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DISINFECTANT-RESISTANT BACTERIA IN BUENOS AIRES CITY HOSPITAL WASTEWATER

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ABSTRACT

Large quantities of disinfectants are used in hospitals, externally on human skin or to eliminate microorganisms from inanimate objects. After use, residual quantities of these products reach the wastewater, exposing the bacteria that survive in hospital wastewaters to a wide range of biocides that could act as a selective pressure for the development of resistance. Increasing attention has been directed recently to the resistance of bacteria to disinfectants. The aim of this paper was to determine the disinfectant bacterial resistance pattern of the microflora released to the urban sewer system by hospital effluents. The characterization of the waste water microflora was performed by determination of the CFU of heterotrophic bacteria, fecal indicator bacteria, *Pseudomonas* sp. and *Staphylococcus sp.*, in a Buenos Aires hospital effluent. The bacterial resistance to the disinfectants more frequently used in the hospital practice, glutaraldehyde, chlorhexidine and povidone-iodine, was then evaluated. Disinfectant resistant bacterial strains were isolated and typified. Between 10^3 and 10^6 chlorexidine resistant bacteria/100 mL were isolated from the samples. Bacteria resistant to other disinfectants ranged between 10^3 and 10^4 /100 mL. The bacterial population resistant to desinfectants to was mainly composed by *Enterobacteriaceae*, *Staphylococcus* spp, and *Bacillus* spp, which are highly associated to nosocomial infections. The results obtained show that the hospital effluents are of importance in the bacterial resistance selection process, particularly in the case of disinfectants.

Key words: disinfectant, resistance, hospital effluent

INTRODUCTION

A variety of substances such as pharmaceuticals, radionucleides and solvents are used in hospitals for medical diagnostics, disinfection and research. After application many non-metabolized drugs are excreted by the patient and enter into wastewater. The microflora of hospital wastewaters is composed by saprophytic bacteria from the atmosphere, soil, medical devices and water employed in the hospital practice; the pathogens are mainly released with the patient excreta.

Large quantities of disinfectants are used in hospitals for a range of purposes. Usually disinfectant compounds are used externally on human skin to prevent or limit microbial infection or for preoperative skin disinfection. They are also used to eliminate microorganisms from inanimate objects (surfaces, instruments). After use residual quantities of disinfectants reach the wastewater (9,22). The hospital effluents are discharged, usually, in the urban sewer system were they mix with other effluents and finally reach the sewage treatment plant. The last step of this process is the release of purified wastewaters to a river, a lake, or to seawaters. Some of these water bodies are sources of drinking water.

Antimicrobial resistance is usually found in the hospital environment. When the antimicrobial drugs attack disease-causing bacteria, they also affect non-pathogenic bacteria in their course. They exterminate these bacteria and make room for more resistant bacterial growth.

The bacteria that survive in hospital wastewaters may be exposed to a wide range of biocides that could act as a selective pressure for the development of resistance. Acquired resistance to biocides may arise by cellular mutation or by the acquisition of genetic elements in the form of plasmids or transposons (21).

Bacterial resistance to antibiotic is a long- established, widely studied problem (6,8,19). Increasing attention has been directed

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recently to the responses of bacteria to biocides. Griffiths *et al.* (5) have observed that *Micobacterium chelonae* isolated from endoscope washer disinfector may be significantly less sensitive to glutaraldehyde in comparison with the type strain. Studies with glutaraldehyde-resistant *M. chelonae* strains suggest that the arabinogalactan/arabinomannan component of the cell wall is associated with resistance (13). Bacterial isolates have been cultured from industrial plants where triclosan and parachlorometaxylenol were manufactured (10). The prevalence of bacteria with reduced susceptibility to benzalkonium chloride was detected in the effluent of a municipal sewage treatment plant (7). Disinfectants and antiseptics are extensively used in hospital practice around the world, nevertheless the profile of resistance to biocides in the hospital effluents has been barely studied.

The aim of this paper was to determine the disinfectant bacterial resistance pattern of the microflora released to the urban sewer system by the hospital effluent. In a first stage, the characterization of the waste water microflora was performed by determination of the number of colony forming units, CFU, of heterotrophic bacteria, fecal indicator bacteria, *Pseudomonas* sp. and *Staphylococcus* sp., in hospital effluent samples. In the next step, the bacterial resistance to disinfectants was studied. The disinfectants more frequently used locally in the hospital practice, glutaraldehyde, chlorhexidine and povidone-iodine, were selected for this study. The resistant strains were isolated and typified for the study of their disinfectant resistance level.

The San Martin hospital is a public health center that provides hospital care in a broad category of illnesses and injuries. This hospital operates with 400 licensed beds (maximum number of beds that the facility can operate) and approximately 300 set-up beds (number of actual beds that are in operation and available for patient use). The Hospital Laboratory conducts test in the following areas: hematology, chemistry, urinalysis, microbiology, blood bank, pathology, and cytology, for both in-patient and out patient services. The San Martin hospital releases daily approximately 560 cubic meters of effluents to the urban sewer system (17).

In Argentina few characterizations of hospital effluents were carried on. One of the main problems of the Buenos Aires city area is the lack of sewer treatment plant. The wastes of the urban sewage system are released to the Rio de la Plata, at a rate of 1.9 millions of m^3/day, with no treatment at all. This river is also the source of water for the plants that provide drinking water to the city.

MATERIAL AND METHODS

Collection of samples

The source of test samples was wastewater obtained from the sewer test chamber, in a representative point located just before the release of the San Martin hospital effluents to the urban sewer system. The sampling was performed during a period of 8 h taking a sample every two hours. The same volume of each partial sample was mixed at the end of the day to obtain the composite sample submitted to biological test. The results reported in this paper correspond to ten representative samples taken monthly during the period October 2003- December 2004.

Microbiological analyses

Total heterotrophic count- Serial 10-fold dilutions of samples were prepared in physiological saline, and 0.1 mL aliquots were streak plated on Tryptic soy agar (TSA). Plates were incubated for 48 h at 28ºC before bacteriological counts were preformed.

Fecal indicator (Total coliform, *Escherichia coli* and *Enterococci*)- One mL aliquot of appropriate dilution were pour plated in duplicate on CHROMagar ECC for coliform and *Escherichia coli*, and Slanetz Bartley agar as a selective media for enterococci. The colony count was carried out after incubation at 35ºC for 48 h.

Pseudomonas sp. and *Staphylococcus* sp. Tenfold dilution of each sample was plated on Cetrimide agar and Baird Parker agar, used for enumeration of *Pseudomonas* and s*taphylococci*, respectively.

Antimicrobial susceptibility testing

The iodine–resistant subpopulation of effluent was selected from the bacterial population by treating wastewater samples 4 minutes with iodine 0.1% (18).

The effluent samples were tested for their resistance patterns determined by dilution agar methods with chlorhexidine 50 mg/L as breakpoint (20).

To evaluate the percentage of glutaraldehyde-resistant bacteria, the effluent samples were added to glutaraldehyde to reach a final concentration of 2% and treated sixty minutes with the disinfectant.

Bacterial identification and test of sensitivity to the biocides

Bacteria tolerant to each disinfectant were identified by different biochemical test, according to the *Manual of Clinical Microbiology* (15).

The susceptibility of the isolated strains to each disinfectant was analyzed:

MIC values of chlorhexidine were determined on tryptic soy agar (TSA) plates containing a range of chlorhexidine concentrations (500-250-125-100-50-25 ppm), inoculated with 0.1 mL of bacterial suspension. All plates were incubated for 48 h at 35ºC. The MIC was the lowest concentration that prevented bacterial growth (23).

Iodination was performed on suspension *ca* 10^7 CFU/mL. Povidone iodine was added to a final concentration of 1%.

Samples were removed at intervals, sodium thiosulphate added (final concentration 0.1%) and bacterial dilutions were plated on Tryptic soy agar (TSA).

For the determination of tolerance level to glutaraldehyde 0.1 mL of the suspension was added to 9.9 mL of glutaraldehyde 2%. Samples were removed at intervals (5-20-60 minutes) and mixed with neutralization medium (5). Bactericide activity was expressed as reduction factors, that is, logarithmic reductions in viable organisms:

Reduction factor = $\log_{10}$ CFU/mL $_{(control)}$ - $\log_{10}$ CFU/mL $_{(treated)}$

RESULTS

The CFU detected in the samples from hospital wastewater are shown in Table 1. Bacterial counts ranged between 1 x 10^2 CFU to 1 x 10^8 CFU/100mL for coliforms; 1 to 4.8 x 10^5 CFU/100mL for *E. coli* and 44 to 1.5 x 10^6 CFU/100mL for enterococci. The proportion of enteric group varied from 58% to 0.75% of the total bacteria.

Pseudomonas was detected in all but two samples. The count of *Pseudomonas* varied from 2 to 800 CFU/mL, Ferreira La Rosa *et al.* (4) detected higher concentration of *Pseudomonas* in hospital de Clínicas de Porto Alegre wastewater. *Staphylococcus* sp. ranged from 2 to 300 CFU/mL.

Median value for the chlorhexidine resistant bacterial population was 6.8 x 10^5 per 100 mL. For other disinfectants, the median values were around 10^3 per 100 mL. The bacterial population showed high resistance to chlorhexidine, a relatively little resistance to glutaraldehyde and to povidone-iodine (Table 2). The resistant bacterial strains for each biocide were isolated and could be identified to at least the genus level (Tables 3 and 4). The MIC of chlorhexidine for the resistant bacteria showed 2 fold increase compared with the sensitive strains. Only Gram-negative rods showed resistance for chlorhexidine, among them *Aeromonas hydrophila* was the most frequently isolated (Table 3). Table 4 shows the activity of glutaraldehyde against resistant strains. Sixty minutes exposure was insufficient to achieve a 5 $\log_{10}$ reduction of isolated *Staphylococcus.* On the other hand the *Staphylococcus* sp. wildtype strain, obtained from Facultad de Farmacia Culture Collection, was susceptible to glutaraldehyde, i.e a 5 log reduction was achieved following exposure for 1minute to 2% glutaraldehyde. Most of the resistant species to glutaraldehyde and povidone-iodine were identified as Gram-positive bacteria.

Table 2. Bacterial resistance to disinfectant.

	Povidone-iodine UFC/100 mL	Glutaraldehyde UFC/100 mL	Chlorhexidine UFC/100 mL
Mean	7.7 x 10^3	5.0 x 10^3	6.8 x 10^5
Minimum	2.0 x 10^3	2.0 x 10^3	1.0 x 10^3
Maximum	2.0 x 10^4	2.4 x 10^4	6.0 x 10^6

Table 3. Bacterial resistance to clorhexidine.

Organism	Tolerance level MIC (mg/L)
Shigella disenteriae	65
Pseudomonas stutzeri	60
Salmonella sp	60
Proteus vulgaris	60
Shigella flexneri	125
Aeromonas hydrophila	125
Alcaligenes sp	80
Aeromonas hydrophila	150
Acinetobacter sp	50
Aeromonas hydrophila	100
Pseudomonas aeruginosa	80

Table 1. Bacteriological profile of hospital wastewater. Data from Hospital San Martín (Buenos Aires) and Hospital de Clínicas (Porto Alegre).

	Total Viable Count UFC/100 mL	Faecal Coliforms UFC/100 mL	*Escherichia coli* UFC/100 mL	Faecal Enterococci UFC/100 mL	*Pseudomonas sp* UFC/mL	*Staphylococci sp* UFC/mL
Mean	1.8 x 10^7 1.0 x 10^7*	1.2 x 10^6	1.1 x 10^5	3 x 10^5	112	85
Minimum	5.3 x 10^5 1.0 x 10^7*	1 x 10^2 4.5 x 10^7**	5 x 102	44 6.6 x 10^6**	<1 1.6 x 10^2**	<1
Maximum	1.4 x 10^8 2.0 x 10^7*	1 x 10^8 1.0 x 10^9**	4.8 x 10^5	1.5 x 10^6 7.5 x 10^8**	800 7.0 x 10^3**	300

Reported by * Ferreira La Rosa *et al.* (1) ** Reported by Ortolan (7).

Table 4. Bacterial resistance to glutaraldehyde and povidone-iodine.

Organism	Tolerance level (Reduction Factor)
Glutaraldehyde-tolerant isolates	
Coagulase negative staphylococci	2.86 log-1 h
Aerocococcus viridans	3.45 log-1 h
Coagulase negative staphylococci	3.56 log-1h
Bacillus circulans	2.30 log- 1 h
Staphylococcus aureus	4.16 log-1h
Povidone-iodine-tolerant isolates	
Staphylococcus epidermidis	2.25 log-5 min
Bacillus coagulans	3.30 log-5 min
Bacillus sphaericus	1.14 log- 5 min
Microbacterium arborescens	3.14 log-5 min
Pseudomonas alcaligenes	1.84 log-5 min
Bacillus sp	2.57 log-5 min

DISCUSSION

The counts of heterotrophic bacteria and fecal indicator bacteria in the hospital samples were similar to those found by Ortolan (8) in the Porto Alegre Hospital de Clínicas wastewater, Brazil. Similar results were reported by Leprat (10) in hospital wastewaters in France. Lucena *et al.* (11) reported the results obtained in the microbiological characterization of municipal sewage plants in Argentina. Considering these results, the San Martín Hospital wastewater showed approximately 10 times lower CUF media than those found in municipal sewage systems. On the other hand, oscillations between maximum and minimum values for faecal indicator bacteria were considerable (Table 1). Emmanuel *et al.* (2) attributed to the presence of disinfectants and antibiotics the high variation in the fecal coliform populations found in hospital wastewater.

Even when the counts of heterotrophic and fecal indicator bacteria in the San Martin effluent were low, the biological risk of the bacteria actually present in the effluents must be high. This risk is mainly associated with the presence of bacteria resistant to antibiotics and disinfectants. In the San Martin wastewater samples an important group of bacteria, namely Gram negate bacilli, resistant to chlorhexidine, was found.

Chlorhexidine is an extensively used handwashing antiseptic. The MIC increase could be the result of different resistance mechanisms, mainly changes in outer membrane proteins (24) or efflux of the disinfectant from the cell (3). The study by Thomas *et al.* (25) lends some experimental support to the idea that repeated exposure to a subinhibitory concentration increases chlorhexidine MICs of *P. aeruginosa.* It is reasonable to assume that in a clinical environment and in the hospital effluents bacteria will be exposed to sub-inhibitory amounts of biocides remaining in the environment. Actually clorhexidine has demonstrated to have a persistent or residual effect after application (24).

Iodine has long been considered an effective antimicrobial agent, especially when used in the form of povidone-iodine. Several instances have been described in which iodophors have been found to be contaminated with *Pseudomonas* sp (1). Pyle *et al*. (18) found that the bacterial susceptibility to iodine was regulated by nutrient status. The resistance increased in nutrient restricted environments like the oligotropic aquatic system found in the hospital wastewaters. Other mechanisms, including cell aggregation, adhesion to surfaces, usually observed in sewage biofilms, may also be involved in halogen resistance.

Little information had been found on the mechanisms of glutaraldehyde resistance. In this paper we report the first isolation of cocci and bacilli glutaldehyde resistants in sewage. The processes involved in glutaraldehyde resistance are not well understood at the present. The main studies performed in Mycobacteria demonstrated that resistance to this compound was related with the cell wall structure. The importance of the wall in the resistance of cocci and bacilli will deserve further studies on the mechanisms of resistance.

Disinfectants present in the hospital wastewater may act as selective pressure for the retention of the plasmids that often contain the genes for resistance to numerous antibiotics (14,22,23,26). It is generally agreed that the selection and dissemination of resistant bacteria in nature should be avoided in order to ensure effective treatment against infectious diseases in humans and maintain an ecological balance that favors the predominance of a susceptible bacterial flora in nature. The indiscriminate use of antimicrobial agents may disrupt the microbial balance in favor of resistance bacteria. In particular, wastewater from hospital plants could play a role in the selection of resistance bacteria in sewage (6,16).

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RESUMO

Resistência bacteriana a desinfetantes em efluentes de um hospital em Buenos Aires, Argentina

Os hospitais utilizam uma grande quantidade de desinfetantes para eliminar microorganismos tanto da pele humana como de superfícies inanimadas. Após sua utilização, esses produtos podem chegar ao esgoto em quantidades

residuais. A pressão seletiva exercida pelos antimicrobianos nos efluentes hospitalares propicia a disseminação de linhagens resistentes. Além dos antibióticos, os desinfetantes podem atuar como agentes seletivos de linhagens resistentes aos antimicrobianos. Este trabalho teve como objetivo o estudo do perfil da resistência aos desinfetantes das bactérias lançadas na rede de esgoto pelo efluente hospitalar. Na caracterização microbiológica do efluente do Hospital de Clínicas Buenos Aires, determinou-se a concentração de bactérias heterotróficas, bactérias indicadoras fecais, *Pseudomonas* sp. e *Staphylococcus* sp. presentes. A resistência aos desinfetantes empregados no hospital, glutaraldeído, iodo povidona, e clorexidina foi então avaliada. Verificou-se a existência de bactérias resistentes à clorexidina em número variando de 10^3 a 10^6 bactérias/100 mL e de bactérias resistentes a outros desinfetantes em uma faixa de variação de 10^3 a 10^4 bactérias/100 mL. Bactérias dos gêneros *Staphylococcus* e *Bacillus*, e da família *Enterobacteriaceae*, envolvidas em infecções hospitalares, apresentaram resistência aos desinfetantes testados. Estes resultados indicam que as águas residuárias de hospitais desempenham um papel de grande relevância na disseminação de linhagens bacterianas resistentes aos desinfetantes no meio aquático.

Palavras-chave: desinfetantes, resistência, efluente hospitalar

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