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# Esbl Incidence and Plasmid Antimicrobial Resistance

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## Abstract

Gram-negative bacilli resistance has been a cause for concern in Intensive Care Units (ICUs) and clinical laboratories. Resistance to cephalosporins and monobactams is associated with the production of enzymes known as beta-lactamases, which are of own by plasmids. This study's objective was to determine the incidence of ESBL-producing bacteria in ICUs in the city of Goiânia Brazil. The methods evaluate the presence of plasmids in bacteria that were known to produce ESBL and as well as to verify the antibiotics resistance conferred by the plasmids. The isolates were tested using antibiotics diffusion disk by Kirby-Bauer method, for ceftriaxone, ceftazidime, aztreonam and cefepime drugs. The plasmid DNA was extracted using Flexiprep Pharmacia Kit and cleaved with *Eco* R1, *Bam* HI and *Pst* I enzymes. The results showed that from the 200 strains analyzed, 74.5% were found to bacteria. From these, 21.5% were identified as *Klebsiella* sp. from which 8% were ESBL positive. The data show that 52.4% of the enterobacteriaceae were resistant to carbapenems, among the carbapenems studied, imipenem represented 97 (65.1%) and meropenem represented 95 (63.75%); 46.3% were cefepime sensitive. Suggesting that almost half of the bacteria presented resistance to some of the antibiotics tested. Almost all samples with plasmid presented countless number of colonies. However, few species presented plasmid, suggesting with the bacterial resistance may be induced by chromosomal factor. Of the samples studied, 10.74% were ESBL-positive, this percentage resistance was suggested by beta-lactamase production.

***Keywords:*** *Beta-Lactamase; Plasmid; Drugs; Bacteria; Genetic Engineering*

## Introduction

Gram positive bacilli are responsible for nearly all infections acquired in ICUs, including urinary tract infections, pneumonia, meningitis and septicemia. They may or may not be glucose fermenters. A bacillus that ferment glucose is the major cause respiratory infections, intestinal infection and urinary diseases [1]. Bacilli that do not ferment glucose or lactose due to high resistance, isolated from hospital, are considered alert factors when present in hemodialysis units and immuno-compromised patients [2].

Prokaryotes can transfer genetic material from one cell to another by conjugation, transformation, transduction and transposition. Gene transfer in transposition process, occurs through plasmids, chromosomes and phages [3]. Plasmids may be responsible for bacterial survival and communication, originating in the transfer of genetic material and explaining bacterial resistance, leading to new cell characteristics [4].

One of the most diagnostic important and concerning resistance mechanisms is the production of enzymes called chromosomal beta-lactamases or plasmids through Gram-negative bacilli that hydrolyze the beta-lactamic rings of penicillins, cephalosporins and mono-

bactams, rendering them inactive and leaving carbapenems as an alternative treatment, as these and cephamycins are not degraded by this resistance mechanism [1].

Beta-lactamases do not depend on an inducing agent [5]. Gram-negative bacteria have a large number of beta-lactamases (ESBL), which appear with the use of penicillin and with the launch of new drugs by the pharmaceutical industry [4]. The emergence of beta-lactamase-producing bacteria and the increasing prevalence of multi-resistant have limited the use of anti-microbial agents. With the use of carbapenems as an alternative treatment, some enzymes have begun to cause resistance to this class of antibiotics as well. Meropenem and imipenem are the most widely used carbapenems for clinical use in Brazil, the United States and Europe. Meropenem has greater *in vitro* activity against Gram negatives while imipenem is slightly more active against Gram positives [3].

The use of carbapenems as an alternative to inhibiting the growth of beta-lactamase (ESBL) producers, resistance to these antibiotics, has begun to emerge and some resistances have been described in carbapenems in the glucose- and lactose- fermenting bacteria through changes in PBP (penicillin binding protein) affinity and ESBL phenotype in addition to loss of porin. Carbapenase production in strains described as KPC has a type of inducible beta-lactamase expression capable of degrading first- and third-generation cephalosporins [1].

Bacteria that do not ferment glucose or lactose have ESBL survival mechanisms and promote the hydrolysis of carbapenems and cephalosporins that have been designated as carbapenases. Enterobacteria pathogens appeared to have been ESBL, examples include *Escherichia coli, Klebsiella pneumonia* [6]. This study assesses the types of bacteria that are commonly found in Intensive Care Units as well as their resistance mechanisms to antibiotics and outlines susceptibility profiles for the main pathogens involved in the infection process.

## Materials and Methods

We evaluated 200 random clinical samples obtained from secretions such as sputum, bronchial alveolar lavage, surgical wounds, noble liquids and others from patients admitted in four hospitals in the city of Goiânia, Brazil. The patients were with clinical indication of infection and lodged in examining applications in the clinical laboratory. This material was stored in the clinical laboratory as biorepository and was used in this study. All microorganisms were identified to the species level by standard methodology and stained using the Gram technique: size, morphology and ink reaction [7,8].

The antibiotic susceptibility tests were performed in a laboratory and all strains with bacterial growth were isolated, identified and marked by sensitivity profile according to the Determination of Minimum Inhibitory Concentration (MIC) done with a MicroScan® Walk-Away (DADE) automation device with NC32 panels. The antimicrobials studied included the broad spectrum used in hospital environments (ceftazidime, cefepime, cefotaxime, imipenem, meropenem and aztreonam).

All isolates were subjected to screening tests for ESBL (ghost zone), regardless of the ESBL automation software alert. According to the Kirby-Bauer [9] disc diffusion technique and based on the standard procedures of the National Committee for Clinical Laboratory Standards [10], the tests were performed using discs impregnated with fixed concentrations of each antibiotic (ceftazidime, cefepime, cefotaxime, imipenem, meropenem and aztreonam) - the last of which has not yet been standardized by the committee). These were placed around a plate at a distance of 30 mm from center in relation to the central disc of amoxicillin/clavulanic acid [10,11].

Gram negatives were considered to be potential ESBL producers, according to the automation device reading and subsequent Kirby-Bauer disc diffusion technique, they had an inhibition zone reduced for at least one of the assessed antibiotics based on the NCCLS document, using standardized cut offs for *Escherichia coli, Klebsiella pneumoniae* and *Klebsiella oxytoca* - ceftriaxona (30 µg) ≤ 25 mm, ceftazidime (30 µg) ≤ 22 mm, aztreonam (30 µg) ≤ 27 mm and ≤ 27 mm for ceftazidime or cefotaxime in *Proteus mirabilis.*

The appearance of the ghost zone formation phenotype and an increase of 5 mm in the sensitivity zone in the presence of the inhibitor (amoxicillin/clavulanic acid) between any antibiotic markers were considered positive for ESBL. The non-formation of a ghost zone was considered negative for ESBL [8,10].

ESBL detection has only been standardized for *E. coli, K. pneumoniae* and *K. oxytoca* and there is no standardization for other members of the Enterobacteriaceae family, such as *Citrobacter freundii, Enterobacter* spp., *Serratia* spp., *Providência* spp. and *Pseudomonas aeruginosa* (CESP group), making detection difficult. Clavulanic acid can induce Amp-C formation, making it difficult to observe synergy (ghost zone) among the antibiotic markers, leading to them having resistance profiles. The CESP group is resistant to cefoxitin and does not require special detection tests [8]. We used approximately 30mm of imipenem as a ceftazidime marker for chromosomal and non-enterobacterial AmpC given that carbapenems are strong inducers of this enzyme [11].

The antibiotics resistant isolated and suggestive of the presence of the plasmids were selected and maintained in the laboratory using nutrient agar at 4°C temperature, after the samples were sub cultured every week. The extraction of plasmid DNA was performed using the Pharmacia FLEXIPREP extraction kit®, following manufacturer's information. According to Sambrook and collaborators [12], plasmid DNA samples were analyzed in 1% agarose gel (w/v) stained with ethidium bromide (0.2 µg / mL), dissolved in 0.5x TEB (Tris/Borato/EDTA). The gel was subjected to an amperage of 30 mA until the sample entered the well, being subsequently adjusted to 60 mA. The plasmid DNA band were visualized by UV irradiation of low intensity.

Based on method of Summers and Sherratt [13], were inoculated *E. coli* cells containing the cryptic plasmid obtained of ESBL bacteria, the bacteria were fused with pUC18 plasmid in nutrient medium, supplemented with specific antibiotic, the ESBL have resistance and will be subsequently incubated at 37°C overnight. After 5 µL of this culture were transferred to 5 mL of nutrient medium without antibiotic and aliquots were placed on 1.0% (w / v) nutrient broth supplemented with tetracycline antibiotics and were also plated in the same medium without antibiotic, the readings were made after 24 hour read. This procedure was repeated for five days following.

## Results and Discussion

Were found 74.5% of contamination in the analyzed samples. Regarding bacterial diversity, Figure 1 shows that Klebsiella sp. was the predominant species found, with 32 samples (21.48%), followed by P. aeruginosa with 29 (19.46%), Enterobacter sp. with 8 (5.37%) and Enterobacter aerogenes with 8 (5.37%) (Figure 1).

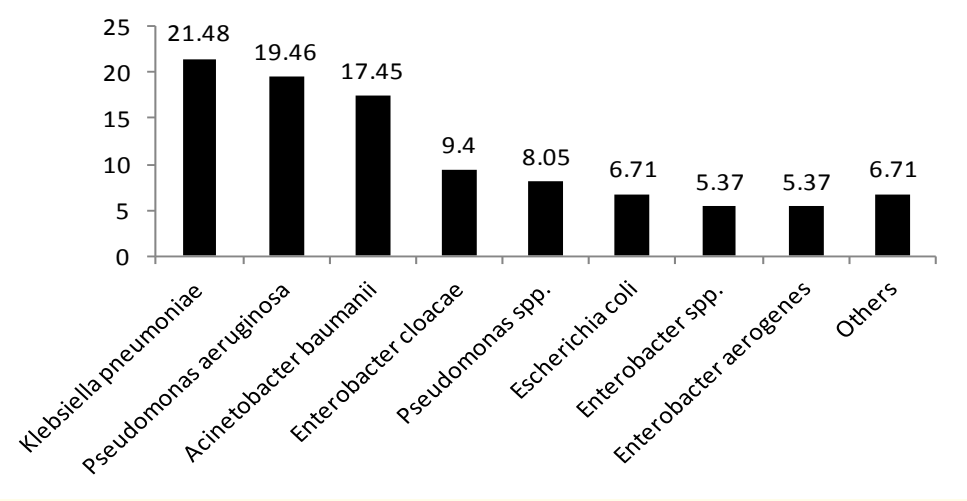


***Figure 1:*** *Percentual of bacteria species isolated in ICUs in Goiânia.*

The clinical specimens in the ICU - Intensive Care Units were secretions from the upper and lower limbs (42 samples, or 28.18%); torso (28 samples, or 18.79%), trachea (21 samples, or 14.09%), abdomen (6 samples, or 4.026%), noble liquids (2 samples, or 1.342%) and other sources (16 samples, or 10.73%) representing other secretions amounting to 1% or less (Table 1).

| Bacterial species incidence in selected samples (n = 200) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Bacterial species** | **Abdominal secretion** | **Catheter tip** | **Secretion trunk** | **Secretion burn** | **Secretion of upper limb and lower limb** | **Tracheal secretion** | **BAL** | **Secretion of wound** | **Other** | **Noble liquids** | **Total occurrences by bacteria** | **Occurrence of species of bacteria in%** |
| *NHCB* | 2 | 23 | 1 | 0 | 9 | 5 | 1 | 0 | 3 | 7 | 51 | 25.5 |
| *Klebsiella pneumoniae* | 3 | 3 | 10 | 1 | 4 | 5 | 0 | 4 | 2 | 0 | 32 | 16 |
| *Pseudomonas aeruginosa* | 0 | 3 | 4 | 1 | 14 | 4 | 0 | 0 | 2 | 1 | 29 | 14.5 |
| *Acinetobacter baumannii haemolyticus* | 1 | 4 | 6 | 0 | 7 | 0 | 1 | 0 | 2 | 1 | 22 | 11 |
| *Enterobacter cloacae* | 0 | 1 | 5 | 1 | 4 | 1 | 0 | 0 | 2 | 0 | 14 | 7 |
| *Pseudomonas sp.* | 0 | 3 | 0 | 0 | 2 | 2 | 2 | 1 | 2 | 0 | 12 | 6 |
| *Escherichia coli* | 2 | 2 | 0 | 0 | 1 | 2 | 0 | 1 | 2 | 0 | 10 | 5 |
| *Enterobacter aerogenes* | 0 | 0 | 0 | 0 | 2 | 5 | 0 | 0 | 1 | 0 | 8 | 4 |
| *Enterobacter sp.* | 0 | 0 | 2 | 0 | 2 | 1 | 2 | 0 | 1 | 0 | 8 | 4 |
| *Proteus mirabilis* | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 3 | 1.5 |
| *Klebsiella sp.* | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 3 | 1.5 |
| *Serratia marcescens* | 0 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 1.5 |
| *Citrobacter freundii* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0.5 |
| *Enterobacter agglomerans* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0.5 |
| *Klebsiella oxytoca* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0.5 |
| *Proteus mirabilis* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0.5 |
| *Proteus sp.* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0.5 |
| Total de ocorrência com crescimento bacteriano por material | 6 | 18 | 28 | 3 | 42 | 21 | 6 | 7 | 16 | 2 | | |

***Table 1:*** *Percentage of clinical specimens in the ICUs of Goiânia.*

Twelve strains of *Klebsiella* spp. were identified as ESBL producers. These data are confirmed in a study conducted by Sousa and collaborators [14], 2004, which reports that ESBL exists most frequently in *Klebsiella* spp., followed by *E coli*. According to the Newsletter of the National Microbial Resistance Monitoring in Health Services [15], *K. pneumoniae* isolates accounted for 13% of total notifications, followed by *P. aeruginosa* (11%), *Acinetobacter* (11%), *Enterobacter* (6%), and *E. Coli* (3%) in primary bloodstream infections in patients undergoing intensive therapy. Thus, we find that the data found in this study (Figure 1) have a bacterial species distribution equivalent to that of the newsletter.

In general, of the 149 samples tested for cephalosporins, cefepime (represented by 69, or 46.3%) was the most sensitive antibiotic, followed by ceftazidime (68, or 45.63%), ceftriaxone (50, or 33.55%), cefotaxime (46, or 30.87%) (Figure 2).

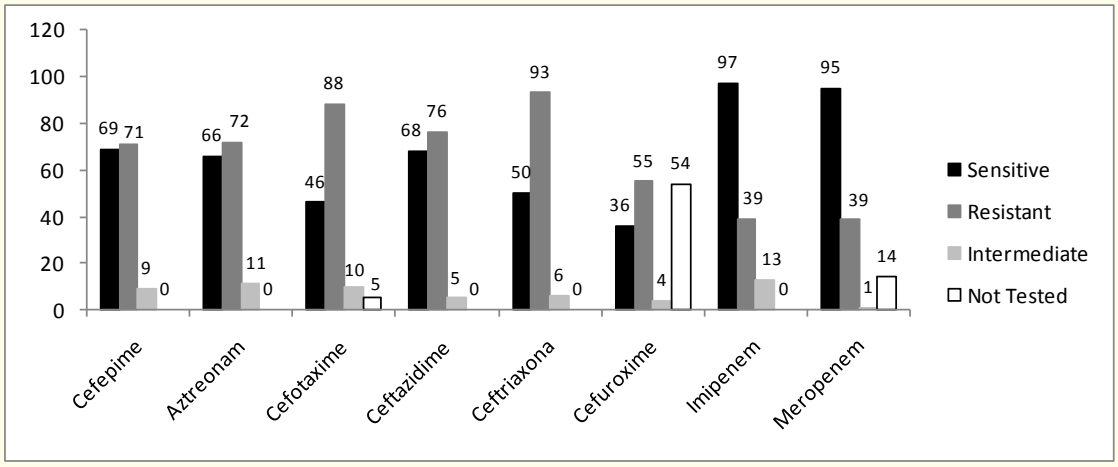


***Figure 2:*** *Antimicrobial action of the tested samples collected from ICUs in Goiânia.*

Of the carbapenems studied, imipenem and meropenem proved to be effective *in vitro* but were not confirmed by the Hodge test in cases of resistance and possible carbapenemase production. Consequently, 52.4% of the carbapenems were not shown to be active for use in patients (Table 2).

| Espécies bacterianas | Cefepime | Aztreonam | Cefotaxime | Ceftazidime | Ceftriaxone | Cefuroxime | Imipenem | Meropenem |
|---|---|---|---|---|---|---|---|---|
| *Pseudomonas sp.* | 4 | 7 | 9 | 6 | 10 | 3 | 6 | 6 |
| *Morganella morgannii* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Proteus mirabilis* | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| *Proteus sp.* | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 |
| *Pseudomonas aeruginosa* | 7 | 6 | 22 | 9 | 21 | 3 | 13 | 11 |
| *Serratia marcescens* | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 |
| *Klebsiella oxytoca* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Klebsiella pneumoniae* | 20 | 20 | 21 | 21 | 21 | 21 | 4 | 4 |
| *Klebsiella sp.* | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 |
| *Enterobacter aerogenes* | 5 | 5 | 5 | 5 | 5 | 5 | 2 | 3 |
| *Enterobacter agglomerans* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| *Escherichia coli* | 1 | 1 | 2 | 1 | 2 | 2 | 1 | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Enterobacter cloacae* | 4 | 4 | 4 | 4 | 4 | 8 | 0 | 1 |
| *Enterobacter sp.* | 3 | 3 | 3 | 3 | 3 | 4 | 1 | 1 |
| *Acinetobacter baumanni* | 22 | 21 | 17 | 22 | 22 | 0 | 11 | 12 |
| *Citrobacter ferundii* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Total* | 71 | 72 | 88 | 76 | 93 | 55 | 39 | 39 |
| *Total em %* | 47.65 | 48.32 | 59.06 | 51 | 62.41 | 36.91 | 26.17 | 26.17 |

***Table 2:*** *Incidence of antimicrobial-resistant bacteria found in ICUs in Goiânia.*

The Figure 2 shows the relationship between the cephalosporins and sensitivity to antibiotics and Table 2 shows carbapenem activity in patients. Carbapenems are used to treat infections caused by Gram-negative bacteria because they are resistant to beta-lactamase hydrolysis. However, it is recommended that these antibiotics be used prudently as they may induce the development of bacterial resistance mechanisms as is the case with the metallobetalactamases in *P. aeruginosa* and the ESBL in *K. pneumoniae* [16].

Occurrence of ESBL-positive in the studied samples was 10.74% (Figure 3). This percentage was assumed due to betalactamase production viewed phenotypically by the disc approximation method, with Klebsiela spp. being the principal species producing ESBL.

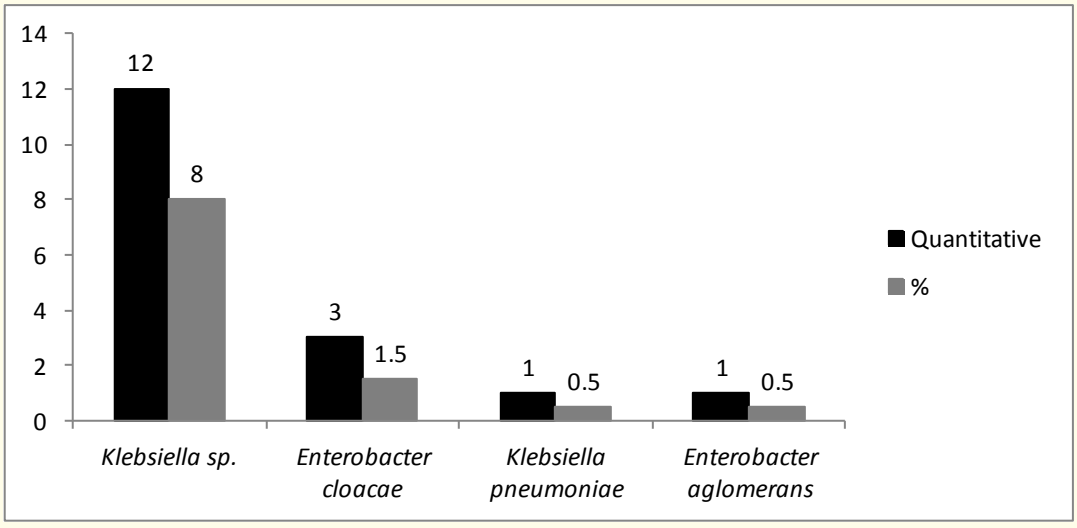


***Figure 3:*** *Incidence of ESBL-producing bacteria found in ICUs in Goiânia.*

The Figure 4 shows the incidence of cAMP producing bacteria in the ICU. The main species producing AmpC-C was *Pseudomonas* spp. (3.35%, being the most prevalent pathogen resistant reported).

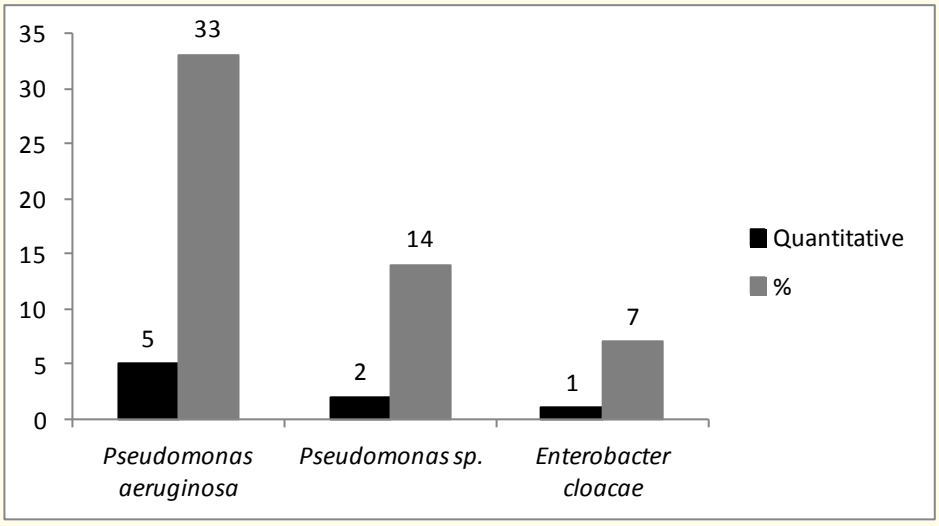


***Figure 4:*** *Incidence of AmpC-producing bacteria in the ICUs.*

In this study 24 samples were analyzed to observe the presence of plasmid DNA, and in ten of the samples, were isolated bacteria that hosted plasmid. By correlating with resistance of bacteria to antibiotics is suggested that the plasmid can be a selective advantage factor (Figure 5).

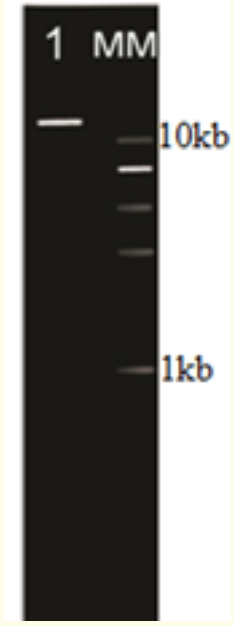


***Figure 5****: Plasmids isolated from antimicrobial resistant bacteria.*

*The number 1 is a representation of plasmid of 10 Kb and the MM letter represent the molecular weight standard.*

Plasmid stability analyzes, using the antibiotic tetracycline, were performed during five generations of bacterial growth. Bacterial growth was detected using atomic absorption spectrophotometer methodology, the results show that bacteria expired the plasmid, therefore the plasmid stability does not remain, the data are in Table 3.

| Samples | Samples without tetracycline | Samples with tetracycline | Samples | Samples without tetracycline | Samples with tetracycline |
|---|---|---|---|---|---|
| 1 | countless | $10^1$ | 21 | countless | 0 |
| 2 | countless | Countless | 22 | countless | $10^2$ |
| 3 | countless | $10^1$ | 23 | countless | $10^3$ |
| 4 | countless | $10^1$ | 24 | countless | $10^1$ |
| 5 | countless | Countless | 25 | countless | $10^2$ |
| 6 | countless | $10^1$ | 26 | countless | $10^2$ |
| 7 | countless | $10^5$ | 27 | countless | $10^3$ |
| 8 | countless | $10^3$ | 28 | countless | $10^2$ |
| 9 | countless | $10^1$ | 29 | $10^5$ | 0 |
| 10 | countless | $10^7$ | 30 | countless | $10^1$ |
| 11 | countless | $10^5$ | 31 | countless | $10^2$ |
| 12 | countless | $10^1$ | 32 | $10^7$ | $10^1$ |
| 13 | countless | $10^7$ | 33 | countless | $10^2$ |
| 14 | countless | $10^3$ | 34 | countless | $10^1$ |
| 15 | countless | Countless | 35 | countless | $10^1$ |
| 16 | $10^7$ | $10^2$ | 36 | countless | $10^1$ |
| 17 | $10^7$ | $10^1$ | 37 | countless | 0 |
| 18 | countless | $10^6$ | 38 | $10^1$ | 0 |
| 19 | $10^7$ | $10^7$ | 39 | $10^7$ | $10^3$ |
| 20 | $10^7$ | Countless | 40 | $10^7$ | $10^3$ |

***Table 3:*** *Bacterial count of the plasmid stability.*

The plasmidial curing process was observed in 10 samples, using the absorbance method, was noted low bacterial growth, when cultured in the presence of antibiotic. These results are suggesting that resistant samples are from the plasmid expired (Table 4).

| Samples | Absorbance | Samples | Absorbance | Samples | Absorbance | Samples | Absorbance |
|---|---|---|---|---|---|---|---|
| 1 | 0.823 | 11 | 0.898 | 21 | 0.22 | 31 | 0.228 |
| 2 | 0.13 | 12 | 0.897 | 22 | 0.103 | 32 | 0.246 |
| 3 | 0.52 | 13 | 0.252 | 23 | 0.137 | 33 | 0.159 |
| 4 | 0.107 | 14 | 0.298 | 24 | 0.242 | 34 | 0.125 |
| 5 | 0.22 | 15 | 0.318 | 25 | 0.192 | 35 | 0.199 |
| 6 | 0.394 | 16 | 0.275 | 26 | 0.178 | 36 | 0.255 |
| 7 | 0.23 | 17 | 0.327 | 27 | 0.109 | 37 | 0.178 |
| 8 | 0.15 | 18 | 0.29 | 28 | 0.388 | 38 | 0.271 |
| 9 | 0.238 | 19 | 0.48 | 29 | 0.332 | 39 | 0.286 |
| 10 | 0.944 | 20 | 0.3 | 30 | 0.17 | 40 | 0.212 |

***Table 4:*** *Bacterial growth after free plasmid on antibiotics presence.*

According the Figure 4 the main species producing AmpC-C was *Pseudomonas* spp. Briceño and collaborators [17], thus suggesting that resistance is related to the overproduction of cAMP-C.

Regarding the ESBL test, ANVISA [18] reported that *K. pneumonia* isolates were tested for the presence of ESBL in 37 (44%) of the 78 hospitals that reported the occurrence of these microorganisms in hospitalized patients. Thus, the test's performance is not systematic and 11 of the 37 hospitals have performed the ESBL test for *K. pneumoniae* isolates.

The Newsletter of the National Microbial Resistance Monitoring in Health Services Network [15] also reports that *K. Pneumonia* sensitivity to ceftazidime or ESBL detection were absent in 55 (21%) of the 257 notifications analyzed through December 2007. This diagnosis may be indicative of possible errors in the antibiogram reading, data entry and/or supervision/monitoring of microbiological results by the laboratory and/or the Hospital Infection Control Committee.

Regarding AmpC detection, five *Pseudomonas* spp. isolates tested positive for inducible chromosomal AmpC when antimicrobial ceftazidime was placed at a distance of 30 mm from imipenem. However, these results proved to be inconclusive in the research as there are no standard procedures for detecting this enzyme and thus antibiograms from the CESP group which despite being sensitive *in vitro*, cephalosporins, penicillins and aztreonam *in vivo* can be a cause of treatment failure) were released in the report according to the antibiogram reading, but while noting the potential for therapeutic failure for first, second and third generation cephalosporins, penicillins and aztreonam [1].

In regard to the sensitivity test, cefepime was found to be the most sensitive antibiotic, according study by Gales and collaborators [19]. Their potency and action spectrums were similar against all of the species tested, except *Enterobacter* spp. Cefepime showed greater potency than ceftazidime against this species. According to Torres and collaborators [20], ceftazidime and the improper use of third-generation antibiotics may have been precursors of the resistance mechanisms developed by the bacteria (ESBL, AmpC e carbapenemases), which shows us the lack of sensitivity of *K. pneumonia* to imipenem demonstrated for seven strains from seven different hospitals. However, the metallo-beta-lactamase and carbapenemase tests were not performed for these isolates.

## Conclusion

This study underscores the need to search for new antimicrobial agents with greater activity against Gram-negative bacilli as the emergence of multi-resistant bacteria limits the range of antibiotic options available to doctors and that some drugs such as carbapenems may compromise the compound's activity, causing instability in the antibiotic and leading to a false result and consequent failure of the treatment used.

In this study 24 samples were analyzed to observe the presence of plasmid DNA, and in ten of the samples, were isolated bacteria that hosted plasmid. By correlating with resistance of bacteria to antibiotics is suggested that the plasmid can be a selective advantage factor. The resistance of *K. pneumoniae* have relation with the plasmid and is the most frequent resistance as ESBL production, this can more easily spread resistance gene.

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## Conflict of Interest

The author no conflict of interest exists.

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