Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1806-2563
ISSN on-line: 1807-8664
Doi: 10.4025/actascitechnol.v35i1.12951

Chlorine and antibiotic-resistant bacilli isolated from an effluent treatment plant

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ABSTRACT. Resistance to different concentrations of chlorine and the susceptibility to antibiotics by bacteria isolated from the final effluent of the Pici Campus wastewater treatment plant of the Federal University of Ceará (UFC) is evaluated. Twelve strains, morphologically and biochemically identified as belonging to the genus *Bacillus*, were selected. The strains were submitted to sodium hypochlorite at different contact times and tested against the antibiotics amoxicillin, erythromycin, chloramphenicol, tetracycline, and vancomycin. All strains were resistant to concentration 0.1 ppm chlorine up to 30 minutes, but bacteria resistant to concentrations up to 5,000 ppm for 10 minutes were detected. Bacterial growth was impaired in 10,000 ppm concentration. The strains presented three antibiotic resistance profiles, 50% were sensitive to all antibiotics, 25% were resistant to one antibiotic and 25% were resistant to two antibiotics.

Keywords: bacteria, susceptibility, hypochlorite, antimicrobial.

Bacillus resistentes ao cloro e antibióticos isolados de uma Estação de Tratamento de Efluente

RESUMO. O presente trabalho teve como objetivo avaliar a resistência ao cloro bem como a susceptibilidade a antibióticos de bactérias isoladas do efluente final da Estação de Tratamento de Esgoto do Campus do Pici (ETE-PICI) da Universidade Federal do Ceará (UFC). Foram selecionadas 12 cepas, identificadas morfológica e bioquimicamente como pertencentes ao gênero *Bacillus*. Estas cepas foram submetidas à ação de Hipoclorito de Sódio em diferentes concentrações e tempos de contato e frente aos antibióticos amoxicilina, eritromicina, cloranfenicol, vancomicina e tetraciclina. Todas as cepas resistiram a concentrações de 0,1 ppm de cloro por até 30 minutos, sendo detectadas bactérias resistentes a concentrações de até 5.000 ppm, por 10 min. Na concentração de 10.000 ppm não foi observado crescimento bacteriano. Evidenciaram-se três perfis de resistência para os antibióticos testados constatando-se que 50% das cepas foram sensíveis para todos antibióticos, 25% apresentaram resistência a um antibiótico e 25% resistiram a dois antibióticos em duas diferentes combinações.

Palavras-chave: bactérias, susceptibilidade, hipoclorito, antimicrobianos.

Introduction

Intense urbanization has resulted in the production of large amounts of wastewater and sludge. In fact, a water crisis has been caused by pollution from domestic sewers and industrial spills. Residuary waters represent a complex mixture of organic and inorganic substances with a great variety of microorganisms which include bacteria, fungi and protozoa (WILLIAMS; BRAUN-HOWLAND, 2003; SUTHAR, 2009).

Among the wastewater treatment stages relevance is given to disinfection by chlorine due to its relatively reduced concentrations and residual activity required, low costs and easy application (SOUZA; DANIEL; 2005; TREE et al., 2003). The chemical agent acts on the enzymes of the microorganisms, inactivates them, alters the permeability of the membrane and affects several metabolic processes (LE DANTEC et al., 2002).

During wastewater treatment the potentially pathogenic microorganisms are removed, although the more resistant ones may remain. Taylor et al. (2000) state that different microorganisms and sometimes different strains of the same species may present variations in their resistance to chlorine disinfection. Antibiotic-resistant microorganisms from humans and animals are released into the sewage by contaminated materials, such as feces present in wastewater. In fact, the latter is probably the major source of pathogenic and antibiotic-resistant organisms released into the environment

(BAQUERO et al., 2008; ABREU et al., 2010). The presence of antibiotics at low concentrations may enrich the resistant microorganism population and reduce the population of susceptible microbiota (MARTINEZ, 2009).

Chapman (2003) reports that this resistance may have a significant impact on health and on the economy. Murray et al. (1984) verified that bacteria isolated from the chlorinated effluent presented greater resistance to antibiotics than isolated bacteria from environments without previous chlorination. This fact actually suggests that chlorine treatment may contribute towards the spread of antibiotic-resistant bacteria. Shrivastava et al. (2004) argue that this crossed resistance is related to the employment of concentrations above the minimum required and to the use of sub-effective concentrations.

Adelowo et al. (2008) also reported the prevalence of co-resistance to disinfectants and antibiotics of relevant clinical use in isolated bacteria of sewer treatment plants of hospitals in Nigeria. This verification has significant reflection on human health, particularly in the effective treatment of human infections, as well as serious environmental consequences in relation to an ecological imbalance that favors the predominance of a resistant micro biota in nature.

On the other hand, there is an increasing interest in isolated microorganisms proper to extreme environments, such as acidity, alkalinity, temperature, osmotic pressure, chlorinated compounds. Interest also focuses on the isolation of enzymes of extraordinary catalytic capacity, naturally stable in extreme environments, which would supply the industries' demand, since, in a certain manner, industrial enzymes have always been at a disadvantage (CARDOSO et al., 2003; DEMIRJIAN et al., 2001). The use of these microorganisms, featuring such characteristics, may also be a viable alternative to accelerate biodegradation, bioremediation and industrial processes (TONDO et al., 1998).

Although the strategies for molecular survival in inhospitable environmental are not totally known, these organisms have enzymes adapted to such atmospheres. Actually their analysis has been the goal of surveys and research in health and environmental areas due to their biotechnological use and permanence in the environment (CARDOSO et al., 2003). Current assay isolates and characterizes bacteria from the final effluent of the Pici Campus Sewer Treatment Plant (ETE-ICI) so that the resistance of strains to chlorine and antibiotics may be evaluated.

Material and methods

Collection of samples

Samples were collected in the final chlorination tank of the Sewer Treatment Plant of the Pici Campus of the Federal University of Ceará, Fortaleza, Ceará State, Brazil, between November and June. Sludge from the treatment plant is activated by slow aeration and receives domestic sewage-like wastewater from the campus premises. Sampling consisted of monthly collections of approximately 200 mL of material from loci previously established in the chlorination tank and placed in previously sterilized flasks. Immediately after collections, pH of samples was checked with a digital pHmeter (Digimed, DMPH-2) and the concentration of residual chlorine (APHA, 1998) and temperature (ºC) were determined.

Isolation and characterization of the bacterial strains

The collected samples were inoculated in Agar Plate Count medium (PCA) (DIFCO) and incubated at 37ºC for 48 hours. After that period, colonies with different cultural characteristics were selected. These colonies were used for the obtaining of pure cultures in PCA medium and incubated at 37°C for 24-48 hours. The cultural and morphologic characterization of isolates was followed by Gram staining, spore coloration, motility, proof of biochemical catalase, indole and fermentation of carbohydrates (glucose, sucrose, maltose and lactose) (COLLINS et al., 1989; HOLT et al., 1994).

Determination of in vitro resistance to chlorine

Initially the concentration of cells was standardized at 10^7-10^8 cells mL^{-1}. Previously isolated bacteria cultures were inoculated in a nutrition broth (DIFCO) and incubated at 37ºC for 24 hours so that the young cultures could be in the exponential growth phase. Cultures were standardized for an optic density of 0.5 using a 600 nm spectrophotometer (Genesys 10uv, Spectronic). At the same time, a total count of viable cells was undertaken with the spread plate technique, following APHA (1998), for approximately 10^7-10^8 cells mL^{-1}. A sodium hypochlorite solution (MERCK) 10% or 100,000 ppm in distilled sterile water was prepared. Dilutions of 10,000, 5,000, 1,000, 100, 50, 10, 1, 0.5 and 0.1 ppm were undertaken from this solution with the nutrition broth as diluting medium (MERCK).

Cultures of isolated strains of the established concentrations were then inoculated in tubes containing sodium hypochlorite. The cells remained in contact with the substance for 10, 20, and

30 minutes; the cultures were then inoculated in plates with Nutrition Agar (MERCK) and incubated at 37ºC for 24 hours. The presence of growth was considered a positive result.

Antibiotics

Resistance pattern of isolated strains for different antibiotics was determined by the disk diffusion method, following Bauer et al. (1966). Five antimicrobial agents were chosen as important representatives of the antibiotic groups due to their application in medical clinic practice and because they present two mechanisms with different actions: Amoxicillin (AM) and Vancomycin (Va) inhibit the synthesis of the cellular wall, while Erythromycin (E), Chloramphenicol (Cl), and Tetracycline (Te) inhibit the protein synthesis. Disks (Oxoid) containing the antibiotics in the concentrations specified by the National Committee for Clinical Laboratory Standards (NCCLS, 2005), AM (10 μg), Va (30 μg), E (15 μg), Cl (30 μg) and Te (30 μg) were used.

Three colonies of each strain were incubated at 37°C for approximately 6 hours in a Müller-Hinton broth (MERCK) until reaching concentration 10^8 cells per mL. Cultures were then sown in Agar Müller-Hinton plates (DIFCO) by means of a swab. Antibiotics disks were put in plates incubated antiseptically at 37°C for 18 hours. A strain of *Staphylococcus aureus* from the American Type Culture Collection ATCC25923 was used as control for the antimicrobial test. Readings were done by measuring the inhibition halos and then compared with rates of table standards and susceptibility to tested antimicrobials (sensitive or resistant) (NCCLS, 2005).

Results and discussion

The concentration of residual chlorine in the final effluent treatment of ETE-PICI varied between 3.7 and 0.9 ppm from the first to the final chlorination tank, respectively. Since the biocide activity of the chlorine depended, among other factors, on pH and temperature (BEUCHAT et al., 2005), these controlled parameters were respectively 6.6 and 28ºC. Conditions were favorable for an efficient disinfection since the Brazilian Ministry of Health, Regulation No. 518/2004 (BRASIL, 2004) guarantees disinfection process when pH strip lies between 6.0 and 8.3, with a maximum of 8.5.

Starting with the collected samples of the final chlorination tank, twelve colonies of bacteria were selected with different cultural characteristics that presented themselves morphologically as sporulating Gram-positive bacilli. All of the strains were characterized as negative indol, positive motility, positive catalase and fermented glucose. Except for strain 4, all fermented the maltose, whereas the exceptions were strains 11 and 12 in the case of sucrose. Lactose fermentation was observed in strains 1, 4, 6, 7 and 9. Results distinguished five biochemical profiles (Table 1).

Table 1. Biochemical profiles of isolated strains.

Profile	Isolates	Fermentation of carbohydrates 24/48 hours							
		Glucose		Lactose		Maltose		Sucrose	
1	1, 6, 7, 9	+	+	+	+	+	+	+	+
2	2, 3	+	+	-	-	-	+	+	+
3	4	+	+	+	+	-	-	+	+
4	5, 8, 10	+	+	-	-	+	+	+	+
5	11, 12	+	+	-	-	+	+	-	-

(+) presence of growth, (-) lack of growth.

Analyzing the cultural, morphologic and biochemical results in line with the proof of mobility and comparing them with those described in Bergey's *Manual of Determinative Bacteriology* (HOLT et al., 1994), the isolated bacteria were classified as *Bacillus* spp. Even though the results obtained were insufficient to identify the isolated strains, the distinct biochemical profiles may be treated as different species. Adelowo et al. (2008) found a predominance of the genus (33%) among the 55 lineages of isolated bacteria from residue water from the laboratories of three hospitals in Nigeria and identified the species as *B. cereus*, *B. subtilis*, *B. firmus*, *B. macerans* and *B. licheniformis*. In a study on a sewer treatment center of a stabilization lake, Oliveira et al. (2006) identified 96.9% belonging to the genus *Bacillus* among the Gram-positive bacteria group.

Regarding the susceptibility of these strains to sodium hypochlorite in different concentrations and exposure times, it was verified that all bacteria were sensitive to 10,000 ppm concentrations for 10 minutes (Table 2).

Table 2. Profile of resistance to chlorine in concentrations 5,000, 10,000 ppm.

Isolates	Concentrations (ppm) / Time Exposure (minutes)					
	5,000			10,000		
	10	20	30	10	20	30
1, 2, 3, 4, 7, 10, 11	+	-	-	-	-	-
9	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	-	-	-	-
8	-	-	-	-	-	-
12	-	-	-	-	-	-

(+) presence of growth, (-) lack of growth.

The resistance presented by the seven strains (58.3%) when exposed to 5,000 ppm of chlorine for 10 minutes should be underscored. On the other hand, eight strains (66.4 %) were resistant to 1,000 ppm for up to 30 minutes (Table 3).

Table 3. Profile of resistance to chlorine in concentrations 50, 100, 1,000 ppm.

Isolates	Concentrations (ppm) / Time Exposed (minutes)								
	50			100			1000		
	10	20	30	10	20	30	10	20	30
1, 2, 3, 4, 7, 10, 11	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+
5	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
8	+	+	+	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-

(+) presence of growth, (-) lack of growth.

Strains 1, 2, 3, 4, 7, 10 and 11 resisted up to 5,000 ppm for 10 minutes; the isolate 9 resisted 1,000 ppm for 30 minutes and failed to grow in 5,000 ppm. Strains 5, 6, 8 and 12 exhibited different behavior patterns with regard to chlorine tolerance. In decreasing order, strains 8, 5, 6 and 12 may be underscored among the more susceptible strains. Strain 8 presented resistance until 50 ppm concentration for 30 minutes, with no evidence of any growth as from 100 ppm chlorine. The isolate 5 was resistant up to 5.0 ppm for 30 minutes but did not survive in higher concentrations of chlorine (Table 4).

Table 4. Profile of resistance to chlorine in concentrations 5 and 10 ppm.

Isolates	Concentrations (ppm) / Time Exposure (minutes)					
	5			10		
	10	20	30	10	20	30
1, 2, 3, 4, 7, 10, 11	+	+	+	+	+	+
9	+	+	+	+	+	+
5	+	+	+	-	-	-
6	-	-	-	-	-	-
8	+	+	+	+	+	+
12	-	-	-	-	-	-

(+) presence of growth, (-) lack of growth.

On the other hand, growth of bacterium 6 occurred until 10 minutes of exposure at concentration 1 ppm and resistance starting at 20 minutes; bacterium 12 resisted up to 20 minutes at concentration 0.5 ppm, but failed to grow at 30 minutes (Table 5).

Table 5. Profile of resistance to chlorine in concentrations 0.1; 0.5 and 1 ppm.

Isolates	Concentrations (ppm) / Time Exposure (minutes)								
	0.1			0.5			1		
	10	20	30	10	20	30	10	20	30
1, 2, 3, 4, 7, 10, 11	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	-	-
8	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	-	-	-	-

(+) presence of growth, (-) lack of grow.

Results suggest that these bacteria may present specific mechanisms for survival in extremely high chlorine concentrations. The literature reports the resistance of microorganisms which were isolated from sites that had undergone the effects of chlorine or its derivates, especially spore-producing bacteria. Rudolph and Levine (1941) demonstrated that a concentration of 25 ppm of sodium hypochlorite and pH 7.0 are necessary to destroy 99% of *Bacillus* spores in 2.5 minutes, while Mercer and Somers (1957) determined that 15 ppm of sodium hypochlorite destroy 99% of the *Bacillus* spores with pH 6.0 in 8.5 minutes. In their experiments with spore-forming Gram-positive bacilli, Ridgway and Olson (1982) verified that the same bacilli resisted 10 ppm concentration of free chlorine for a 2-minute exposure. In studies carried out at a Water Treatment Plant, Diab et al. (2001) isolated and identified bacteria that resisted to chlorine concentrations up to 5 ppm, 30% of which belonged to the *Bacillus* genus. The same authors highlighted, among other mechanisms, the formation of spores as a factor to explain the bacterial resistance to chlorine.

The resistance of *Bacillus* spores to environmental stress, such as actions against toxic substances or nutrient limitation, is due to several factors including an external layer of protection and the relative impermeability of the internal spore membrane (MAUGHAN et al., 2009; YOUNG; SETLOW, 2003). These layers are composed of approximately 50 proteins in *B. subtilis* and the production of most of these proteins is related to specific genes (LAI et al., 2003). The low content of water and the degree of spore DNA saturation with a group of small acid-soluble proteins should also be considered (SETLOW, 2006). The same authors suggest that the mechanism of chlorine activity may be related to the breaking of the spore's internal membrane, impeding the germination. It should be noted that this fact did not occur with the selected strains since they grew within the established experimental conditions.

Le Dantec et al. (2002) argued that resistance to chlorine's bactericidal action may be related to the composition of the cell membrane, especially the outside layer, which influences the amount of lipids, polysaccharides and proteins. The temperature and readiness of the nutrients should also be related to the bacteria's resistance and adaptation to chlorine, because these bacteria present a greater resistance to chlorine when cultivated with poor nutritional needs and at low temperatures. This characteristic is probably linked to the total amount of lipids in the cell, which may duplicate when the microorganisms are submitted to growth conditions.

These adaptive metabolic processes favor the production of enzymes that are active under extreme conditions. In fact, extremophile enzymes are being used more and more to increase industrial processes and for biodegrading and bioremediation of polluted areas, thus constituting great technological progress on behalf of the planet's health (DEMIRJIAN et al., 2001).

Regarding the resistance to antibiotics, Murray et al. (1984) studied the influence of chlorination in the development of resistance to antibiotics with an isolated bacterium in a sewer treatment plant in Ottawa, Canada. The researchers observed that the chlorination process reduced the total number of bacteria and greatly enhanced the proportion of resistant bacteria for one or more antibiotics, with the possible transfer of resistance to other bacteria strains, especially pathogens. However, it has not been shown whether chlorination selects or induces changes in its resistance against antibiotics in bacteria populations. Further, Le Dantec et al. (2002), Randall et al. (2004), Shrivastava et al. (2004) and Gallert et al. (2005) demonstrated the influence of chlorine in the selection of bacteria with multiple resistances to antibiotics. Russel and Path (2001) suggested that the acquired resistance to biocide substances, such as that of chlorine, may contribute towards an increase in cellular mutation or in the acquisition of genetic elements in plasmid forms or transposons. Nuñez and Moretton (2007) reported that biocide substances may act as a selective pressure for plasmid retention that frequently contains resistance genes against numerous antibiotics. Results of the antibiotic susceptibility test of *Bacillus* spp. strains isolated in current research appeared for three resistance profiles. In fact, it has been verified that six strains (1, 2, 6, 7, 8 and 12) were sensitive for all tested antibiotics (Table 6).

Table 6. Profiles of sensibility and resistance of isolated strains to antibiotics.

Profile	Isolates	Antibiotics				
		AM	E	Cl	VA	Te
1	1, 2, 6, 7, 8, 11, 12	S	S	S	S	S
2	5	S	S	R	S	S
3	9, 11	R	S	S	S	S
4	3, 4	R	S	S	S	R
5	10	R	R	S	S	S

R – resistant; S – sensitive.

Strains 5, 9, and 11 presented resistance to only one antibiotic, while strains 3, 4, and 10 were resistant to two antibiotics in two different combinations. It has been observed that 5 strains (41.6%) presented resistance to Amoxicillin. Luna et al. (2007) tested the susceptibility of different species of isolated *Bacillus* of different atmospheres against 24 antimicrobials and found that in the case of species *B. cereus* and *B. thurigiensi*, they were, as a rule, resistant to this antibiotic. On the other hand, all 95 isolates were sensitive to Chloramphenicol, Tetracycline, and Vancomycin. In the above-mentioned research, in spite of the similarity of sensibility to Vancomycin, two strains (16.6%) were resistant to Tetracycline, one (8.3%) resistant to the Chloramphenicol and the other to Erythromycin (8.3%). With regard to the latter antibiotic, the authors reported one strain of *B. cereus* and one of *B. mycoides* with resistance characteristics. Fernández-Fuentes et al. (2012) reported that in a study with bacteria isolated from organic foods, among which eight were strains of the genus *Bacillus*, only one strain of this genus was resistant to Amoxicillin and all were sensitive to Erythromycin.

It should be enhanced that species of the *Bacillus* genus are found in the soil and, except for *B. anthracis* and *B. cereus*, they are clinically only slightly significant (ROWAN et al., 2003), with low interest in medical microbiology in studies on the susceptibility to antibiotics. However, it is possible to transfer that resistance to other bacteria, especially pathogenic strains.

Although it is still not possible to establish a conclusive connection between the resistance to chlorine and resistance to antibiotics, it is possible to speculate that exposure of effluent microbiota to concentrations of the disinfecting agent used may be related to the antibiotic resistance observed. It should be emphasized once more that these microorganisms can be a risk to public health, even if the strains are not pathogenic. In fact, there is always a possibility of transmitting such resistance to other microorganisms. It is also important to underscore that the extreme resistance of isolated strains to chlorine may suggest their use for the production of extremely resistant enzymes with possible biotechnological application.

Conclusion

The isolated bacteria, classified as belonging to the genus *Bacillus*, presented extreme in vitro resistance to chlorine, with 50% of the strains resistant to 1 or 2 antibiotics. Although no definitive conclusion may be reached with regard to the co-resistance prevalence for chlorine and to the co-resistance prevalence for chlorine and antibiotics, the need for environmental precautions is evident. The environment where water is discharged should preserve its ecological balance for human health. There are high possibilities that these strains may have biotechnological applications for the production of extremely resistant enzymes.

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Received on March 23, 2011.
Accepted on February 29, 2012.