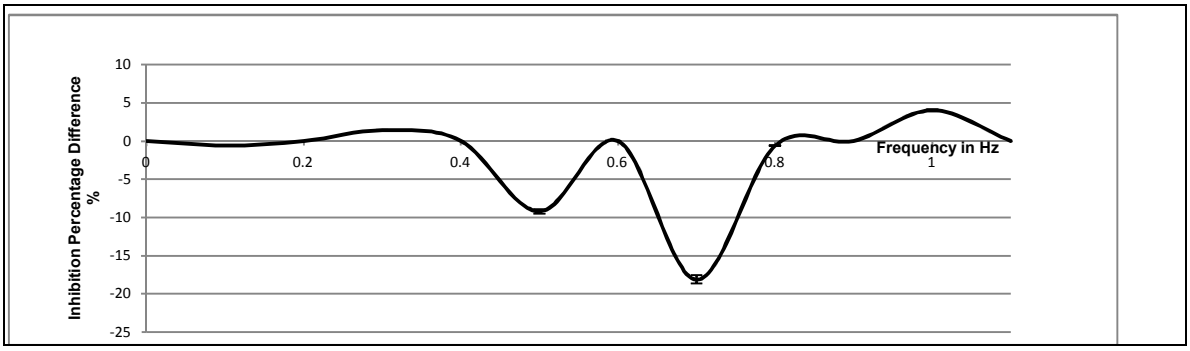


Figure-2: Resonance curve for exposed samples to PEF at different frequencies.

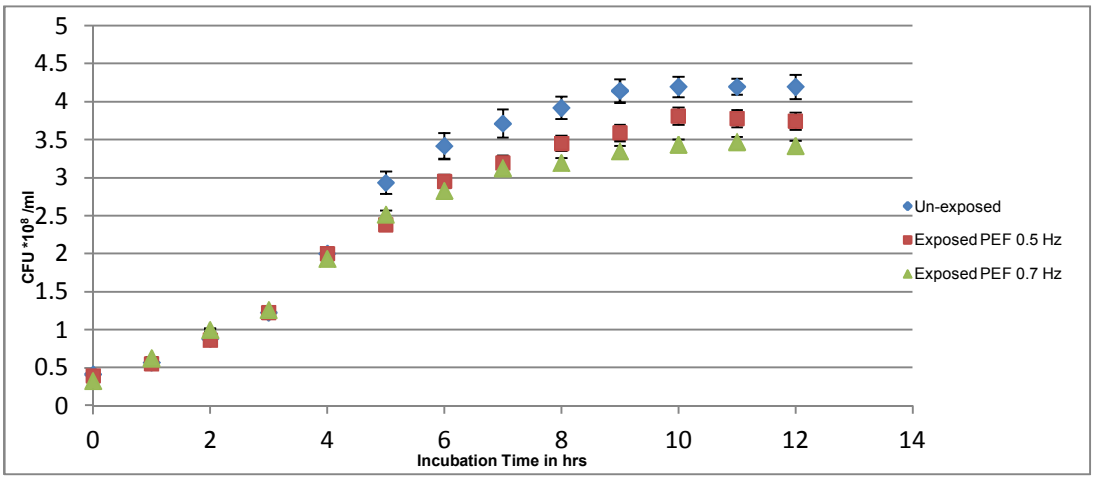


Figure-3: Growth curves for control (unexposed) and 0.5 Hz and 0.7 Hz PEF exposed Samples.

Table-1: The cell count in CFU/ml for control and exposed Samples with its inhibition percentage difference.

Sample / Exposure Frequency	PEF	
	0.5 Hz	0.7 Hz
Control (* 10^9 CFU/ml)	(4.21 ± 0.07)	
Exposed (* 10^9 CFU/ml)	(3.81 ± 0.04)	(3.44 ± 0.04)
Inhibition Percentage Difference D%	(9.2 %)***	(18.1 %)***

^{NS}Not Significant *Significant (P<0.05) **Highly Significant (P <0.01) ***Very Highly Significant (P <0.001)

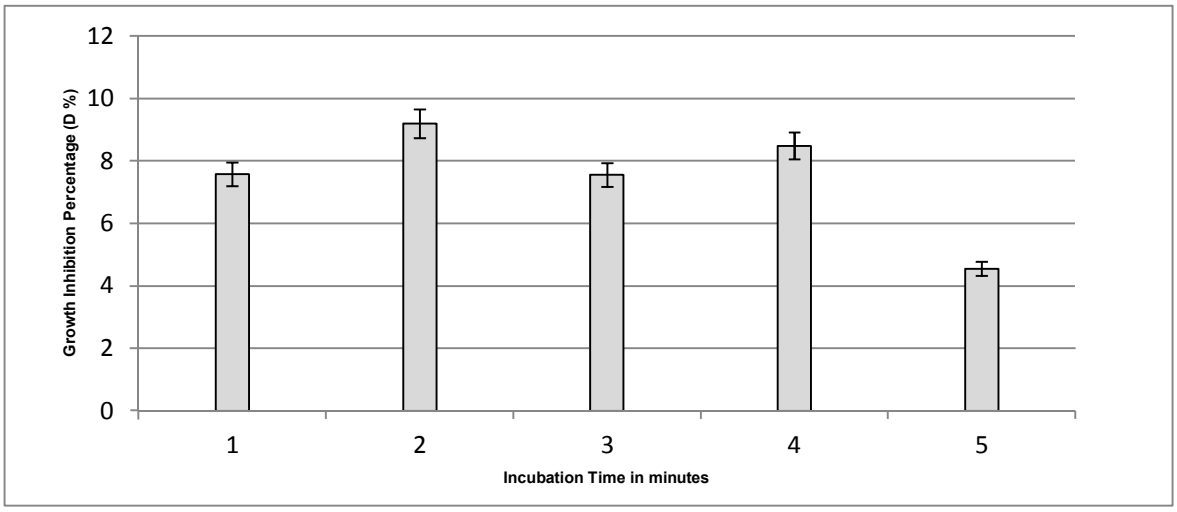


Figure-4: Most effective inhibition time of samples exposed to 0.5 Hz PEF for different exposure periods.

Table-2: The mean inhibition zone diameter of different antimicrobial agents for control and exposed samples.

^{NS}Not Significant *Significant (P <0.05) **Highly Significant (P <0.01) ***Very Highly Significant (P <0.001)

Sample / Antibiotic disc		Mean Inhibition Zone Diameter in mm				
		Cell Wall Inhibitors		Protein Inhibitors		DNA inhibitors
		IPM	CRO	AK	TOB	PF
Control (unexposed)		(20 ± 0.4)**	(18 ± 0.6)***	(18 ± 0.2)*	(20 ± 0.4)**	(20 ± 0.3)**
PEF	0.5Hz	(27 ± 0.8)*	(20 ± 0.3)**	(24 ± 0.2)*	(27 ± 0.9)*	(19 ± 0.3)*
	0.7Hz	(28 ± 0.7)***	(22 ± 0.9)***	(25 ± 0.7)**	(22 ± 0.4)*	(21 ± 0.8)**

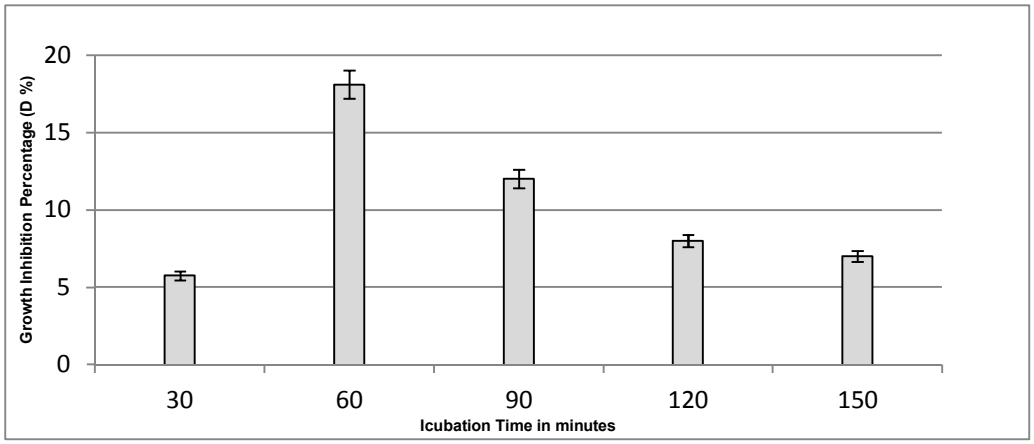


Figure-5: Most effective inhibition time of samples exposed to 0.7 Hz PEF for different exposure periods.

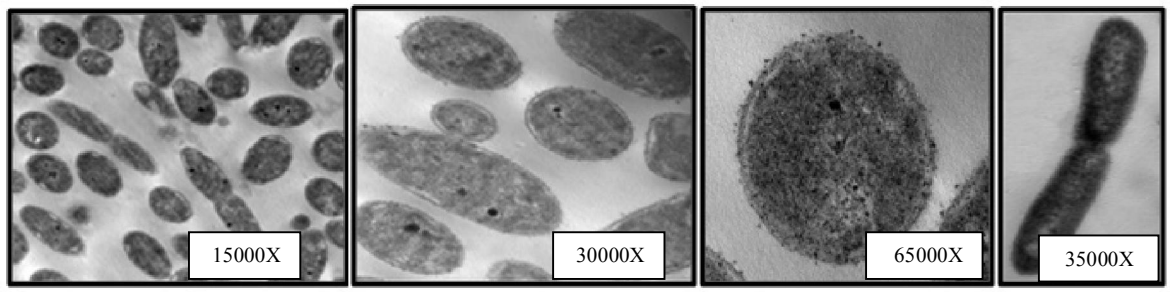


Figure-6: TEM images of control *P. aeruginosa* cells with different field sizes. The cell images revels its uniform outer cell wall and membrane with high density organelles. The binary fission cell image illuminates chromosome spread throughout the entire cytoplasm suggesting an active metabolism.

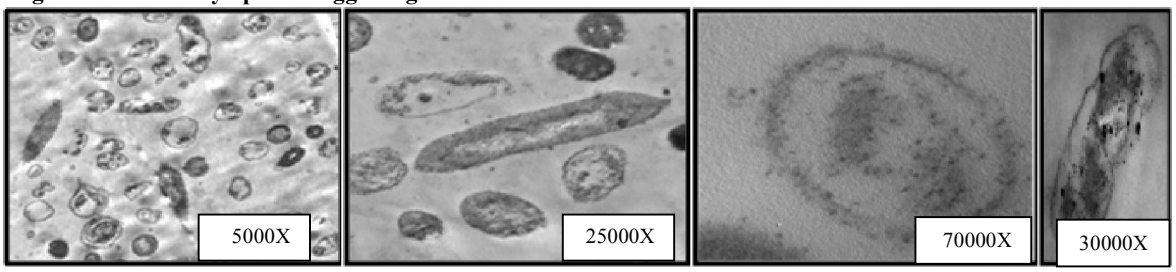


Figure-7: TEM images with different field size of *P. aeruginosa* cells exposed to 0.5 Hz PEF. The micrographs revel uniform outer cell wall and membrane with localized organelles and binary fission cell image illuminates disorganized intracellular content.

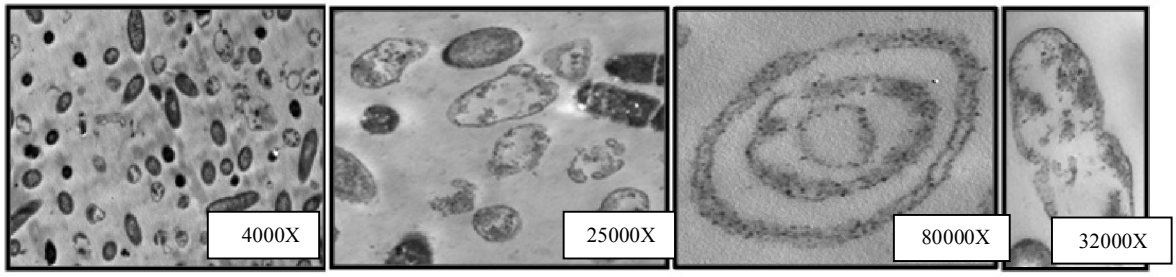


Figure-8: TEM images with different field size of *P. aeruginosa* cells exposed to 0.7 Hz PEF. The micrographs revel preserved outer cell wall and membrane with localized organelles and binary fission cell image illustrates irregularity in cellular contents distribution into the outer cell wall.

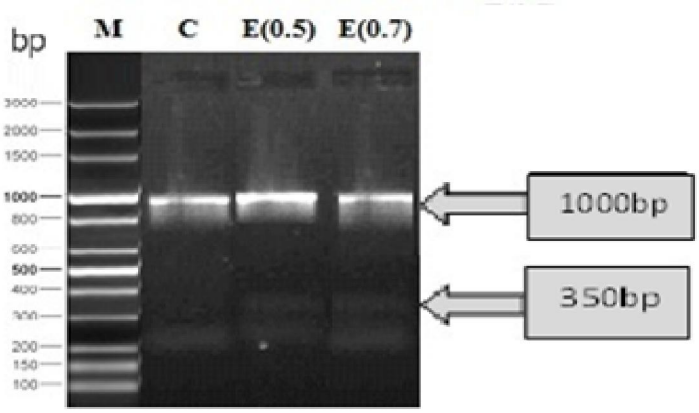
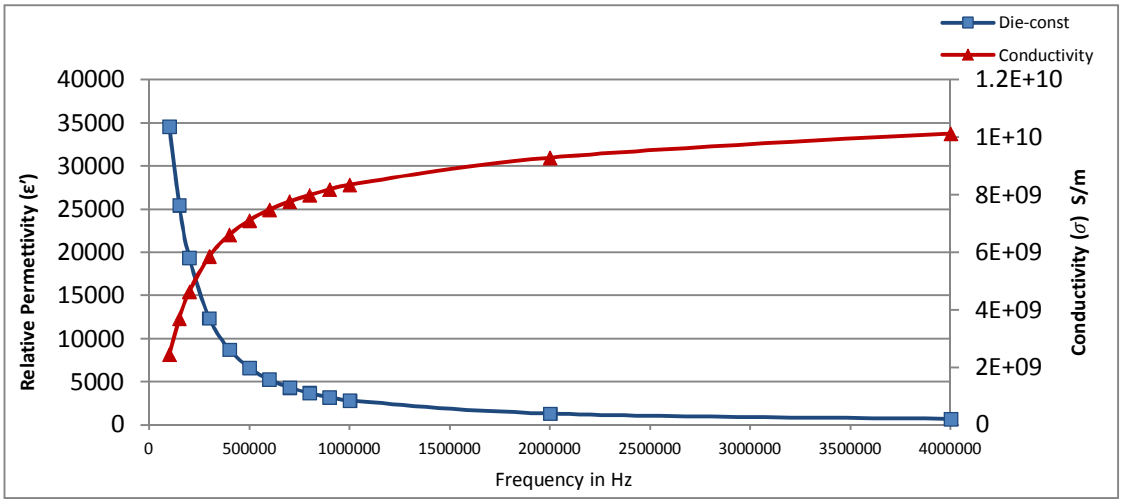
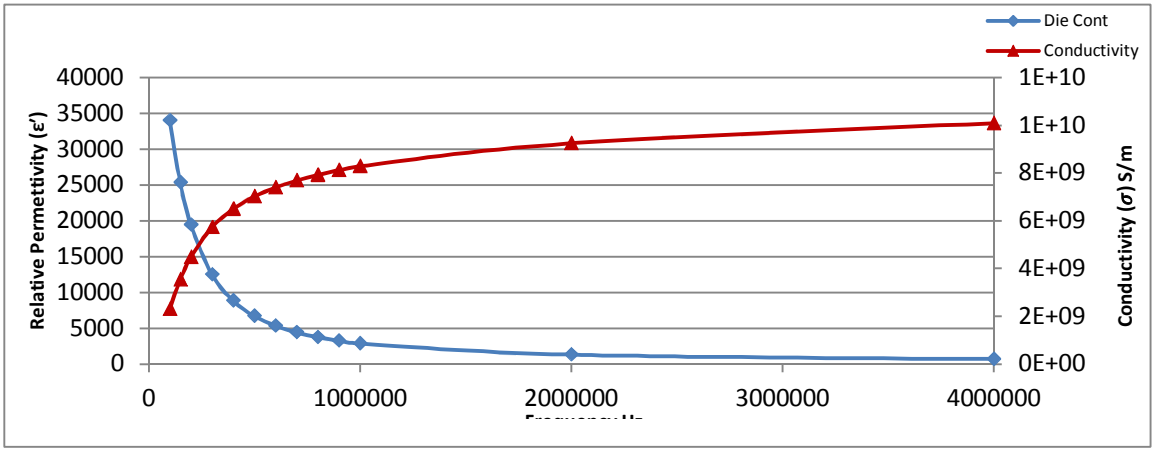


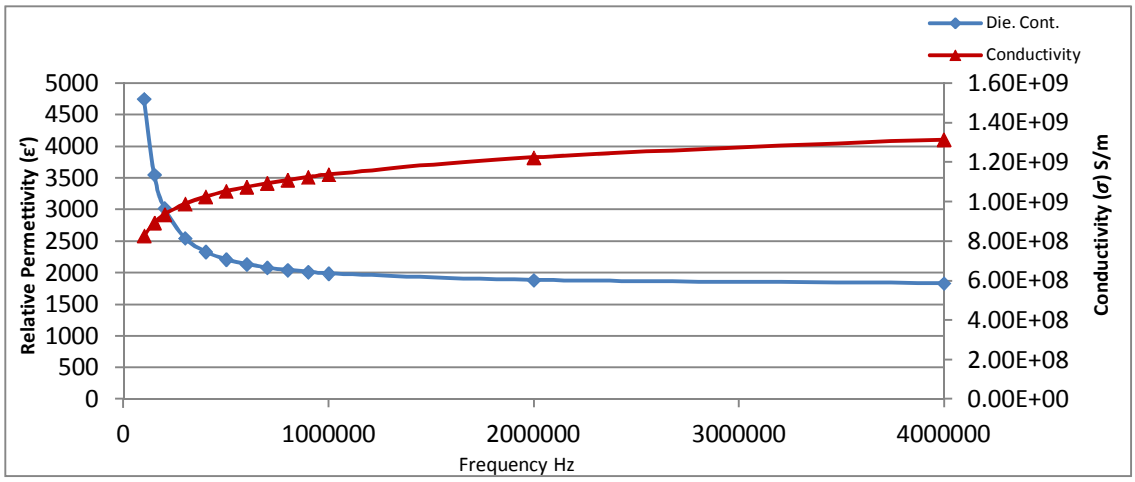
Figure-9: Electrophoretic amplified pattern of *P. aeruginosa* DNA extracted from control and exposed samples to PEF at 0.5 and 0.7 Hz. using The specific primer (PA-SS-F= GGGGGATCTTCGGACCTCA; PA-SS-R TCCTTAGAGTGCCCACCCG) indicated the appearance of new band at 350bp for exposed samples. M= DNA Ladder (DNA Marker). C= control (unexposed) sample DNA. E(0.5)= 0.5 Hz-PEF exposed sample DNA. E(0.7)= 0.7 Hz-PEF exposed sample DNA.



Figurr-10: The variations of the relative permeability ε' and electric conductivity σ as function of frequency in the range 0.1-10MHz for control bacterial suspension.



Figurr-11: The variations of the relative permeability ε' and electric conductivity σ as function of frequency in the range 0.1-10MHz for bacterial suspension exposed to 0.5 Hz PEF.



Figuer-12: The variations of the relative permeability ε' and electric conductivity σ as function of frequency in the range 0.1-10MHz for bacterial suspension exposed to 0.7 Hz PEF.

Table-3: The relaxation time (τ), the dielectric increment (Δέ) and the conductivity (σ) of control and exposed samples to 0.5 Hz and 0.7 Hz PEF for 1 h.

Sample / Dielectric Parameter		Relaxation time τ (x10^{-7}sec)	Dielectric increment $\Delta\varepsilon' = \Delta\varepsilon_o' - \Delta\varepsilon'_\infty$	Conductivity σ at 10 MHz (x10^{9}s/m)
Control (unexposed)		7.50 ± 14	34226 ± 205	11.12 ± 0.4
PEF	0.5Hz	6.47 ± 17	23640 ± 195	7.21 ± 0.4
	0.7Hz	2.58 ± 15	3950 ± 210	3.44 ± 0.5

4.Discussion:

In the present study the effects of different frequencies and different exposure periods of PEF on the growth characteristics of *P. aeruginosa* were investigated in order to determine the resonance frequency that cause maximum growth inhibition. For this the growth characteristics, molecular structure, antibiotic sensitivity, and TEM of the microorganism were studied. The results indicated inhibition of the microbial growth at 0.5 Hz and 0.7 Hz of the influencing field as shown in figure-2 and maximum inhibition of microorganism cellular growth occurred after one hour exposure. It may be presumed from these results that the inhibition effect is due to the interference of the PEF according to its frequency with the bioelectric signals generated from physiological functions of bacterial cells. The results of these interference reactions depend on the mode of interference which may lead to cause changes in the microorganism molecular structure that affect its cellular division.

There are several parameters involved to effect the cellular division of the microorganism such as its cellular membrane packing properties and structure, synthesis, intracellular constituents and DNA. Since changes in permeability or in structure of cellular membrane may lead to the loss of the inter-constituents of the cell and/or the permeation of extracellular components to come inside the cell which may interact with DNA. The dielectric relaxation results indicated pronounced decrease in the average values of the dielectric increment (Δέ), the relaxation time (τ) and the electrical conductivity (σ) for exposed microbial samples. Of note, the relaxation time and electrical conductivity are directly related to the macromolecular electric dipole moment which in tern dependent on the size and charge of the macromolecule and thus, the dielectric relaxation significant changes may be due to cellular and molecular charges redistribution and/or its size changes. In view of the potential role played by ELF-EMF in modulating charge movements on the membrane, it has been verified that ELF-EMF can affect membrane functions not only by local effects on ion fluxes or ligand binding but also by altering the distribution and/or the aggregation of the intra membrane protein (Bersani, *et al.* ,1997, Marchionni *et al.,* 2006).

Thus, the changes in electrostatic surface charges of exposed cells may be related to disorder in the packing properties of the phospholipids macromolecules forming the cellular membrane which

affected membrane permeability and hence ionic pumping mechanism. This phenomena can be noticed from the results of the antibiotic sensitivity tests using different antibiotics of different interaction mechanisms with a microbe. The susceptibility increase against protein synthesis inhibitors may be related to changes on bacterial uptake process of aminolglycoside which are cationic molecules that bind to anionic components of the bacterial cell membrane in a reversible and concentration-dependent manner (Taber, *et al.,* 1987). Also the susceptibility increase of the microorganism to cell wall synthesis inhibitors after exposure may be due to the PEF effect on mucopeptide synthesis in the bacterial cell wall.

The cells that exposed to PEF preserved its cell shape but with quite different than normal due to the rearrangements and low density of its constituent organelles as depicted from the TEM micrographs which in agreement with those reported by (Ayse *et al.,* 2011). It is reasonable to speculate that exposure to PEF has altered the electrostatic balance of the membrane components in a manner that producing loss of integrity and/or disorganization and thus triggering growth arrest or death of bacterial cells. The appearance of new bands in the DNA pattern of exposed bacteria prove that the DNA sequences have been changed under the effect of PEF in a way that the primer used find a new binding sequences which not present in the control bacteria. The result indicated that the genetic sequences of bacterial DNA were modified due to the exposure to (0.5 Hz, 0.7 Hz) PEF, and subsequently protein and enzyme synthesis expressed from these modified sites which may affect the bacterial growth (Lee *et al.,* 2001, Salanoubat *et al.,* 2001, Wolf *et al.,* 2004, Maercker 2005 and Lupke *et al.,* 2006).

One may conclude that exposure to PEF is a new unthermal promising technique as an aid to avoid the use of antibiotics against bacterial pathogen and it is a resonance-frequency phenomenon at which external signal coincides with particular bio-membrane signal and hence resonance occur.

Acknowledgment:

I wish to acknowledge Mr. Asem Abd El-Monem Ali (Physics Specialist) and Mr. Mark Email (Student), from German University in Cairo for helping on manufacturing and making the exposure facilities.

Corresponding author:
Khalil Alaa M.,
Basic Sciences Dep. Faculty of Engineering at Pharos University in Alexandria, Egypt
Alaa.khalil@pua.edu.eg

References:

1. Ayse I., Burak A., Zafer A., Dilek A. *et al.,* (2011), "Effect of extremely low frequency electromagnetic fields on growth rate and morphology of bacteria", Int. J. Radiat. Biol.: 1–8.
2. Belyaev I., (2011) "Toxicity and SOS-response to ELF magnetic fields and nalidixic acid in *E. coli* cells," *Mutation Research*, vol. 722, no. 1, pp. 56–61.
3. Bersani F., Marinelli F., Ognibene A. *et al.,* (1997), "Intramembrane protein distribution in cell cultures is affected by 50Hz pulsed magnetic fields," *Bioelectromagnetics*, vol. 18, no. 7, pp. 463– 469.
4. Cao Y, Askenazi M, Halling C, *et al.,* (2001). Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature; 415:497-502.
5. Cellini L., Grande R., Di Campli E., *et al.,* (2008) "Bacterial response to the exposure of 50Hz electromagnetic fields," *Bioelectromagnetics*, vol. 29, no. 4, pp. 302–311.
6. Dellaporta S.L., Wood J, and Hicks JB (1983): A plant DNA minipreparation: Version II. Plant Mol. Biol. Reporter, 1: 19-21.
7. De Mattei M., Gagliano N., Moscheni C. *et al*., (2005) "Changes in polyamines, c-myc and c-fos gene expression in osteoblast-like cells exposed to pulsed electromagnetic fields," *Bioelectromagnetics*, vol. 26, no. 3, pp. 207–214.
8. Demicheli M., Miranda A. and Silva A. (2007), "Ultrastructural changes in Paracoccidioides brasiliensis yeast cells attenuated by gamma irradiation", *Journal Compilation*; 50: 397–402.
9. Fadel M.A., Ahmed M.A. and El Hag M.A. (2009) "Control of *Sclerotium cepivorum* (Allium White Rot) activities by electromagnetic waves at resonance frequency". *Australian Journal of Basic and Applied Science*, 3(3):1994-2000, ISSN 1991-8178.
10. Goodman R., Lin-Ye A., Geddis M.S., *et al.,* (2009) "Extremely low frequency electromagnetic fields activate the ERK cascade, increase hsp70 protein levels and promote regeneration in Planaria," *International Journal of Radiation Biology*, vol. 85, no. 10, pp. 851–859.
11. Grassi C., D'Ascenzo M., Torsello A. *et al*., (2004), "Effects of 50 Hz electromagnetic fields on voltage-gated Ca2+ channels and their role in modulation of neuroendocrine cell proliferation and death," *Cell Calcium*, vol. 35, no. 4, pp. 307–315.
12. Hoisington D., Khairallah M. and Gonzalez-Leon D. (1994). "Laboratory Protocols: CIMMYT applied molecular genetics laboratory. D.F. Mexico; CIMMYT Press, 1994. 50 p. ISBN 968-6923-30-6.
13. Jonathan K. Lutz 1 and Jiyoung (2011) "Prevalence and Antimicrobial-Resistance of *Pseudomonas aeruginosa* in Swimming Pools and Hot Tubs", *International Journal of Environmental Research and Public Health, 8*, pp. 554-564.

14. Lambert P A, (2002) "Mechanisms of antibiotic resistance in Pseudomonas Aeruginosa", *Journal of Royal Society of Medicine*, 95 (Suppl. 41), pp. 22–26.
15. Lee Y., Fan S., Chiu L. and Hsia K. (2001) Isolation of an insertion sequence from *Ralstonia solanacearum* race 1 and its potential use for strain characterization and detection", Appl Environ Microbiol.; 67(9):3943-50.
16. Lisi A., Ledda M., Rosola E. *et al.,* (2006), "Extremely low frequency electromagnetic field exposure promotes differentiation of pituitary corticotrope-derived AtT20 D16V cells," *Bioelectromagnetics*, vol. 27, no. 8, pp. 641–651.
17. Lupke M, Frahm J, Lantow M, Maercker C. *et al.,* (2006). Gene expression analysis of ELF-MF exposed human monocytes indicating the involvement of the alternative activation pathway. Biochim. Biophys. Acta. 1763: 402-412.
18. Maercker, C. (2005). *In vitro* Gene Expression Studies and Their Impact on High Content Screening Assays in EMF Research. Application of Proteomics and Transcriptomics in EMF Research, Helsinki, Finland, Oct30−Nov1, Available online: www.cost281.org/download.php.fid= 792.
19. Marchionni A., Paffi M. and Pellegrino P. (2006) "Comparison between low-level 50 Hz and 900MHz electromagnetic stimulation on single channel ionic currents and on firing frequency in dorsal root ganglion isolated neurons," *Biochimica et Biophysica Acta*, vol. 1758, no. 5, pp. 597–605.
20. Martin Blank and Reba Goodman, (2004) "Initial Interactions in Electromagnetic Field-Induced Biosynthesis", *Journal of Cellular Physiology* 199:359–363.
21. Morabito C., Rovetta F., Bizzarri M., Mazzoleni G., *et al.,* (2010) "Modulation of redox status and calcium handling by extremely low frequency electromagnetic fields in C2C12 muscle cells: a real-time, single-cell approach," *Free Radical Biology and Medicine*, vol. 48, no. 4, pp. 579–589.
22. Moshe G., Yaara P., Alexandra B., Yoram W., *et al.,* (2008) "Microbial Growth Inhibition by Alternating Electric Fields" *Antimicrobial Agents and Chemotherapy*, Vol. 52, No. 10. pp 3517–3522.
23. Obermeier A., Matl F., Friess W. and Stemberger A. (2009) "Growth Inhibition of Staphylococcus aureus Induced by Low-Frequency Electric and Electromagnetic Fields", Bioelectromagnetics 30:270 – 279.
24. Performance Standards for Antimicrobial Susceptibility Testing; (2007) Seventeenth Information Supplement, Vol. 27, No. 1, M100-S17.
25. Piacentini R., Ripoli C., Mezzogori D., Azzena G.B., and Grassi C., (2008) "Extremely low-freauency electromagnetic fields promote in vitro neurogenesis via upregulation of Cav1- channel activity," *Journal of Cellular Physiology*, vol. 215, no. 1, pp. 129–139.
26. Segatore B., Setacci D., Bennato F., Cardigno R., *et al.,* (2012), "Evaluations of the effects of extremely low-frequency electromagnetic fields on growth and antibiotic susceptibility of Escherichia coli and Pseudomonas aeruginosa ," *International Journal of Microbiology*, Volume 2012, Article ID 587293, 7 pages.
27. Taber H. W., Mueller, J. P., Miller, P. F. & Arrow, A. S. (1987) "Bacterial uptake of aminoglycoside antibiotics," Microbiological Reviews, vol. 51, no. 4, pp. 439–457.
28. Van der Wolf, J.M.; Van Beckhoven, J.R.C.M., De Haan, E.G. *et al.,* (2004). Specific detection of *Ralstonia solanacearum* 16S rRNA sequences by AmpliDet RNA. European J. of Plant Path. 110: 25–33.
29. Vianale G., Reale M., Amerio P., Stefanachi M., Di Luzio S., and Muraro R., (2008) "Extremely low frequency electromagnetic field enhances human keratinocyte cell growth and decreases proinflammatory chemokine production," *British Journal of Dermatology*, vol. 158, no. 6, pp. 1189–1196.
30. Wolf F. I., Torsello A., Tedesco B., *et al.,* (2005), "50Hz extremely low frequency electromagnetic fields enhance cell proliferation and DNA damage: possible involvement of a redox mechanism," *Biochimica et Biophysica Acta*, vol. 1743, no. 1-2, pp. 120–129.
31. X. Q. Ke, W. J. Sun, D. Q. Lu, Y. T. Fu, and H. Chiang, (2008) "50Hz magnetic field induces EGF-receptor clustering and activates RAS," *International Journal of Radiation Biology*, vol. 84, no. 5, pp. 413–420.