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Growth, Morphology, Cross Stress Resistance and Antibiotic Susceptibility of *K. pneumoniae* Under Simulated Microgravity

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Abstract

Spaceflights results in the reduction of immune status of human beings and increase in the virulence of microorganisms, especially gram negative bacteria. The growth of *Klebsiella pneumoniae* is enhanced by catecholamines and during spaceflight, elevation in the levels of cortisols occurs. So it is necessary to know the changes in physiology, virulence, antibiotic resistance and gene expression of *K. pneumoniae* under microgravity conditions. The present study was undertaken to study effect of simulated microgravity on growth, morphology, antibiotic resistance and cross stress resistance of *K. pneumoniae* to various stresses. The susceptibility of simulated microgravity grown *K. pneumoniae* to ampicillin, penicillin, streptomycin, kanamycin, hygromycin and rifampicin were evaluated. The growth of bacteria was found to be fast compared with normal gravity grown bacteria and no significant changes in the antibiotic resistance were found. The bacteria cultured under microgravity conferred cross stress resistance to acid, temperature and osmotic stress higher than the normal gravity cultured bacteria but the vice versa was found in case of oxidative stress.

Key Words : Simulated microgravity, *K. pneumoniae*, growth analysis, antibiotic sensitivity, TEM, SEM

1. Introduction

Long term spaceflights affects not only the various functions of human systems like demineralization of bones, skeletal atrophy, anaemia, renal lithiasis and suppression of immune systems but also leads to occurrence of diseases by the microorganisms (Guegionou et al., 2009). It is important for micro-organisms to sense the various environmental changes and produce appropriate cellular responses in order to survive. Microorganisms survive even under extreme conditions and they are able to adapt to various physical stresses like pH, temperature, oxygen levels, osmotic pressure and nutrient availability by sensing the changes through sensors and receptors (Nickerson et al., 2004**).** Loss of anaerobic, gain in aerobic microorganisms was observed in Apollo and Skylab crews and opportunistic pathogens like *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacteriaceae* were isolated from cosmonauts (Taylor, 1974). *Citrobacter*sp., *Enterobacter*sp., and *Klebsiella* sp., were found to be the frequent isolates isolated from crew members after post-flight. It was also found the flight travel leads to bifido and lactoflora deficiency leading to disbacteriosis (Pierson, 1993; Castro et al., 2004; Ilyin, 2005). *Staphylococcus*, *Bacillus*, *Micrococcus*, *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Enterococcus*, *Haemophilus*

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and many other genus were found in Mir environment (Novikova, 2004).

Microgravity is a unique environment and the impact of microgravity on physiology, virulence and gene expression on microorganisms can be effectively studied using ground based models with the high aspect rotating vessel. Previous studies by Nickerson et al., (2000) revealed that the microgravity induces changes in virulence, stress resistance, protein expression of *Salmonella enterica* serovar typhimurium compared with the *Salmonella* grown under normal gravity. The growth was found to be higher and changes in gene expression were observed with *Escherichia coli* under the conditions of modeled reduced gravity (Vukanti, 2008). *Staphylococcus aureus* grown under simulated microgravity decreased the production of proteins and virulence determinants (Rosado et al., 2010). An increase in alginate production and increased heat and oxidative stress resistance was noticed with the *Pseudomonas aeruginosa* PAO1 to low shear modeled microgravity (Crabbe et al., 2010).

Klebsiella pneumoniae is a gram-negative, lactose fermenting, facultative anaerobe and it is found in mouth, skin and intestines of the human beings. *K. pneumoniae* is responsible for a wide range of infections like pneumonia, urinary tract infections, cholecystitis, diarrhoea, upper respiratory tract infection and osteomyelitis. *Klebsiella* infections are found commonly in the immuno compromised people. As the immune systems of humans are compromised in space and *K. pneumoniae* was found to thrive in the space, the present study was undertaken to analyze physiological changes and stress responses of bacteria *K. pneumoniae* cultured under simulated microgravity. The growth of bacteria under normal gravity and simulated microgravity were measured and Transmission Electron Microscopy and Scanning Electron Microscopy were performed to analyze changes in the morphology of bacteria. The antibiotic resistance was found to be increased in both gram-positive and gram negative bacteria based on the experiments conducted on Soviet and American Vessels (Tixador et al., 1985; Lapchine et al., 1985). The antibiotic sensitivity assay was performed using five different antibiotics with cultures cultured under normal and simulated microgravity. The ability of bacteria *K. pneumoniae* to resist various other stresses like acid, thermal, osmotic and oxidative stress were examined using the cultures from simulated microgravity and compared with the control normal gravity grown cells.

2. Materials and methods

2.1. Bacterial strain and growth conditions

All experiments were performed using *Klebsiella pneumoniae* subsp. *pneumoniae* (KACC 11402) obtained from Korean Agricultural Culture Collection. Pure cultures were maintained on petriplates for the experiments. Bacterial cultures were cultured overnight in the nutrient broth at 37ºC in a shaking incubator shaking at 150 rpm. The cultures were diluted to 1:100 and then inoculated into High Aspect Ratio Vessel (HARV)reactor and same dilution was used for culturing cells under normal gravity. HARV reactor creates reduced sedimentation, low shear and low turbulence conditions similar to the environment conditions exhibited during space flight. The HARV reactor was completely filled with nutrient broth and care was taken to ensure the complete removal of air bubbles. The incubation temperature for both normal and microgravity was maintained at 37°C and a rotation of 25 rpm. All the experiments were performed in triplicates.

2.2. Growth kinetics

The cell density was measured at a regular interval of 4 hours upto 24 hours by using UV-Vis Spectrophotometer at optical density 600 nm. 1 ml of

the culture was removed and simultaneously 1ml of fresh medium was added, to maintain zero head space in the reactor. The total culture volume was maintained as 50 ml during the experiments for both normal and simulated microgravity experiments.

2.3. TEM and SEM analysis

To determine the morphology of bacterial cells cultured under normal gravity and simulated microgravity TEM and SEM analysis were carried out. The bacteria were recovered by centrifugation and the recovered cells were washed twice with phosphate buffered saline. The washed cells were fixed using 1.5% glutaraldehyde for two hours at room temperature. The fixed cells were then centrifuged, washed once with 70% ethanol and twice with 100% ethanol. These cells were then dispersed into sterile distilled water and TEM analysis was performed using HITACHI-JP/H7600 instrument functioning at an accelerating voltage of 100kV. The air dried cells were mounted on metallic stub, sputtered using Osmium and observed under Bio-LV SEM S-3000N at an accelerating voltage of 10,000 volts and magnification of 18,000 X.

2.4. Acid stress survival test

The ability of bacterial cells cultured under normal gravity and simulated microgravity to survive in acid stress was determined using citrate buffer of pH 3.5. The *K. pneumoniae* cells were cultured for 14 hours to reach the mid-log phase, the cells were retrieved and used for the experiment. The bacterial cells were introduced into citrate buffer and incubated at room temperature under static conditions. Samples were taken immediately for counting the colony forming units at t_0 min. Samples were then collected at every 10 min of interva lupto 60 min, diluted and 100 µl of culture plated onto nutrient agar and colonies were counted.

2.5. Thermal stress survival test

The thermal stress analysis were carried out at three different temperatures 45ºC, 50°C and 55ºC. The 14 hr grown bacterial cultures were immediately incubated statically at 45ºC, 50°C and 55ºC for 30 min. The samples were obtained at 0 min and after 30 min of incubation and 100 µl serial diluted samples were spread onto the nutrient agar plates. The colonies were counted after 12 hr of incubation at 37ºC.

2.6. Osmotic stress survival test

Sodium chloride of three different concentrations 1.5 M, 2.0 M and 2.5 M were used to analyze the ability of bacteria to withstand the osmotic stress. Briefly the mid-log phase cells were collected and sodium chloride was added to make the desired concentrations 1.5 M, 2.0 M and 2.5 M. The cells were then incubated at 37°C for 6 hr, samples were collected at 0 min and after 6 hr of incubation, diluted, spread plated 100 µl of culture and counted the colonies after 12 hr.

2.7. Oxidative stress survival test

The oxidative stress was given to bacterial cultures by addition of required amount of 30% hydrogen peroxide to make a final concentration of 30 mM. Cultures were then incubated at room temperature for 30 min. Samples were retrieved at 0 min and 30 min and plated 100 µlof serially diluted culture and colony counting was done after overnight incubation at 37°C.

2.8. Antibiotic susceptibility test

The disc diffusion method was employed for determining the antibiotic susceptibility of *K. pneumoniae* cultured at normal gravity and simulated microgravity. Solid plates were spread with 100 µl of normal and microgravity grown cultures. Whatman 6 mm filter paper discs were prepared and sterilized. The discs were loaded with 50, 100, 150, 200 µg of

five different antibiotics rifampicin, β- lactam antibiotics ampicillin, penicillin, aminoglycoside antibiotics kanamycin, rifampicin and dried under sterile conditions. The dried discs were placed on *K. pneumoniae* inoculated plates. The plates were then incubated at 37°C for 12 hours. The diameters of inhibition zones were measured in millimeters. The tests were performed in triplicates.

3. Results

3.1. Effect of microgravity on the growth of *K. pneumoniae*

The *K. pneumoniae* was cultured on nutrient broth and growth rate was measured at every four hours of interval in UV-Vis spectrophotometer. The growth curve plotted using triplicates values of experiment performed were presented in Fig. 1. The growth of *K. pneumoniae* under simulated microgravity was observed to be higher compared with bacteria grown under normal gravity. Comparison of growth curves of normal gravity grown culture and simulated microgravity in Fig. 1 indicated shortened lag phase with the microgravity grown cultures and cell density was observed to be higher with microgravity grown *K. pneumoniae* than the normal gravity grown bacteria.

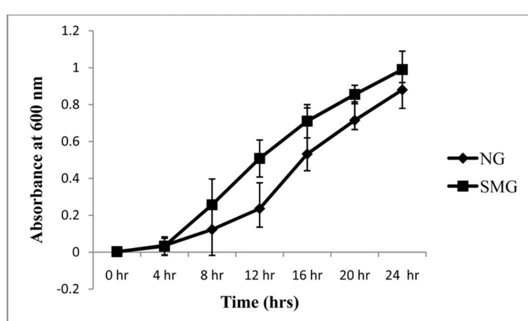

Fig. 1. Growth of *K. pneumoniae* in nutrient broth measured at 600nm using UV-Vis Spectrophotometer grown in normal gravity (NG) and simulated microgravity (SMG). Errors bars represent the standard error of the mean of the triplicates.

3.2. Morphological analysis

The microbial cells cultured under simulated microgravity and normal gravity were studied under transmission electron microscope; no differences in their structure could be noted as seen in the Fig. 2. The scanning electron microscopic analysis revealed difference in their external structure and clumping of cells as in the Fig. 3.

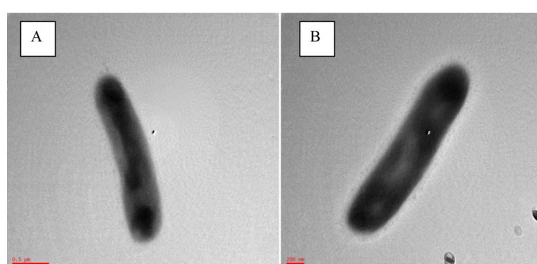

Fig. 2. Transmission electron microscopic photographs of *K. pnuemoniae* grown under A) normal gravity and B) simulated microgravity. (Magnification: X62600).

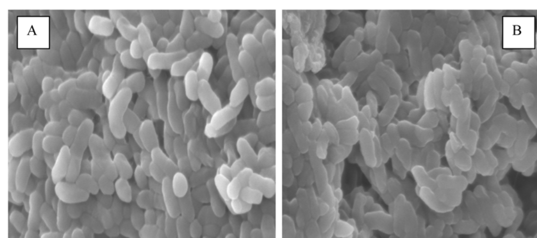

Fig. 3. Scanning electron microscopic images of *K. pnuemoniae* grown under A) normal gravity and B) simulated microgravity. (Magnification: X18000).

3.3. Acid stress analysis

K. pneumoniae grown under simulated microgravity was able to resist the acid stress comparatively to normal gravity cultured cells. The results were presented in Fig. 4. The normal gravity grown *K. pneumoniae* were able to withstand the stress upto 30 min, whereas the microgravity grown cultures can withstand upto a maximum time of 50 min. Complete loss of viable cells were observed at 30 min with normal gravity cultured bacteria as seen in Fig.4.

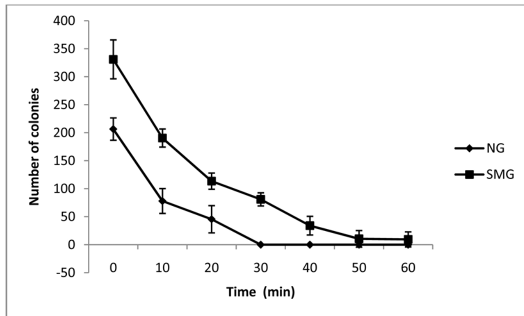

Fig. 4. Colonies formed by *K. pnuemoniae* with normal gravity (NG) and simulated microgravity (SMG) grown cells under acid stress (pH 3.5). Error bars indicate the standard error of the mean.

3.4. Thermal stress analysis

The log phase cells collected at mid log phase were subjected to various temperatures of 45°C, 50ºC and 55°C and the results obtained were presented in Fig.5 and Fig.6. The plates A and C of Fig. 6 represent the colonies formed at 10^{-5} dilution for the normal gravity and microgravity cultured *K. pneumoniae* at 55°C, after the exposure to thermal stress for 30 min the viability of the bacteria was found to be reduced as seen in the plate D for the microgravity cultured bacteria and no bacterial colony was found even at 10^{0} dilution as observed in plate B of Fig. 6. The cells cultured under simulated microgravity were found to withstand thermal stress at all the three temperatures tested. The cells grown under normal gravity were able to withstand the temperatures 45°C, 50ºC whereas they cannot thrive at 55°C, but the cells grown under simulated microgravity can withstand even the 55ºC thermal stress, but the survival rate was quite low compared with 45°C and 50ºC temperatures.

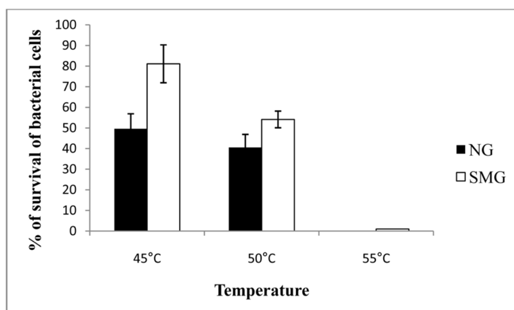

Fig. 5. Cross resistance of *K. pneumoniae* to thermal stress at 45ºC, 50°C, 55ºC grew under normal gravity (NG) and simulated microgravity (SMG). Error bars represents standard error of the mean of triplicates.

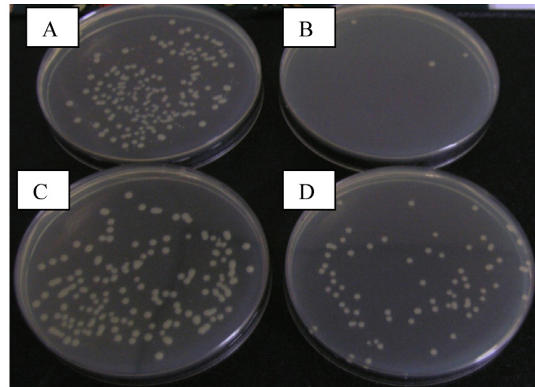

Fig. 6. Spread plating of *K. pneumoniae* culture exposed to 55°C thermal stress, cultured under normal gravity A) 0 min, before exposure to stress (10^{-5} dilution) C) 30 min after exposure to stress and under simulated microgravity (10^{0} dilution) B) 0 min, before exposure to stress (10^{-5} dilution) D) 30 min after exposure to stress (10^{0} dilution).

3.5. Osmotic stress analysis

The osmotic stress was induced by addition of sodium chloride at various concentrations 1.0 M, 1.5 M and 2.0 M to cultures grown at normal gravity conditions and simulated microgravity. Analysis of viability of the cultures revealed that both cultures were not able to withstand higher osmotic stress induced by 2.0 M NaCl but can with stand 1.0 M and 1.5 M NaCl stress. The results were presented in Table 1. and Fig.7. The plates A and C of Fig. 7 represent the colonies formed before osmotic stress and B and D represents after stress of normal and simulated microgravity grown cultures respectively. The colonies formed were found to be reduced from 10^{-5} to 10^{0} under osmotic stress in case with both the cultures but the number of colonies after stress with

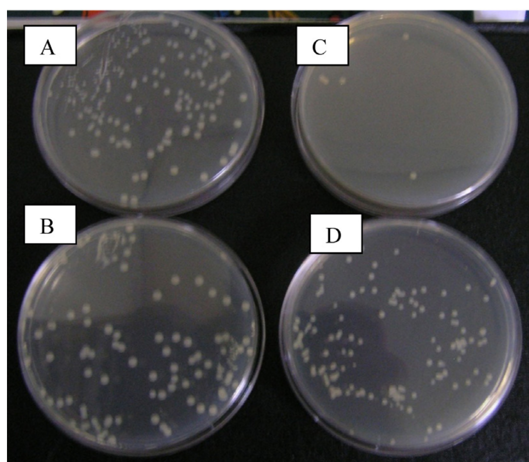

Fig. 7. Growth of *K. pneumoniae* before and after 6 hrs treatment with 2M NaCl A) and C) bacteria cultured under normal gravity (10^{-5} dilution), B) and D) bacteria cultured under simulated microgravity (10^{0} dilution).

Table 1. Tolerance of *K. pneumoniae* to the osmotic stress cultured under normal gravity (NG) and simulated microgravity (SMG)

% of survival after 6 hrs at 37°C	1.5M NaCl		2.0M NaCl	
	NG	SMG	NG	SMG
	0.004	0.03	0.00	0.001

microgravity cultured bacteria was higher when compared with normal gravity grown cultures.

3.6. Oxidative stress analysis

The microgravity and normal gravity grown *K. pneumoniae* cells were subjected to 30 mM hydrogen peroxide stress and results were obtained as in the Fig.8. The results show that bacterial cells cultured under normal gravity and simulated microgravity can withstand oxidative stress but normal gravity cultures can withstand higher oxidative stress comparatively to the simulated microgravity gravity grown bacterial cells with the case of *K. pneumoniae*.

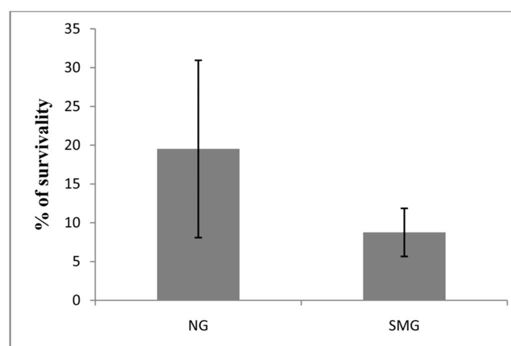

Fig. 8. Effect of hydrogen peroxide on the survival rate of *K. pneumoniae* grown under normal gravity (NG) and simulated microgravity (SMG). Error bars indicates standard error of the mean of triplicates.

3.7. Antibiotic susceptibility test

The antibiotic susceptibility test performed using antibiotics ampicillin, penicillin, streptomycin, hygromycin, kanamycin and rifampicin indicated that *K. pneumoniae* are susceptible to all the antibiotics tested. The antibiotics streptomycin, kanamycin, rifampicin gave higher zone of inhibition. The obtained zone of

Table 2. Influence of simulated microgravity on antibiotic resistance of *K. pneumoniae* at various concentrations (NG: normal gravity, SMG: simulated microgravity). Values are indicated as mean±standard deviation of the triplicates

Antibiotic	Concentration of the antibiotic (µg/disc)							
	50		100		150		200	
	NG	SMG	NG	SMG	NG	SMG	NG	SMG
Ampicillin	15.5±0.7	14.5±07	19.5±2.1	19.0±0.0	21.0±2.8	21±1.4	21.5±0.7	21.0±1.4
Penicillin	8.0±0.0	8.5±0.7	12.0±2.8	11.0±1.4	14.0±1.4	13.5±0.7	14.0±1.4	14.5±0.7
Streptomycin	20±0.0	18.0±2.8	22.5±2.1	21±1.41	23.5±0.7	23.0±1.4	23.5±0.7	24.5±0.7
Hygromycin	17.5±0.7	16.5±0.7	19.5±2.1	19±0.0	21.0±0.0	21.5±0.7	22.0±0.0	22.0±0.0
Kanamycin	19.5±2.1	19.0±0.0	20.0±0.0	21.5±2.1	22.5±2.1	20.5±0.7	24.0±0.0	23.0±1.4
Rifampicin	20.0±1.4	18.0±2.8	20.0±2.8	20.5±0.7	22.0±2.8	21.0±1.4	23.0±1.4	23.0±1.4

inhibition for six antibiotics at four different concentrations against *K. pneumoniae* grown under normal gravity and simulated microgravity are presented in Table 2.

4. Discussion

The effect of simulated microgravity on growth rate of *K. pneumoniae* was studied by measuring optical density at regular time intervals. The growth rate was observed to be similar with *K. pneumoniae* when cultured under simulated microgravity and normal gravity. The lag phase was found to be shortened under simulated microgravityas indicated in Fig. 1., but the exponential phase of growth curve of simulated microgravitywas found to be parallel with the exponential phase of normal gravity grown cultures. Very similar growth pattern was exhibited in *Salmonella enterica* Serovar *Typhimurium, Staphylococcus aureus* and higher cell density with *Escherichia coli* (Nickerson et al., 2000; Rosado et al., 2006; Vukanti et al., 2008) but no difference in initial, exponential growth and final cell densities were observed in case of *Pseudomonas aeruginosa.* A strong correlation was found to exist between the effects of space flight and motility on final cell numbers of bacteria cultured as suspension cultures under microgravity. Generally non-motile bacteria grown in suspension cultures under the microgravity analog devices and space flight had increased cell numbers compared with 1g controls (Benoit and Klaus, 2007). An inter relationship was found between the motile and non motile bacteria on cell number of bacteria during flight induced changes. An increased cell number was observed in non-motile *S. typhimurium*, *E. coli, B. subtilis* (Mattoni, 1968; Klaus et al., 1994; Brown et al., 2002; Mennigmann and Heise, 1994) and no changes in cell number in motile *E. coli*.

The observation of simulated microgravity harvested cells under scanning electron microscope revealed some changes in morphology and the cells were found to be clumped together as seen in the Fig. 3B, but no marked difference in size was observed. The normal gravity cell structures were found to be more defined in structure and look rigid compared with the simulated microgravity grown cells. Previous studies by Wilson et al., (2007) on *S. typhimurium* revealed the formation of an extracellular matrix, cellular aggregation and clumping of cells. The *S. aureus* did not showed any differences in their morphology as observed under SEM and TEM (Rosado et al., 2006). The acid stress assay was carried out to find whether the bacteria can withstand acidic conditions prevailing in the stomach and macrophages. The simulated microgravity gravity cultured cells was found to withstand acid stress for long time interval, compared with the cells cultured under normal gravity.

The primary defense produced by host against bacteria is to increase body temperature to inhibit the growth of bacteria. So the response of bacteria cultured under simulated microgravity and normal gravity to thermal stress was carried out at three different temperatures 45ºC, 50°C and 55ºC. The survival of bacteria under thermal stress at 45ºC, 50°C and 55ºC indicated that normal gravity grown *K. pneumoniae* can withstand temperature stress to a limit but very high increase in temperature could not be tolerated. The simulated microgravity grown bacteria are able to withstand very high temperature stress of 55°C, but the survival rate is not too high only less than 1% of survival rate exists. The survival rate of bacteria cultured under normal and microgravity showed 49.62% and 81.13% at 45ºC and 40.55% and 54.15% at 50°C respectively, revealed that the simulated microgravity grown culture have more thermal resistance than to normal gravity grown culture.

Survival of bacterial cells to the osmotic stress created by sodium chloride indicated that simulated

microgravity grown *K. pneumoniae* can bear the osmotic stress. Previous studies on *S. typhimurium* (Wilson et al., 2002) demonstrated that modeled microgravity grown cells are able to tolerate the 2.5 M NaCl stress, but modeled microgravity cultured *K. pneumoniae* could not tolerate high salt stress of 2.5 M NaCl, no colonies were formed after 6 hours of incubation at 37ºC. The analysis performed at 1.5 M and 2.0 M NaCl concentrations were found to formthe colonies after 6 hours of incubation at 37°C, but the cells cultured under normal gravity conditions thrived at 1.5 M NaCl but lost the viability at 2.0 M NaCl concentration.

Oxidative stress is created within the phagocytes to lyse the bacterial cells. To determine the effect of simulated microgravity on bacterial oxidative stress resistance, the bacterial cells cultured under simulated microgravity were exposed to hydrogen peroxide. The Oxidative stress induced by hydrogen peroxide addition into bacterial culture revealed more than two fold difference between the simulated microgravity and normal gravity cultured cells. Normal gravity grown cells resisted the oxidative stress higher as compared with the simulated microgravity grown cells. Normal gravity grown *S. typhimurium* had more resistance to hydrogen peroxide than the 1Xg grown cells (Wilson et al., 2002). The genes involved in the protection of cells against oxidative stress were found to be down regulated under low shear modeled microgravity growth conditions (Wilson et al., 2002a).

The efficacy of *K. pneumoniae* to resist β-lactam antibiotics and aminoglycosides were tested on both microgravity and normal gravity grown cultured cells. The simulated microgravity cultures less produced low zones of inhibition when compared with zone of inhibition obtained from normal gravity cultured *K. pneumoniae*, but the difference was not found to be significant. This could be attributed to the fact that study was conducted only with the bacterial cells obtained from normal gravity and simulated microgravity conditions, not by the addition of antibiotic and prevailing same condition. *E. coli* cultures grown in solid agar plates to eliminate the fluid effects, it was found that the *E. coli* cultures had higher growth compared with the ground controls. The *E. coli* was also cultured with addition of gentamicin to the agar and found that same effect of gentamicin was observed with both cultures on space flight and ground controls (Kacena and Todd, 1999).Previous studies by (Tixador et al., 1985; Lapchine et al., 1994) showed that microorganisms become resistance to antibiotics under space conditions. The study by (Poliokarpov and Bragina, 1989) revealed that factors related to isolated, confined environments contributed to an increase in resistance of antibiotics. The microorganisms cultured in an airtight environment for 96-175 days were found to have increased the resistance spectrum. *S. aureus* cultures obtained from normal gravity and low shear modeled microgravity showed no significant different in the antibiotic susceptibility (Rosado et al., 2006). Long term exposure to microgravity made the bacteria more susceptible to most of the antibiotics challenged but not to all, and their response to antibiotics was found to be different for each species (Juergensmeyer et al., 1999).

5. Conclusion

Simulated microgravity conditions increased growth of *K. pneumoniae* affected the morphology of bacteria. The simulated microgravity cultured bacterial cells were found to confer cross resistance to osmotic, thermal, acid and temperature stresses but susceptible to the oxidative stress. No distinct difference in antibiotic resistance could be detected. Further gene expression studies are necessary to detect the mechanism behind cross resistance of *K. pneumoniae* under simulated microgravity conditions.

Acknowledgement

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