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HOSPITAL EFFLUENT CARE WITH OZONE FOR INACTIVATION OF POTENTIALLY PATHOGENIC BACTERIA

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ABSTRACT

Hospital effluents are characterized as possible vehicles for the dissemination of numerous pathogenic microorganisms and other contaminants and, when left untreated, represent a potential risk to man and the environment. The objective of this research was to evaluate the presence of arable pathogenic microorganisms present and to verify the efficacy of ozone in the disinfection of effluents from a public hospital in the city of Uberlandia-MG. Hospital effluent collections were performed at two points, with a distance of 200 m: the first point, from the Emergency Room, wards, intensive care unit, surgical center and laboratory and the second point, hemodialysis, kitchen, laundry and clothing. Sampling was performed for three months. The samples were evaluated for the presence of total mesophiles, total and thermotolerant coliforms, Escherichia coli and Salmonella spp. The effluent was treated with ozone for different periods of time. The presence of mesophiles, coliforms, Escherichia coli and Salmonella spp. was verified in both effluents and in all months of evaluation. Ozone was effective in decontamination of hospital effluent, requiring 20 minutes to eliminate bacterial load.

Keywords: Coliforms, *Escherichia coli*, *Salmonella*, Sewage, Ozonization.

1. INTRODUCTION

Health services stand out for the production of relevant environmental impacts due to the complexity and breadth of medical-hospital activities, which produce a diversity of chemical, pharmaceutical and infectious residues, constituting important sources of contamination for the internal and external population of the hospital environment[1-4].

Hospital effluents are characterized as possible vehicles for the dissemination of numerous pathogenic microorganisms and other contaminants and, when untreated, represent a potential risk to man and the environment, with the possibility of contamination of drinking water sources, both surface and

underground, and multidrug-resistant strains to antibiotics can present risks to public health if they reach the supply system[5,6].

Hospital effluents come from various activities, such as laundry water, boilers, cafeterias, employee and patient sanitary facilities, cleaning waste, surgical centers, hemodialysis sector, clinical and anatomopathological analysis laboratory, and may present high concentrations of heavy metals, radioisotopes, medications, cytostatic agents, hormones and disinfectants[7]. The presence of pathogenic microorganisms in the sewage may come from excrements from sick individuals[8]. According to Tundisi[9], sewage released *in natura*, or insufficiently treated, contains a high load of pathogenic organisms, which implies a large amount and spatial distribution in regions with high population density and intense human activities. Hospital effluents have higher levels of antimicrobial-resistant bacteria than effluents derived from other sources[2,10-12]. According to Calijuri *et al*.[13], the spread of resistant microorganisms contributes to the increase in hospital and community infection rates.

Sewage treatment is considered as a basic infrastructure requirement of urban centers because the various sewage agents that cause environmental pollution, mainly chemical pollutants or that cause diseases that affect the health of the population, are removed from the liquid environment before they are returned to nature. For the most part, treatment methods involve sophisticated systems due to diversification and quantity of waste[7]. Many disinfection technologies, such as chlorination, ultraviolet (UV) light, electrochemical disinfection (ED) and ozonization, have been used in large wastewater treatment facilities. However, the adaptation of conventional disinfection systems to small-town treatment plants is often difficult due to financial constraints or technological barriers[14-19]. This context aimed to evaluate the presence of arable pathogenic microorganisms and to verify the efficacy of ozone in the disinfection of effluents from a public hospital in the city of Uberlandia-MG.

2. MATERIALS AND METHODS

2.1. Survey place

According to data from the Brazilian Institute of Geography and Statistics[20], the city of Uberlandia is the most populous in the Region of Triangulo Mineiro and the second most populous in Minas Gerais, with an estimated population in 2021, was 706,597 inhabitants, occupying a territorial area of 4,115,206 km^2.

According to the 2018 Basic Sanitation Ranking of Brazil, published by the Trata Brazil Institute[21], the city ranked third among the 100 cities in Brazil for the indicator of urban sewage service and the top second position for the treated sewage index referred to water consumed.

The institution analyzed was a public university hospital located in the city of Uberlandia. It is a large institution with a built area with more than 51,000 m^2. Reference for high complexity care for the micro and macro-region of the state of MG.

Totals 520 beds distributed in various specialties that include General Emergency Room, Adult, Pediatric and Neonatal Intensive Care Unit, Gynecology Ward, Obstetrics, Medical Clinic, Pediatrics, Internal Medicine, Psychiatry, General surgical and obstetric Center, Burn Unit, Hemodialysis,

Tomography Services, X-ray, Clinical Analysis Laboratories, Pharmacy, Outpatient Clinics, Legal Medicine Service, Kitchen, Laundry and Clothing.

2.2. Sample collection

Samples from the hospital effluent were collected directly from the depletion boxes of the General Hospital, carried out in December, January and February, in the morning, in the interval from 7:30 a.m. to 8:30 a.m. on Mondays of a random week referring to the three months of the research. The collections were performed in triplicate.

The samples were collected at two points, with the distance between them being 200m. The first point, coming from the Emergency Room (ER), wards, Intensive Care Unit (ICU), surgical center and laboratory (sample 1) and the second point, coming from hemodialysis, kitchen, laundry and clothing (sample 2).

A collector was used in the sewer mouth to collect the sample. Immediately the material was packed in amber glass jars, sterile 500 mL, which were accommodated in isothermal boxes with ice, transported to the laboratory for microbiological analysis and ozone treatment.

2.3. Microbiological analysis

For the detection of total mesophiles, total coliforms and thermotolerant, *Escherichia coli* (*E. coli*) and *Salmonella spp*. the methodology recommended by the American Public Health Association (APHA)[22] was used.

For the total coliform count, serial dilutions were prepared from the sample in saline solution (NaCL, 0.85%), and a 1mL aliquot was aseptically transferred to a series of test tubes containing inverted Durham tube and lauryl tryptosis broth (OXOID®). The tubes were gently stirred and incubated for 48 hours at 37ºC. Gas production and lactose fermentation were observed as positive reactions. The thermotolerant coliforms were determined using serial dilutions of the sample and an aliquot of 1mL was transferred to tubes containing inverted Durham tube and Bile Bright Green broth (OXOID®). The tubes were gently stirred and incubated for 48 hours at 44.5ºC. Gas production and lactose fermentation were observed as positive reactions.

Pathogenic bacteria isolated from the hospital effluent were identified based on colony appearance, Gram staining, growth in selective media and biochemical tests according to the standard methods for the examination of water and effluents developed by APHA[22].

For the detection of total mesophiles, *E. coli* and *Salmonella spp*., aliquots of 0.1 mL of samples diluted in series (from 10^{-3} to 10^{-6}) were spread in MacConkey agar (OXOID®), *Salmonella-Shigella* agar (OXOID®), EMB Levine agar (OXOID®) and nutrient agar (HIMEDIA®). The plates were incubated at 35°C for 24-48 hours for bacterial growth, when colonies were counting. Results were expressed in colony-forming units (CFU), except for *Salmonella spp*. evaluated by presence or absence. The isolates were identified using the API 20E (Analytical Profile Index, BioMérieux) system to identify potentially pathogenic bacteria. All procedures were performed in triplicate.

2.4. Effluent treatment with ozone

Ozone was produced by means of a corona generator (Ozone & Life®). Pure oxygen was supplied through an oxygen cylinder. The ozone gas constantly produced by the equipment was driven through a silicone tube to the diffuser through poral stone, thus generating 2ppm min. L^{-1}[23,24]. The effluents (100mL) were directly exposed to the ozone through the diffuser, at a controlled temperature of 25ºC.

Before the application of ozone, 0.1 mL of solution was removed to verify the number of CFU of the microorganisms.

Each effluent was exposed to ozone for 5, 10, 15, 20, 25, 30 minutes. To verify the effect of ozone on microorganisms, 0.1mL of sample was collected in each interval. After each collection, the samples were inoculated and cultivated in triplicates, following the methodology recommended by the APHA[22]. The efficacy of ozone in the control of microorganisms was determined by the count of CFU. The treatment was considered effective when no growth of microorganisms was observed.

2.5. Statistical analysis of the results

The results were tabulated and submitted to the following analyses: (1) Descriptive analysis of microbial count of several microorganisms according to the collection site, period and antimicrobial treatment of ozonization; (2) Application of the Variance Analysis test with multiple comparison test of Games-Howell, when $p<0,05$, for the comparison of collection periods in relation to the type of microorganism and the treatment of ozonization; (3) Mann-Whitney test at $p<0.05$ to compare the quantification of microorganisms according to the evaluated site, both for the different microorganisms and for the different ozonization treatments; (4) Line charts for the visualization of the decrease in microbial count resulting from the increase in the period of exposure of the sample to ozone.

All statistical tests were applied with a significance level of 5% ($p<0.05$). Software used was Minitab 17 (Minitab Inc.).

3. RESULTS AND DISCUSSION

Table 1 shows the results of the microbial count of each of the sites evaluated. The results indicate the presence of significant differences in all the analyses made for the months when the same site was evaluated. In relation to total mesophiles, in both locations. In relation to total mesophiles, in both locations. The highest microbial load was in January, differing significantly from February to site 1 (PS, wards, ICU, operating room and laboratory), and from December and February to site 2 (hemodialysis, kitchen, laundry and clothing).

In the case of total coliforms, site 1 presented higher microbial load in February, whereas in site 2 there was a higher count of these microorganisms in January. Thermotolerant coliforms showed higher microbial counts in December and January at sites 1 and 2, respectively. When *E. coli* was considered, higher counts were found in February in both sites, however, for site 2, there were no significant differences between February and December, and these differed significantly only from January (Table 1).

In a way, there is no tendency to observe which microorganisms presented higher microbial counts over the months evaluated, however, in general, it is possible to highlight high counts of total mesophiles,

total coliforms and thermotolerant coliforms in January, while *E. coli* stood out in February. In addition, the results suggest that there are statistically significant differences between the months of analysis for all microorganisms evaluated, showing that, depending on the month of collection, the microbial count can vary significantly.

Several studies have demonstrated the presence of total coliforms, thermotolerant coliforms, *E. coli* and *Salmonella* in hospital, household, industrial, agricultural effluents, among others [1,4,10,12,25-27], confirming the results found in this study.

Studies conducted by Fekadu *et al*.[27] in two hospitals in Southern Ethiopia showed that the wastewater from both hospitals contained pathogenic bacteria of *Salmonella spp*., *Shigella* spp. and *Staphylococcus aureus*, and potentially pathogenic identified as *E. coli*. In addition, this study revealed that the two hospital effluents contained antibiotic-resistant bacteria that were released into the bodies of receiving water, which posed a threat to public health. These authors state that large amounts of antimicrobials are used in hospitals to care for patients. As antibiotics are partially metabolized and residuals reach hospital effluents, exposing bacteria to a wide range of biocides, they can act as selective pressure for the development of resistance.

The results obtained by Amaya *et al*.[26] when evaluating the sanitary quality of different water sources in the city of León (Nicaragua) showed that *E. coli* isolates obtained from hospital effluent presented higher levels of antibiotic resistance compared to *E. coli* isolates from other aquatic samples. These authors state that untreated wastewater is considered hazardous to health and potential vector of infectious diseases transmitted by water.

Table 1. Microbial count of hospital effluent samples from a hospital in the city of Uberlandia-MG.

Microorganism	Place	Months of analysis			P value[1]
		December	January	February	
Total mesophiles	1	$2.6,10^8 \pm 2.64,10^7$ ab	$2.99,10^8 \pm 9.01,10^6$ a	$1.91,10^8 \pm 2.25,10^7$ b	0.010
	2	$5.33,10^8 \pm 3.78,10^7$ b	$6.76,10^8 \pm 1.15,10^7$ a	$3.7,10^7 \pm 1.73,10^6$ c	<0.001
Total coliforms	1	$1.55,10^7 \pm 8.66,10^5$ c	$2.23,10^7 \pm 5.77,10^5$ b	$3.93,10^7 \pm 1.15,10^6$ a	<0.001
	2	$1.43,10^6 \pm 1.15,10^5$ c	$4.43,10^7 \pm 1.52,10^6$ a	$7.3,10^6 \pm 1.73,10^5$ b	<0.001
Thermotolerant coliforms	1	$3.33,10^6 \pm 1.52,10^5$ a	$4.47,10^5 \pm 6.8,10^3$ b	$3.76,10^5 \pm 5.77,10^3$ c	<0.001
	2	$2.23,10^4 \pm 5.77,10^2$ c	$4.13,10^5 \pm 5.77.,10^3$ a	$5.53,10^4 \pm 1.15,10^3$ b	<0.001
E. coli	1	$1.63,10^5 \pm 1.15,10^4$ b	$5.16,10^4 \pm 2.88,10^3$ c	$2.53,10^5 \pm 5.77,10^3$ a	<0.001
	2	$1.52,10^4 \pm 2.52,10^2$ a	$3.36,10^3 \pm 2.31,10^2$ b	$1.66,10^4 \pm 5.77,10^2$ a	<0.001
P. aeruginosa	1	-	$1.23,10^6 \pm 2.51,10^5$	-	-
	2	-	$5.16,10^4 \pm 1.52,10^3$	-	-

[1]Value p referring to the Variance Analysis test at $p<0.05$. Different letters on the same line indicate significant differences by the Games-Howell multiple comparison test at $p<0.05$. Site 1: Emergency Room (ER), wards, ICU, operating room and laboratory. Location 2: Hemodialysis, kitchen, laundry and clothing. **Source:** The authors (2022).

Table 2 presents the results of the quantification of the evaluated microorganisms comparing the studied sites. There were no significant differences in the quantification of total mesophiles (p=0.250), total coliforms (p=0.249) and *P. aeruginosa* (p=0.081) when sites 1 and 2 were compared; however, in the case of thermotolerant coliforms (p=0.006) and *E. coli* (p<0.001), the differences observed were significant, suggesting lower count for site 2 (hemodialysis, kitchen and laundry) and both cases.

As additional analysis, the presence of the microorganism *Salmonella* spp. it was also evaluated at both sites over the three-month study collection period. According to the results obtained, S*almonella spp*. in both places, and there is, in no case, absence of the aforementioned microorganism. Thus, the hospital sewage from site 1 and site 2 showed positive results for *Salmonella spp*.

The presence of pathogenic bacteria in hospital effluents and the impact on public health of the release of resistant bacteria into the receiving environment involves a number of aspects to be considered. First, if resistant bacteria are carrying a transmissible gene, they can transfer these resistance genes to other bacteria in the community, so that the infection caused by these bacteria is usually difficult to treat, and also decreases the antibiotic pool for treating bacterial infections[8,28]. Secondly, this organism can act as a vector or reservoir of resistant genes[12,25-27].

Hospital effluents when untreated are important contaminants of drinking water springs, both superficial and underground, and antibiotic-resistant multidrug-resistant bacterial strains can pose public health risks if they reach the supply system[19].

The methods of treatment of hospital effluents are diverse, however often their implantation becomes difficult due to financial restrictions or technological barriers[15]. In recent years the use of ozone as a disinfectant agent has intensified due to its high oxidative activity. The germicidal effect of ozone consists in totally or partially destroying the cell wall, resulting in lysis of microorganisms[29]. In addition, the breakdown of chromosomes by ozone of nitrogen-carbon bonds between sugar and bases, DNA hydrogen bonds, as well as phosphate sugar bonds lead to depolymerization and leakage of cellular constituents and irreversible enzymatic inhibition[28].

Table 2. Descriptive statistics of the quantification of each of the microorganisms comparing the sites evaluated.

Microorganism	Place		P value[1]
	1	2	
Total mesophiles	$2.5,10^8 \pm 5.0,10^7$	$4.15,10^8 \pm 2.91,10^8$	0.250
Total coliforms	$2.57,10^7 \pm 1.06,10^7$	$1.76,10^7 \pm 2.01,10^7$	0.249
C. thermotolerant	$1.38,10^6 \pm 1.46,10^6$	$1.63,10^5 \pm 1.87,10^5$	0.006
E. coli	$1.56.,10^5 \pm 8.77,10^4$	$1.17,10^4 \pm 6.33,10^3$	<0.001
P. aeruginosa	$1.23,10^6 \pm 2.51,10^5$	$5.16,10^4 \pm 1.52,10^3$	0.081

[1]P value referring to the Mann-Whitney test at p<0.05. **Source:** The authors (2022).

Table 3 shows the results of the antimicrobial action of ozone in the samples obtained over the three months of sampling in relation to the collection sites. The results suggest the existence of significant differences in the microbial count of treatments with and without ozone, and for the treatment without

ozone, the highest counts of microorganisms were observed in January.

From the moment the ozonization treatment began, it was possible to observe that with 5 minutes of ozonization, site 1 presented lower microbial count in December and site 2 presented lower counts in January and February. The antimicrobial action of ozone in 10 minutes had a more significant effect on the microbial count from February to site 1, and in this period, the quantification of microorganisms was lower than the others determined for the other months. For site 2, it was not possible to compare the evaluated months, because the microbial count for the month of February did not present dispersion.

A result similar to the previous one was observed for the treatment with ozone for 15 minutes, evidencing that February was the one with the lowest microbial count for site 1. The statistical test was not possible to be performed in the treatment of ozonization for 15 minutes for site 2 due to the lack of dispersion in February.

For the treatments with ozone at 20 and 25 minutes, the statistical test was not possible to be performed due to the cancellation of the microbial count in both sites and in all months evaluated. Thus, the efficacy of ozone became consolidated at 20 minutes of treatment, that is, the submission of hospital effluent to ozonization for 20 minutes is effective to cancel the microbial count for the evaluated species.

The activity of disinfection by ozone occurs through two mechanisms: one by direct oxidation of compounds by the ozone molecule and the other by the reaction involving the radical products of ozone decomposition, mainly believed to be, the hydroxyl radical. This radical is highly reactive and has a lifespan of only a few microseconds in the water. The predominant reaction will depend on the characteristics of wastewater[30].

At the beginning of the use of ozone as an antimicrobial agent was characterized by the need to achieve a measurable level of dissolved ozone in treated wastewater, which resulted in high gas dosages that were not economically viable. Studies point to the good efficiency of disinfection of effluents with ozone at low doses transferred and very short contact time[31,32].

Table 3. Descriptive statistics of microbial count of sites evaluated according to antimicrobial action of ozone.

Antimicrobial action of ozone	Place	Months of analysis			P value[1]
		December	January	February	
No ozone	1	$2.6,10^{8}\pm2.64,10^{7}$ ab	$2.99,10^{8}\pm9.01,10^{6}$ a	$1.91,10^{8}\pm2.25,10^{7}$ b	0.010
	2	$5.33,10^{8}\pm3.78,10^{7}$ b	$6.76,10^{8}\pm1.15,10^{7}$ a	$3.7,10^{7}\pm1.73,10^{6}$ c	<0.001
Ozone 5 minutes	1	$1.3,10^{3}\pm3.46,10^{2}$ b	$3.56,10^{3}\pm5.51,10^{2}$ a	$2.93,10^{3}\pm1.15,10^{2}$ a	0.012
	2	$4.0,10^{3}\pm1.0,10^{3}$ a	$1.33,10^{3}\pm1.52,10^{2}$ b	$2.3.,10^{3}\pm1.73,10^{2}$ b	0.004
Ozone 10 minutes	1	$1.23,10^{2}\pm2.5,10^{1}$ a	$2.7,10^{1}\pm2.0,10^{0}$ b	$1.76,10^{1}\pm0.57$ c	0.007
	2	$1.66,10^{2}\pm5.77,10^{1}$	$1.13,10^{1}\pm0.57$	$1.60,10^{1}\pm0.00$	-[2]
Ozone 15 minutes	1	1.33±0.57 ab	7.33±2.08 a	0.66±0.57 b	0.025
	2	5.33±0.57	1.33±0.57	1.00±0.00	-[2]
Ozone 20 minutes	1	0.00±0.00	0.00±0.00	0.00±0.00	-[2]
	2	0.00±0.00	0.00±0.00	0.00±0.00	-[2]

Ozone 25	1	0.00±0.00	0.00±0.00	0.00±0.00	-[2]
minutes	2	0.00±0.00	0.00±0.00	0.00±0.00	-[2]

[1]P value referring to the Variance Analysis test at p<0.05. Different letters on the same line indicate significant differences by the Games-Howell multiple comparison test at p<0.05. [2]Comparison test was not possible to be performed due to one or more variables presented null dispersion. **Source:** The authors (2022).

Table 4 presents the results of the quantification of each of the evaluated microorganisms comparing the studied sites according to antimicrobial treatments using ozone.

The results of Table 4 indicate the absence of significant differences between the evaluated sites, since all P values resulted in higher than the significance level applied in the statistical test (p>0.05). In addition, it is possible to notice a considerable decrease in microbial load as microorganisms are exposed to longer ozone. Thus, the longer the exposure time of microorganisms to ozone, the greater the decrease in microbial load[24,33].

Table 4. Descriptive statistics of microbial load according to ozone treatments and the evaluated sites.

Antimicrobial action of ozone	Place			P value[1]
	N	1	2	
No ozone	9	$2.5,10^8$±$5.0,10^7$	$4.15,10^8$±$2.91,10^8$	0.250
Ozone 5 minutes	9	$2.6,10^3$±$1.06,10^3$	$2.54,10^3$±$1.27,10^3$	0.658
Ozone 10 minutes	9	$5.6,10^1$±$5.2,10^1$	$7.0,10^1$±$8.5,10^1$	0.289
Ozone 15 minutes	9	3.11±3.37	2.75±2.18	0.923
Ozone 20 minutes	9	0.00±0.00	0.00±0.00	-[2]
Ozone 25 minutes	9	0.00±0.00	0.00±0.00	-[2]

[1]P-value referring to the Mann-Whitney test at p<0.05. [2]P-value not resulting due to lack of dispersion. **Source:** The authors (2022).

Studies point to the good efficiency of disinfection of effluents with ozone at low doses transferred and very short contact time[3,4,31,32]. Bustos *et al*.[34] evaluated the application of ozone and UV radiation in the disinfection and oxidation of the organic matter of an effluent and verified that total coliforms showed higher inactivation rates than fecal coliforms in all pH values tested, but the lowest bacterial inactivation was observed at pH 7. Ozone presented low oxidation potential and the best results were obtained for ozone doses of approximately 20 mg min^{-1}, with removals of 72% and 78% of thermotolerant and total coliforms, respectively. In the present study, it was verified that the removal of all microorganisms from the hospital effluent was obtained with ozonization (O_3= 2ppm) for 20 minutes. Kozusny-Andreani *et al*.[24] they evaluated the bactericidal effect of ozone on different concentrations of pathogenic bacteria and different times of ozone exposure and found that efficacy was proportional to bacterial cell concentration and time of exposure to ozone.

Currently there is growing interest in the evaluation of ozone efficiency, in the oxidation of microorganic pollutants present in hospital effluents, since conventional treatment is of high cost, a large area is required, long time and the final result leaves a new residue known as iodine. While the ozonization

of hospital effluents is a new technology, more efficient, less costly and does not generate residues harmful to the environment and human and animal health[3,6,18,19]. Silva[35] states that the use of ozone can also increase the concentration of oxygen in water, reduce organic matter, oxidize ammonia, remove color, nutrients and suspended solids.

According to Vecchia *et al*.[5], from the universe of hospitals, a small minority are endorsed with sewage treatment plants; the absence of treatment of these effluents allows the dissemination of pathogenic microorganisms from the hospital environment to different aquatic matrices, which represents risks to public health.

The samples analyzed in this research prove the presence of several pathogenic microorganisms, demonstrating the importance of managing monitoring and proper disposal of hospital effluents, since they can reach and contaminate a larger geographic area.

The possibilities of treatment of hospital effluents should not be overconsidered, because, with the reduction of microbial load, effluents may cause less impact when discarded in the urban exhaustion system.

4. CONCLUSION

Hospital effluents present survival and reproduction conditions to pathogenic microorganisms as presented in this research, so the need for improvements in the management of these residues is fundamental.

The results suggest that the hospital effluent constitutes an object of analysis with a wide diversity of pathogenic microorganisms with possibilities of reaching water bodies causing impacts to the environment and public health. Therefore, study strategies to expand knowledge about the subject are necessary, and it is important that other methods of treatment of hospital effluents are evaluated, to analyze its efficacy as an alternative in reducing and preventing the spread of multidrug-resistant bacteria.

Conflict of Interest

The authors declare no conflict of interest.

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