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An Investigation on the Heavy Metal Tolerance and Antibiotic Profile of the *Pantoea agglomerans* UCP1320

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ABSTRACT

The resistance of bacteria to antibiotics is an emerging public health concern due to antibiotics being widely available and used without proper prescription. The introduction of heavy metals in various forms in the environment may cause considerable changes in the structure and function of microbial communities. In the last decade, several studies reported that the resistance of bacteria to antibiotics can occur in the environment because of multidrug resistance or cross-resistance to metals and co-regulation of airway resistance. The aim of this study is to determine the antimicrobial resistance profile patterns to 15 antibiotics and heavy metals (Zn^{+2}, Cu^{+2} and Cd^{+2}) by *Pantoeaagglomerans* bacteria. The (MIC) of the heavy metals was varied from 200 µg /mL to 2200 µg/mL. The results showed that the bacteria were resistant to Zn^{+2}, Cu^{+2} and Cd^{+2}, considering the MIC values compared with the strain *Escherichia coli* K-12 used as control. *P. agglomerans*showed an antibiotic profile of resistance to Cefepime, Cefotaxime, Cefpodoxime, Clindamycin, and Amikacin, and sensitivity to Penicillin, and other antibiotics, thus suggesting that genetically-determined systems for resistance to toxic heavy metals was observed.

Introduction

Bacteria present in the environment, both aquatic and in the soil, may be indigenous or result from hospital and sewage contamination, such as human and animal feces, which is usually discharged into the aquatic environment. Polluted sewage contains large amounts of pathogenic bacteria.

These bacteria present various ways of infecting humans, and can be ingested, inhaled or come into contact with wounds (Schlusener and Bester, 2006; Matyar, 2012).

There are also several antibiotics used in animal feed to promote weight gain. Many countries have implemented antimicrobial resistance and antimicrobial surveillance programs to monitor these factors in animals raised for meat (Akinbowal *et al.,* 2007).

The potential for antibiotic-resistant bacteria developing has raised social concerns that has led to the intensive investigation of the influence of antibiotics on human health and ecosystems (Kim*et al.,* 2011; Matyar, 2012). In the last decade, several studies have reported that patterns of antibiotic resistance are becoming a global problem (Stachowiak *et al.,* 2011; Matyar, 2012).

Studies have demonstrated an additional mechanism that keeps bacteria resistant to antibiotics in the environment due to multi-drug or cross-resistance to metals or co-regulation of resistance pathways (Stepanauskas *et al.,* 2005).

Therefore, it seems likely that exposure to metal may directly select the bacteria resistant to metals, as a co-selection for antibiotic resistant bacteria. Metals, such as copper and zinc and their chemical derivatives, also have antimicrobial activity (Antunes *et al.,* 2003). Animal feed is often supplemented with copper and/or zinc salts because they promote growth. There is concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance (Baker-Austin *et al.,* 2006). Heavy metals can enter the food chain; in particular fish and crustaceans, and these contaminants can be introduced into the aquaculture system when fish meal bases are used as these can produce soluble contaminants such as heavy metals and polychlorinated biphenyls (Erickson, 2002).

There are three main strategies by which microorganisms can develop resistance to drugs: they produce enzymes that are capable of rendering the antimicrobial unfeasible; they prevent the drug reaching its target, through efflux pumps or membrane permeability and; they alter the molecular target of the antimicrobial (Freitas *et al.,* 2017). In general, after the microorganism develops a better resistance strategy, the new genes that confer resistance are disseminated between organisms of the same species or different species by means of different gene transfer strategies (March-Rosselló, 2017).

Mutations can spread horizontally among bacteria by processes such as conjugation or transduction. Drug resistance is often carried by plasmids or by small segments of DNA called transposons, which can jump from one piece of DNA to another. Some resistance plasmids can be transferred between bacterial cells in the same population and between different but closely related bacterial populations (De Maayer *et al.,* 2012).

Being resistant to antimicrobial agents, including heavy metals, is important for the survival of bacteria in contaminated environments. Resistance genes are exchanged between bacteria living in areas contaminated by heavy metals. Therefore, it can be concluded that the natural selective pressure imposed by heavy metals can, indirectly, develop bacterial resistance to antibiotics (Fard *et al.,* 2011). This study sets out to to determine the resistance profile of *Pantoea sp.*to antibiotics and heavy metals in order to investigate the resistance relationship to antimicobrials.

Materials and Methods

Identification of Microorganism

The isolate of a bacterium was characterized by phenotypic, biochemical and Gram staining, oxidase and catalase, motility, glucose reactions and gelatin liquefaction tests (Mardaneh and Dallal, 2013).

Antibacterial Susceptibility Test

Antibacterial susceptibility testing was performed by agar diffusion (Bauer *et al.*, 1996) using Müller-Hinton medium (Difco). During the tests, the bacterial isolate was inoculated in LB medium (Tryptone, 10g, Yeast Extract, 5g, NaCl, 19g, 1000mL distilled water) at 30°C for 24h, respecting the turbidity of the MacFarland 0.5 scale (approximately 1.5x108 CFU.mL-1). A sterile swab was soaked in the culture, removing excess liquid, and seeded uniformly on plates containing Müller-Hinton agar. The antimicrobial discs were deposited equidistantly on the surface of the inoculated medium. A total of 15 antibiotic disks belonging to 9 different classes were used in this study, including Ertapenem (ETP, 10µg), Oxacillin (OXA, 1µg), Cefotaxime (CFX, 5µg), Cefepime (CPM, 30µg), Cefpodoxime (ERI, 15 µg), Nalidixic Acid (10 µg), Gentamicin (GEN, 10 µg), Amicacin (AMI, 30 µg), Erythromycin (ERI, 15 µg) NAL, 30µg), Ciproflaxin (CIP, 5µg), Tigecycline (TGC, 15µg) and Clindamycin (CLI, 2µg). The plates were incubated at 37°C for 24 h and after that period the inhibition halos were measured, in millimetres (mm), by the diameter of the zone of inhibition around the disks, and characterized as sensitive (S), intermediate (I) and resistant (R) according to the Clinical and Laboratory Standards Institute/ 2007. Control strains were Escherichia coli ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Minimal Inhibitory Metal Concentration Test (MIC)

Minimal inhibitory concentration (MIC) tests on the heavy metals were conducted using the Akinbowale methodology (2007). The inoculum was prepared as described above and used for dilution tests on Müller-Hinton Agar containing different concentrations of Cd2, Cu2, and Zn2 in the form of the salts of Cadmium Chloride, Copper Sulphate and Zinc Sulfate, respectively. The stock solutions of the metals were made in distilled water and sterilized using a 0.22 µm syringe to filter them into sterile glass vials which were then stored at room temperature. Dilutions in Müller-Hinton Agar media followed the concentrations of 200 µg/ ml to 2200 µg/ ml of each metal. The plates were incubated with 10 µL of the inoculum at 30°C for 24h. Samples were considered resistant when MIC values exceeded those of the control organism, *Escherichia coli* K-12, described by Akinbowale *et al.*, (2007) and Ansari and Malik (2007).

Results and Discussion

The genus *Pantoea* belongs to the family Enterobacteriaceae and currently comprises nineteen species of Gram-negative bacteria, with yellow or beige pigmentation and mobility. Members of this genus have been isolated from a wide variety of environments including soil, water, dust, dairy products, meat, fish, insects, humans and animals. Most often they are found associated with a wide variety of host plants, such as nonpathogenic endophytes or epiphytes, the leaves, stems and roots of which they colonize (De Maayer *et al.,* 2012).

The bacteria were grown on nutrient agar (AN) for 24 hours at 30°C. The colonies obtained had the following macroscopic characteristics: circular, smooth colonies, irregular and flat borders, 1 mm in diameter and had a yellow pigment. Microscopic examination revealed there to be a Gram-negative bacillus with rounded ends. They were seen to be alone or in pairs. It is mobile, catalase +, facultative anaerobic, non-fermenting glucose. These results corroborate those found by Silini-Cherif *et al.*, (2012) in the identification of a lineage of *Pantoea agglomerans* Ima2 isolated from wheat rhizosphere.

Fujikawa and Akimoto (2011) also show similar results for *Pantoea agglomerans*. Both studies present yellow pigment production by microorganisms. These results are also common to the strains of *P. ananatis*, *P. dispersa and P. stewartii* (Delétoile *et al.*, 2009).

The results of antibiotic susceptibility showed that *Pantoeasp* was sensitive to most antibiotics and intermediate to ertapenem and erythromycin and resistant to the three antibiotics tested in the class of cephalosporins (cefotaxime, cefepime, cefpodoxime), an aminoglycoside antibiotic (Amikacin) and a licosamide (clindamycin) (Table 1). In heavy metal tolerance tests, *Pantoea sp.*showed resistance to the three Cu>Zn>Cd tested metals (Table 2).

Nath *et al.,* (2013) presented results, where antibiotics of the cell-phosporins and aminoglycyses groups were inefficient at controlling bacterial isolates of the genera *Pseudomonas*, *Klebsiella* and *Bacillus*, resistant to zinc, copper and lead. Akimbowale *et al.*, (2007) on isolating strains of *Pseudomonas* and *Aeromonas* found that these were also resistant to drugs in the cephalosporin group, and also showed similarities in resistance to metals.

Table.1 Susceptibility to antibiotics of *Pantoea agglomerans* isolated from laundry effluent

Antibiotic Class	Antibiotic	Disk [C] µg/mL	R	I	S	Results (Halo)
	Penicillin	10	≤ 28	-	≥ 29	30 mm (S)
	Ertapenem	10	≤ 15	16-18	≥ 19	17 mm (I)
Penicillins	Oxacillin	1	≤ 10	11-12	≥ 13	18 mm (S)
	Nalidixic Acid	30	≤ 13	14-18	≥ 19	24 mm (S)
Quinolones	Ciproflaxin	5	≤ 15	16-20	≥ 17	30 mm (S)
Cephalosporins	Cefotaxime	5	≤ 14	15-17	≥ 18	(R)
	Cefepime	30	≤ 14	15-17	≥ 18	(R)
	Cefodoxime	10	≤ 17	18-20	≥ 21	(R)
	Gentamicin	10	≤ 12	13-14	≥ 15	24 mm (S)
Aminoglycosides	Tobramyicin	10	≤ 12	13-14	≥ 15	20 mm (S)
	Amikacin	30	≤ 12	15-16	≥ 17	(R)
Glycopeptides	Vacomicin	30	≤ 14	15-16	≥ 17	20 mm (S)
Glycylcycline	Tigecycline	15	≤ 19	20-27	≥ 28	30 mm (S)
Macrolides	Erythromycin	15	≤ 13	14-22	≥ 23	15 mm (I)
Amphenicol	Chloramphenicol	30	≤ 12	13-17	≥ 18	24 mm (S)
Lincosamides	Clindamycin	2	≤ 14	15-20	≥ 21	(R)

Reference: (CLSI, 2006). R- resistant; I- Intermediate; S- sensitive

Table.2 Resistance of *Pantoea agglomerans* to different concentrations of heavy metals

Heavy metal	**MIC** (µg/mL)*Pantoea*							
	100	200	400	600	800	1200	1600	2200
Cadmium	a		MIC					
Zinc	a			MIC				
Copper	a						MIC	

MIC (Minimum Inhibitory Concentration).
(a) MIC of the strain *Escherichia coli* K12 (Akinbowale *et al.*, 2007)

Sharma *et al.,* (2012), when analyzing a case of septic arthritis caused by *Pantoea agglomerans*, found that this species did not respond to treatment with amikacine, gentamicin, cotrimoxazole, ciprofloxacin, tobramycin, ampicillin and ceftamizine.

The resistance of Enterobacterium species to a broad spectrum of cephalosporins is already known, and because it is mediated by a chromosomal overproduction of AmpC [beta] -lactamases (Aibinu *et al.,* 2012).

Such enzymes are normally encoded on the chromosome of Gram-negative bacteria, including Citrobacter, Serratia, and Enterobacteria species in which their expression is usually inducible, but may also occur in *Escherichia coli*. However, AmpC [beta] -lactamases can also be transported in plasmids (Philippon*et al.,* 2002). The selection of resistance determinants in the environment could occur even in the absence of the antimicrobial.

Many multiple-resistance determinants are capable of simultaneously conferring resistance to compounds belonging to various classes of chemical compounds, such as detergents and antiseptics (Chadha, 2012).

Other studies have shown that selection of antimicrobial resistance determinants could occur due to heavy metal pollution and chemicals (Getanda *et al.,* 2017). Therefore, the selection of resistant bacteria could occur by selecting resistance to compounds that are not antimicrobial, but that make this selection with the same mechanism of resistance (Chadha, 2012).

The various ecological niches occupied by species of *Pantoea*, including plant and animal hosts, and their distinct lifestyles such as epiphytes and endophytes, are indicative of the diversification within the genus *Pantoea* and even among individual strains belonging to the various species of the genus. One means by which this diversification takes place is exactly because plasmids between bacteria are acquired. These plasmids carry genes that confer various phenotypes on the bacterium, including toxin production; hormone production; and virulence factors that contribute to host pathogenesis and specificity; antibiotic and heavy metal resistance and survival under adverse conditions; catabolism of Amino acids and organic acids, carbohydrates and inorganic ions; and the colonization and dissemination of these species (De Maayer *et al.,* 2012).

The strain of *Pantoea agglomerans* presented resistance to the antibiotics cefotaxime, cefepime and cefpodoxime of the cephalosporin group. The group of aminoglycosides presented resistance to amikacin and clidamycin from the licosamides group. In heavy metal tolerance tests, *P.agglomerans* showed crossing resistance to the three metals tested at the higher levels for Cu, followed by Zn and by Cd.

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Conflict of Interest

The authors confirms that this article content has no conflict of interest.

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