African Journal of Microbiology Research Vol. 6(3), pp. 611-616, 23 January, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1005
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

In vitro effect of ozonated saline on microorganisms involved in pancreatic and peripancreatic necrosis infection in severe acute pancreatitis

Al Hajjar Nadim[1], Flaviu Pitu[1], Emoke Pall[2], Florina Pitu[1], Ilie Suarasan[3], Calin. Popa[1] and Nicodim Fiţ[2]*

[1]Institute of Gastroenterology-Hepatology, University of Medicine and Pharmacy Cluj-Napoca,"Prof. O. Fodor", 19-21 Croitorilor Street, Cluj-Napoca, Romania.
[2]University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, 3-5 Manastur Street, 3400 Cluj-Napoca, Romania.
[3]Technical University of Cluj-Napoca, 400020, 15 C. Daicoviciu Street, Cluj-Napoca, Romania.

Accepted 18 November, 2011

The unsatisfactory results obtained in the treatment of severe acute pancreatitis or the "great abdominal drama"; with pancreatic and peripancreatic infected necrosis require new therapeutic methods. This paper aims to evaluate the antimicrobial effect of ozonated saline on bacterial and yeast species commonly involved in pancreatic and peripancreatic necrosis infection in severe acute pancreatitis. Tests were conducted on four bacterial species (*Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 10536, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* ATCC 27853) and one *Candida albicans* ATCC 90028 strain. Ozonated saline and Imipenem (antibiotic) was used for all microbial strains. Two types of tests were used: the first on solid medium to determine the number of colonies (CFU/mL) and the second type of tests was the liquid medium for MIC determination. The results show that ozonated saline caused a reduction in the number of bacteria and yeast colonies even in small concentrations in plates. Minimum inhibitory concentrations obtained were very small for the strains of *S. aureus* ATCC 6538P (0.001563 mg/mL) and *E. coli* ATCC 10536 (0.003125 mg/mL) and; somewhat higher for *K. pneumoniae* (0.05 mg/mL), *P. aeruginosa* ATCC 27853 (0.1 mg/ml) and *C. albicans*. These results are encouraging in terms of possible use by peritoneal lavage of ozonated saline for pancreatic necrosis infection in acute pancreatitis.

Key words: Ozonated saline, antimicrobial effect, acute pancreatitis.

INTRODUCTION

Severe acute pancreatitis or the "great abdominal drama", as named by Dieulafoy represents about 20% of all cases of acute pancreatitis (Al Mofleh, 2008). Once primed, severe disease is followed by the development of pancreatic and peripancreatic necrosis leading to serious complications, both local and systemic. In 30-70% cases, necrosis infection takes place (Hans and Bettina, 2007), the main late complication in severe acute pancreatitis, which despite intensive treatment, is followed in up to 70% of cases by death (Linhua et al., 2010). Unsatisfactory results of classic medical and surgical treatment of severe acute pancreatitis with pancreatic and peripancreatic infected necrosis require new therapeutic methods, able to reduce the unacceptably high mortality rate of this disease (Farkas et al., 2006; Adler et al., 2003; Wig et al., 2004).

In recent years, several studies have attempted to evaluate the effectiveness of ozone therapy in the treatment of severe local and systemic infections (Białoszewski and Kowalewski, 2003; De Souza et al., 2010;

*Corresponding author. E-mail: nfit@usamvcluj.ro.

Yamanel et al., 2011). Although controversial, this method of treating patients with a mixture of oxygen and ozone has been used for years as auxiliary method of conventional anti-infectious therapy, especially in those cases where traditional treatment has proved ineffective or had variable results.

In gas phase, ozone has an obvious antimicrobial effect, being one of the most powerful oxidants, but its use in medicine is limited because of the strong toxic effect on the respiratory system, with consequences on the whole body (Dyas et al., 1983; Bocci et al., 2009).

In order to reduce the toxicity of ozone, but maintaining the antimicrobial effect, various ways of use were tested, bubbling ozone in aqueous medium and topical application of these solutions to avoid main adverse effects (Ozmen et al., 1993; Białoszewski et al., 2010).

The present study aimed to evaluate the *in vitro* antimicrobial effect of ozone saline solution on the main microorganisms involved in pancreatic and peripancreatic necrosis overgrowth of severe acute pancreatitis.

MATERIALS AND METHODS

Microorganisms and culture mediums

Four bacterial strains were used for the experiment: *S. aureus* ATCC 6538P, *E. coli* ATCC 10536, *K. pneumoniae* and *P. aeruginosa* ATCC 27853 and a strain of *C. albicans* - ATCC 90028, the species of microorganisms were selected on the basis that, they are among the most frequently reported pathogens involved in pancreatic and peripancreatic necrosis overgrowth of acute pancreatitis. The chosen culture mediums were in accordance with the technique in use. Mueller Hinton Agar (Merck) was used for initial bacterial strains growth and for *C. albicans* Sabouraud dextrose agar (Merck). Saline (0.9% NaCl) was used in the dilution medium in order to obtain a standard optical density (OD), and as liquid medium for the growth of bacteria, Mueller Hinton broth and Sabouraud broth together with dextrosis were used for *Candida*.

Ozonated saline

Ozonated saline was used with a concentration in ozone of 4 g/1000L obtained extemporaneously with the ozone device solutions by corona discharge method. Ozone concentration in saline was measured using an ozonometer (Anseros). The advantages of this method offers the production of ozone in elevated concentrations and ozone concentration adjustment. Antimicrobial effect of ozonated saline was tested by direct contact similar to difusimetric method of germs sensitivity determination and the determination of minimum inhibitory concentration (MIC) microdilution method similar to the protocols recommended by the Clinical and Laboratory Standards Institute.

Technique of microorganism sensitivity testing in ozonated saline

Determination of antimicrobial effect of ozonated saline by microorganisms growing on solid medium: the technique has been achieved relying on the bactericidal/ inhibitory effect of ozone from saline in direct contact with microbial cells. To this end each bacterial strain was grown in Petri plates with Mueller Hinton agar at 37 ± 1°C for 24 h. A suspension of microorganisms in saline with 10^8 UFC/ml was obtained from 24 h cultures of each bacterial strain. Plates were flooded with a quantity of 1 ml of microorganism suspension and after drying the surface, 4 g/1000L ozonated saline was added in quantity of 5 ml (0.02 mg O_3) which was maintained for 10 min at laboratory temperature, and was removed. After drying the surface, the plates were incubated at 37 ± 1°C for 24 h. In the case of *C. albicans* strain, we used the same technique by using the appropriate medium, incubated to a temperature of 28 ± 1°C for 48 h. Subsequently the plates were read by counting colonies with a semiautomatic Colony Counter.

For each sample we used double testing. We also used a positive control using the antibiotic Imipenem. The same technique was applied for the antibiotic, flooding the plates with 5 ml (100 mg Imipenem).

Determination of minimum inhibitory concentration (MIC) using microdilution technique

This technique was performed in microplates with 96 wells using Mueller Hinton broth dilution medium and for each microorganism ozonated saline with the following concentrations in mg/mL:0.2, 0.1, 0.01, 0.05, 0.025, 0.0125, 0.00625, 0.00625, 0.003125, 0.0015625, 0.000781 and 0.000390. Antibiotic (Imipenem) was used in the test with the following concentrations in mg/mL: 25000, 12500, 6250, 3125, 1562.5, 781.25, 390.62, 195.31, 97.65, and 48.82. Plates inoculated with bacteria (10^8 CFU/ml) were incubated at 37 ± 1°C for 24 h and those inoculated with *Candida* were incubated at 28 ± 1°C for 48 h. Results interpretation was performed with a microplate reader at a wavelength of 450 nm after priorly mixing the plates. Limit in establishing MIC was ≥ 0.1DO difference.

Statistical analysis

For each sample we used positive and negative control optical density. The experiment was repeated twice and each sample was read 3 times (n = 2x3). Results were expressed as average data from media. Test t-test was performed between test samples and blanks yielding statistically significant results. Differences between averages were considered significant when p value <0.05 and distinct significant when p<0.001. The program used for statistical processing was GraphPad InStat 3.

RESULTS AND DISCUSSION

Determination of antimicrobial effect of ozonated saline by microorganisms growing on solid medium

The results of this study show that ozonated saline had an inhibitory effect on all the tested microorganism strains. It is noted that low levels of 0.02 mg O_3 in plates determined microorganisms development inhibition (Table 1). The most sensitive bacterial specie to ozone solution was *E. coli* ATCC 10536 which, after treatment with ozonated saline 10^6 UFC/mL compared with positive control of 10^8UFC/mL (Figure 1). On *C. albicans* strain, an inhibitory effect similar to that obtained in case of bacteria 10^7 UFC/mL was observed. Tests conducted by the same method with imipenem but with a greater amount of active ingredient (100 mg Imipenem/plate) shows that this product resulted in inhibition of bacterial

Table 1. Sensitivity test results (CFU/mL) of microbial strains to ozone saline and Imipenem, by the method of cultivation on solid medium.

Species	CFU/mL		
	Ozone (0.02 mg O_3/plate)	Imipenem (100 mg Imipenem/plate)	C+
Staphylococcus aureus ATCC6538P	10^6	0	10^8
Escherichia coli ATCC 10536	10^5	10^2	10^8
Klebsiella pneumoniae	10^7	10^3	10^8
Pseudomonas aeruginosa ATCC 27853	10^7	10^3	10^8
Candida albicans ATCC 90028	10^7	10^8	10^8
P value	p<0.05	p<0.05	-

C+ = positive control.

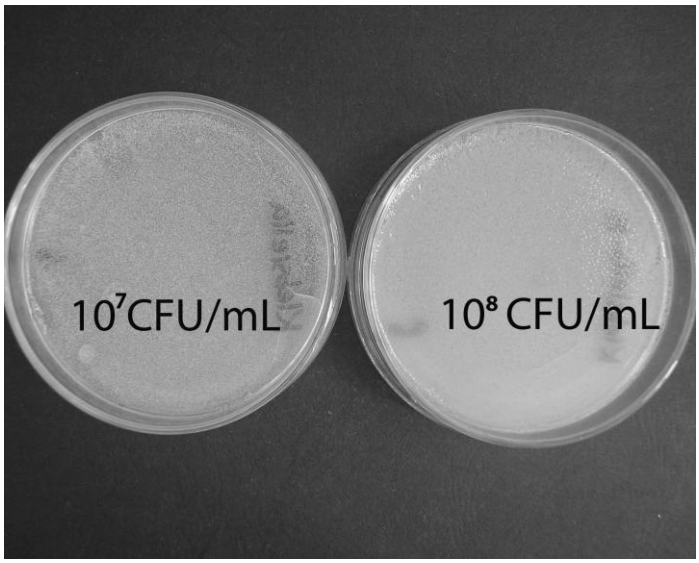


Figure 1. Culture of *Klebsiella pneumoniae* treated with ozone (left) and untreated (right).

growth in plates, totally inhibiting strain of *S. aureus* ATCC6538P. Compared to the other bacterial strains, antibiotic had a decreased effect resulting in inhibition of colony development in plates of 10^2 CFU/mL for *E. coli* ATCC 10536 and 10^3 CFU/mL for *K. pneumoniae* and *P. aeruginosa* ATCC. There was no inhibitory effect on *C. albicans* strain. After analyzing the results, a significant difference between samples treated with ozonated saline or Imipenem and untreated samples (positive control) can be observed, aspect supported by the values of p<0.05.

Determination of minimum inhibitory concentration (MIC) using microdilution method

Analysis of the results show that data on antimicrobial effect of ozonated saline is similar to that achieved in sensitivity test by cultivation on solid medium. This can be seen from the data processing of optical densities and extrapolated in very small minimum inhibitory concentrations (Figure 2) on *S. aureus* ATCC 6538P and *E. coli* ATCC 6538P strains. For the other tested strains, higher MIC were obtained, which proves that they are not as sensitive to ozone. Imipenem tests on the same microbial strains shows that strains of *S. aureus* ATCC6538P and *E. coli* ATCC 10536 had a good *pneumoniae* strain, higher MIC were obtained, ranging 6250 µg/mL, being less sensitive to the antibiotic. In case of *P. aeruginosa* ATCC 27853 strain a value of 390.62 µg/mL was obtained. For *C. albicans* ATCC 90028 strain we observed that the concentrations used in the experiment are not affecting the development (Table 2).

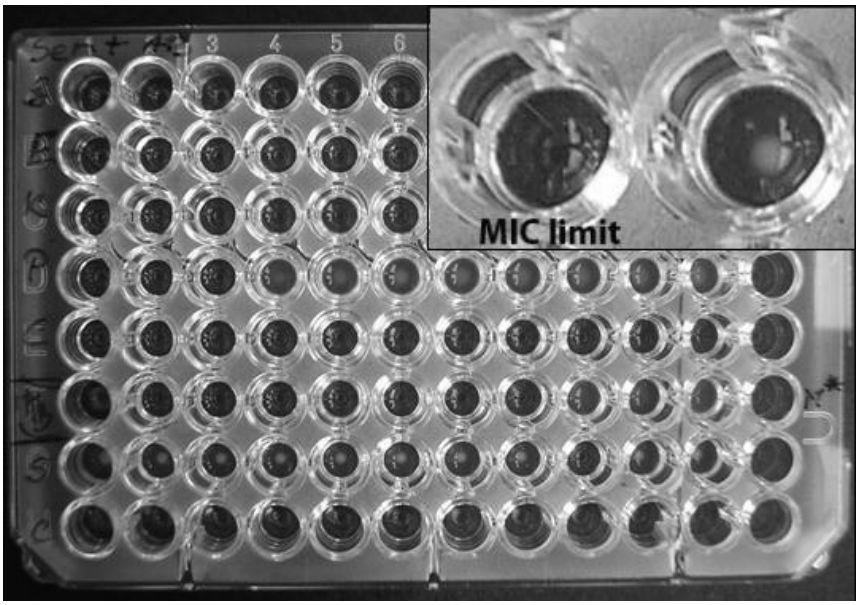


Figure 2. Picture of MIC micromethod: MIC determination limit setting.

Table 2. Results obtained in case of MIC (µg/ml) test with ozonated saline and imipenem on microbial strains.

Species	MIC µg/mL	
	Ozonated saline	Imipenem
Staphylococcus aureus ATCC 6538P	0.001563	195.31
Escherichia coli ATCC 10536	0.003125	< 48.82
Klebsiella pneumoniae ATCC	0.05	6250
Pseudomonas aeruginosa ATCC 27853	0.1	390.62
Candida albicans ATCC 90028	0.1	> 25000 *

* = does not affect development.

By analyzing OD obtained with ozonated saline as seen in Appendix A, we concur there is a significant difference between the OD which represent MIC for each microbial strains and the positive control OD $p<0.001$. Appendix A also shows strains of *S. aureus* ATCC6538P, *E. coli* ATCC 10536 and *C. albicans* ATCC 90028 were inhibited by the activity of ozonated saline in extremely small quantities.

Analyzing optical densities (Appendix B) obtained with different concentrations of imipenem on microbial strains of the experiment we observed that at concentrations between 390.62 and 48.82 µg/mL, all bacterial strains were inhibited in their development. However, the MIC for these bacterial species fall within CLSI standards as strains resistant to this product compared to the minimum standard of ≥ 8 µg/mL. Differences of optical density setting MIC obtained from bacteria also in this case very significant compared with positive control samples $p<0.001$. It is clearly visible that similar to previous test, the strain of *C. albicans* is not sensitive to the antibiotic, OD obtained for this strain being similar to positive control.

The studies demonstrate antibacterial and antifungal effect of ozonated saline. Minimum inhibitory concentration of ozonated saline was demonstrated at low concentrations of 0.001563 µg/mL for *S. aureus* and somewhat higher than 0.1 for *P. aeruginosa.* Antifungal effect is present on the strain of *C. albicans* at 0.1 µg/mL. Our studies are consistent with other authors who report minimal bactericidal concentrations from 1 ppm or between 20 and 40ppm (Hamelin and Chung, 1974; Masaka et al., 1982). Hibben and Stotzkey, (1969) examined the effect of ozone on the germination of fungi and compared with bacteria, observed a lower sensitivity of yeasts. *Aspergillus spp*. appears to be moderately sensitive to ozone. Dyas et al. (1983) observed a reduced sensitivity of *Aspergillus* spp. and *C. albicans* to ozonated solutions. The same authors mention a decrease

in the number of bacterial colonies of approximately 95% after exposure to ozone of bacterial cultures.

Recent research by Rodrigo Altenfelder Silva et al. (2009) also shows good antibacterial effect on *E. coli*. Data recorded by authors in vivo show a decrease in the number of colonies (CFU/mL) from an average of 19 000 at a concentration of ozone 42 µg/mL to 850 CFU/mL concentration of 62 mg/mL. This demonstrates a more intensive antimicrobial effect of high ozone concentrations. Our studies demonstrate by the determination of colony forming units and by determining the minimum inhibitory concentration, good antibacterial effect in small quantities of ozonated saline on bacterial species frequently involved in pancreatic necrosis infections. A similar effect on *C. albicans* strain tested with the same ozonated solution was observed. Using these low concentrations of ozone in solution can explain the limited antimicrobial effect by decreasing the number of colonies from plates and not their total disappearance.

Conclusions

Tests regarding the sensitivity of microorganisms to ozonated saline revealed that low concentrations of this solution has inhibitory effect for some bacteria and yeasts species most frequently isolated in pancreatic and peripancreatic necrosis in acute pancreatitis. The inhibition effect to the development of microorganisms could be demonstrated both by determining CFU/ml and by determining the MIC using microdilution technique.

ACKNOWLEDGEMENT

This work was supported by PNCDI - IDEI Nr. 829/2008 grant.

REFERENCES

Al Mofleh IA (2008). Severe acute pancreatitis: pathogenetic aspects and prognostic factors. World J. Gastroenterol, 7:14(5):675-84.

Hans G Beger, Bettina M Rau (2007). Severe acute pancreatitis: Clinical course and management. World J Gastroenterol. 14: 13(38): 5043-5051.

Linhua Y, Xiayue Huang, Yuqin Li, Ruihua Shi, Guoxin Zhang (2010). Prophylactic Antibiotics Reduce Pancreatic Necrosis in Acute Necrotizing Pancreatitis: A Meta-Analysis of Randomized Trials. Dig. Surg., 27:442–449.

Farkas G, Marton J, Mandi Y, Leindler L (2006). Surgical management and complex treatment of infected pancreatic necrosis: 18-year experience at a single center. J. Gastrointest. Surg., 10: 278-285.

Adler DG, Chari ST, Dahl TJ, Farnell MB, Pearson RK (2003). Conservative management of infected necrosis complicating severe acute pancreatitis. Am. J. Gastroenterol., 98: 98-103.

Wig JD, Mettu SR, Jindal R, Gupta R, Yadav TD (2004). Closed lesser sac lavage in the management of pancreatic necrosis. J. Gastroenterol. Hepatol., 19: 1010-1015.

Białoszewski D, Kowalewski M (2003). Superficially, longer, intermittent ozone theraphy in the treatment of the chronic, infected wounds. Ortop. Traumatol. Rehabil., 30: 5(5):652-8.

De Souza YM, Fontes B, Martins JO, SannomiyaP, Brito GS, Younes RN, Rasslan S (2010). Evaluation of the effects of ozone therapy in the treatment of intra-abdominal infection in rats. Clinics, 65(2):195-202.

Yamanel L, Kaldirim U, Oztas Y, Coskun O, Poyrazoglu Y, Durusu M, Cayci T, Ozturk A, Demirbas S, Yasar M, Cinar O, Tuncer SK, Eyi YE, Uysal B, Topal T, Oter S, Korkmaz A (2011). Ozone therapy and hyperbaric oxygen treatment in lung injury in septic rats. Int. J. Med. Sci., 3; 8(1): 48-55.

Dyas A, Boughton BJ, Das BC (1983). Ozone killing action against bacterial and fungal species: microbiological testing of a domestic ozone generator. J. Clin. Pathol.. 36: 1102-4.

Bocci V, Borrelli E, Travagli V, Zanardi I (2009). The ozone paradox: ozone is a strong oxidant as well as a medical drug. Med. Res. Rev. 29(4): 646-82.

Ozmen V, Thomas WO, Healy JT, Fish JM, Chambers R, Tacchi E, Nichols RL, Flint LM, Ferrara JJ (1993). Irrigation of the abdominal cavity in the treatment of experimentally induced microbial peritonitis: efficacy of ozonated saline. Am. Surg., 59(5): 297-3.

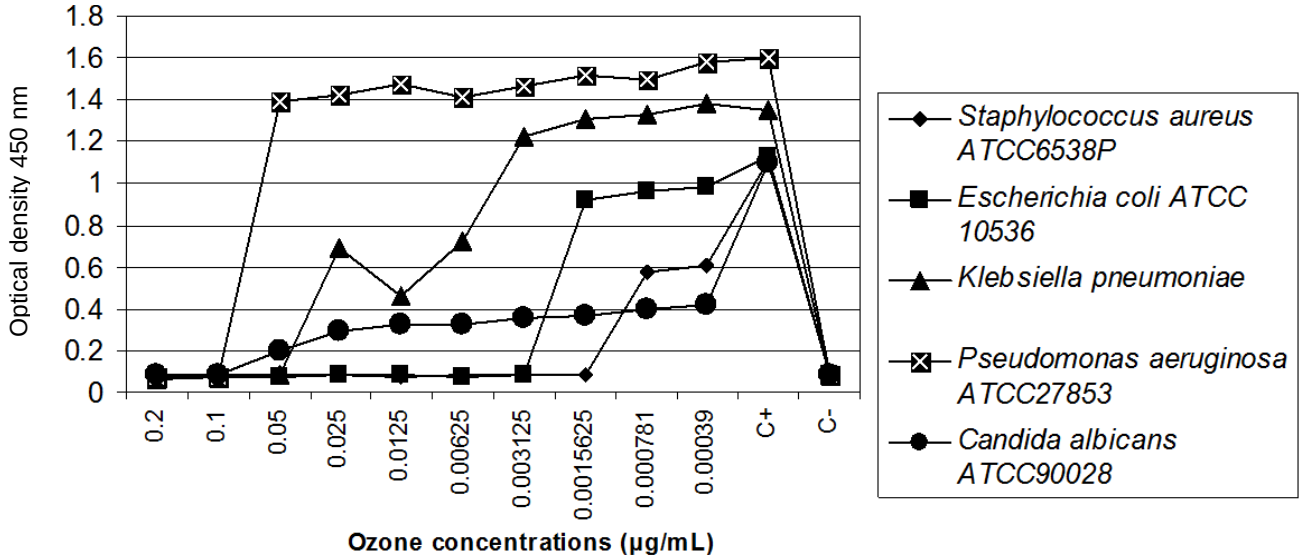
Białoszewski D, Bocian E, Bukowska B, Czajkowska M, Sokół-Leszczyńska B, Tyski S (2010). Antimicrobial activity of ozonated water. Med. Sci. Monit. 16(9): MT71-5.

Hamelin C, Chung YS (1974). Optimal conditions for mutagenesis by ozone in E coli K12. Mutat. Res., 24: 271-9.

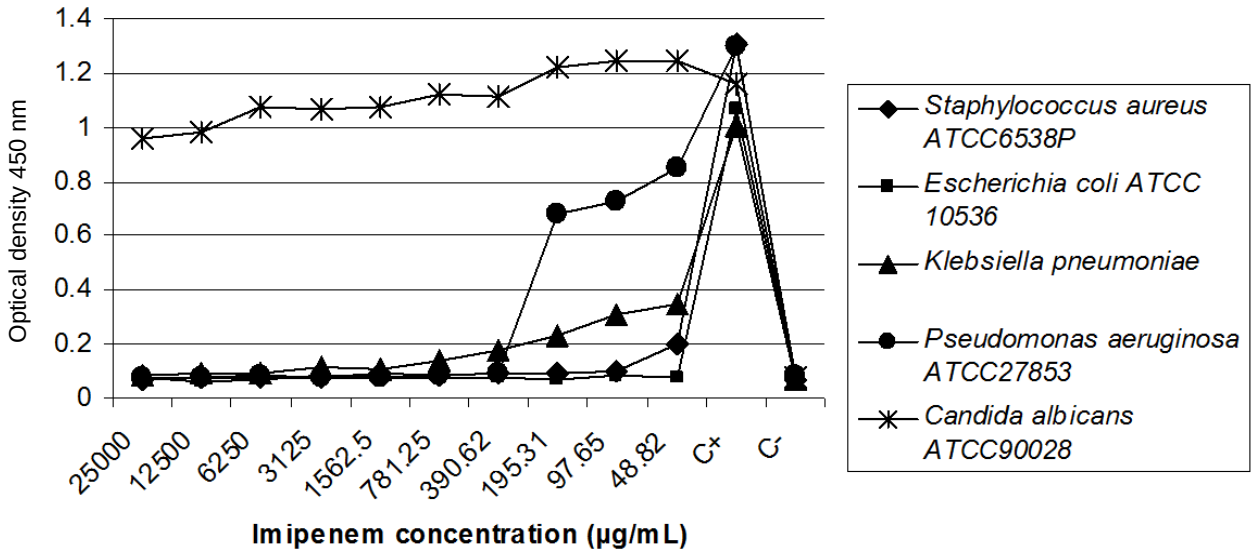
Masaka T, Kubota Y, Namiuchi S (1982). Ozone decontamination of bioclean rooms. Appl. Environ. Microbiol. 43: 509-13.

Hibben CR, Stotzky G (1969). Effects of ozone on germination of fungus spores. Can J Microbiol;15:1187-96.

Rodrigo Altenfelder Silva, José Eduardo Rosseto Garotti, Renata Santos Bittencourt Silva, Alessandra Navarini, Adhemar, Monteiro Pacheco Jr. (2009). Analysis of the bactericidal effect of ozone pneumoperitoneum. Acta. Cirúrgica Brasileira, 24(2): 124.



Appendix A. The value of optical density (450 nm) read according ozonted saline concentration on each tested microbial strain using microdilution method.



Appendix B. The value of optical density (450 nm) read depending on Imipenem concentration for each microbial strain tested by microdilution method.