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ARTICLE

- Pharmaceutical care supported by the evidencing of “*in vitro*” interactions with the use of *aminoglycosides* and *penicillins* in patients affected by *Enterococcus* sp** 271
Amouni Mohmoud Mourad, Solange Aparecida Petilo de Carvalho Bricola,
Suzethe Matiko Sasagawa, Eitan Naaman Berezin and Suely Mitoi Ykko Ueda

Full Length Research Paper

Pharmaceutical care supported by the evidencing of “*in vitro*” interactions with the use of *aminoglycosides* and *penicillins* in patients affected by *Enterococcus sp*

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Since antimicrobials are used to fight infectious diseases, their proper use is a major world concern. The use of more than one medicine in the treatment of infectious disease is controversial hence the association therapy is used empirically to treat severe infections when one antimicrobial alone is not sufficient to account for all possible pathogens involved in the infection. The importance of understanding the mechanisms of gentamicin and ampicillin antibiotic action, as well as their interactions has important therapeutic implications. This research aimed to verify isolated and simultaneous antimicrobial action “*in vitro*” of gentamicin and ampicillin on strains of *Enterococcus sp.* in patients with *Enterococcus sp.* Fifty strains of *Enterococcus sp.* were used to determine the minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of ampicillin and gentamicin, both isolated and associated, from which the fractional inhibition coefficient (FIC) was determined. A modified checkerboard method was also used for evaluating the association of the two antimicrobial agents. The results revealed that the antibiotics have a time- and dose-dependent response, and ampicillin has better results isolated than when in association. The results indicate a potential therapeutic failure in 32% of the associations in these agents. Thus, the most effective pharmaceutical care is facilitated by determining the antibiotics concentrations. Determining the MIC, MBC and FIC can be a powerful tool for guiding strategies to prevent bacterial resistance through the rational use of antibiotics, thereby averting and avoiding therapeutic failures.

Key words: Drug antagonism, drug synergism, *Enterococcus sp.*, microbial sensitivity tests.

INTRODUCTION

Pharmaceutical Care is a set of actions aimed at the promotion, protection and recovery of health, both of individuals and society, relying on the use of medicines

and drugs as an essential resource and aiming at their access and rational use. This set entails research, development and production of medicines and supplies,

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as well as their selection, programming and evaluation of their use, with a view to achieving concrete results and improving the population's quality of life (MS, 2004).

The success of a pharmacological treatment is linked to the interrelation of various events such as drug interactions, players and conditions, in a complex and not always predictable manner. Pharmacotherapy succeeds when results such as disease prevention, control, cure, normalization of laboratory parameters and/or relief of symptoms are achieved as expected (Correr et al., 2011).

To achieve this pharmacotherapeutical improvement, we must remain alert against the misuse of antimicrobials in Medicine, both in human and animal care, which plays a key role in selecting resistant bacteria (MS, 2004).

Drug interaction is a clinical event in which the effects of a drug are altered by the presence of another drug, phytomedicine, food, beverage or any environmental chemical agent. When two drugs are administered concurrently to a patient, they can either act independently or interact with each other, thereby increasing or decreasing the therapeutic or toxic effects of one or both agents. Sometimes, drug interaction reduces the effectiveness of a drug and can be as harmful as the increase in its toxicity (Oliveira et al., 2006).

Aminoglycosides are antibiotics with broad clinical use due to their efficacy against gram-negative bacilli and positive synergism with other antibiotics in treating infections caused by gram-positive organisms. They are very commonly used for the prevention and treatment of postoperative infections following cardiac surgery. The main side effect of this class of antibiotics is nephrotoxicity, which can occur in up to 20% of patients (WHO, 2001).

Although usually reversible, renal damage leads to longer hospital stays and, consequently, higher costs. Additionally, what is still more important is the fact that nephrotoxicity is associated with higher mortality rates in these patients (Avorn and Solomon, 2000).

The use of antimicrobials is an extremely useful tool in the hospital setting, especially in intensive care centers or units but, as any drug class, one cannot minimize the problems that can arise from drug interactions, which are occasionally beneficial if little is known about them (Jacomi and Silva, 2011).

Bacterial resistance is a global concern, being the object of current publications on antimicrobials; antibiotics constitute the main drugs affecting not only the patient receiving treatment, but also the entire ecosystem in which he or she lives, with important repercussions (Avorn and Solomon, 2000).

In view of the need for rational use of antibiotics to minimize the alarming growth of bacterial resistance, Brazil's National Agency of Sanitary Surveillance (ANVISA, acronym for Agência Nacional de Vigilância Sanitária) published the Resolution RDC 20/11 that regulates the commercialization of antibiotics, requiring

the retention of the prescription in pharmacies as a strategy to avoid the easy antibiotics consumption (ANVISA, 2011).

Antimicrobials are used to fight infectious diseases and their proper use is a major world concern (Mandell et al., 1996). The use of more than one medicine in the treatment of infectious disease is controversial. The association therapy is used empirically to treat severe infections when one antimicrobial alone is not sufficient to account for all possible pathogens involved in the infection. This occurs in some types of pneumonia, sepsis, endocarditis, and meningitis (Castro et al., 2002).

The scientific community has been watchful over the inappropriate use of antibiotics and the growing prevalence of highly resistant microorganisms (CDC, 2017).

Antibiotics are used in three general ways: empirical, permanent and preventive or prophylactic therapy. When used as an empirical or initial therapy, the antibiotic must provide coverage for all likely pathogens, since the microorganism or infective microorganisms have not yet been identified. One can use an association therapy or, preferably, a treatment with a single broad-spectrum agent. Nonetheless, once the infecting microorganism has been identified, one should establish the definitive antimicrobial therapy, with a narrow-spectrum and low-toxicity agent to complete the treatment (Horner et al., 2005).

Considering the very definition of pharmaceutical care, the importance of fully understanding the action of the drug becomes clear when one is to identify the best strategies for achieving pharmacotherapeutical success and particularly the approach regarding the use of antibiotics. Gentamicin and aminoglycosides were thus selected, and their possible interactions in the treatment against *Enterococcus sp* were investigated.

This study consists therefore in experimental research on bacterial resistance, prospective in nature, aimed to investigate the isolated and simultaneous antimicrobial action, "in vitro" of gentamicin and ampicillin, on *Enterococcus sp.* strains in patients with *Enterococcus sp.*

MATERIALS AND METHODS

This study was conducted from February 2011 to March 2012 at the laboratory of the Microbiology Division at the Pathology Sciences Department, School of Medical Sciences, *Santa Casa de São Paulo (FCMSCSP)* and at the laboratory of Pharmaceutical Sciences – CCBS – Mackenzie Presbyterian University – UPM (acronym for *Universidade Presbiteriana Mackenzie*).

This study was approved by the Scientific Committee at the Department of Pathology Sciences, FCMSCSP, thus rendering unnecessary the development and implementation of a FICF (Free and Informed Consent Form) given that the studies to be conducted did not involve human beings.

Fifty strains of *Enterococcus sp.* from the collection of the Microbiology Division at the Department of Pathology Sciences, School of Medical Sciences, *Santa Casa de São Paulo*

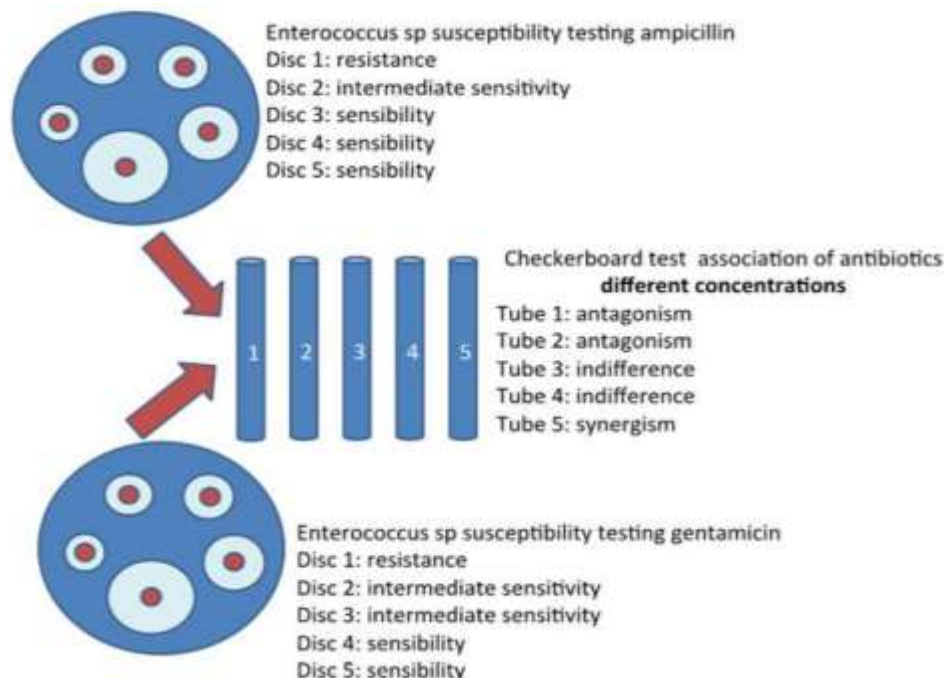


Figure 1. Scheme of activity evaluation of antibiotics, isolated or in association, by simple antibiogram and by the checkerboard method, with the determination of MIC and FIC.

(FCMSCSP) were used. A 0.5 McFarland inoculum, that is, a 1.5×10^8 CFU/mL (colony-forming units per ml) was used for conducting the tests.

One of the methods for testing the antimicrobial activity of drug associations employs serial double dilutions of antibiotics in inoculated culture medium with a standard number of tested microorganisms, in a checkerboard arrangement, which allows the simultaneous evaluation of a large number of antibiotic concentrations at different ratios (Koneman et al., 2001) (Figure 1),

Minimum Inhibitory Concentration (MIC)

The MIC in a culture system refers to the lowest drug concentration capable of inhibiting bacterial growth. CBM refers to the lowest drug concentration able to kill at least 99.9% of microorganisms in culture.

Minimum bactericidal concentration (MBC)

It is the lowest concentration of a drug, expressed in $\mu\text{g/mL}$, which causes the death of 99.9% of the bacterial cells analyzed. We used a series of six tubes for determining the MIC and Müller-Hinton agar plates, in which subcultures were made. Decreasing concentrations of antibiotic were placed in tubes 2 through 5, inoculated with enterococci (0.5 McFarland, or 1.5×10^8 CFU/mL), and incubated for 24 h. After 24 h, bacterial growth was monitored by observing the turbidity in the tubes (Starling and Biscotto 2001).

Tube number 1 contained only antibiotics (negative control), and Tube number 6 contained only bacteria (positive control). Subcultures of all tubes were grown in Müller-Hinton agar medium and evaluated for the development of colonies. The plate where there was a 99.9% reduction of the inoculum was considered as the MBC, whereas the tube where there was no turbidity was deemed

as the MIC.

Checkerboard method assay

According to the method described by Satish et al. (2005), the initial concentration of ampicillin and gentamicin used in the study was 0.4 mg/ml. We used 10 mL capacity test tubes, into each of which 8 mL antibiotic were poured at an initial concentration of 0.4 mg/mL into tubes from number 4 onwards, 2 ml of saline were poured for diluting the antibiotics. Four milliliters were pipetted from tube 1 to tube 2, whose subsequent shaking yielded a $\frac{1}{2}$ dilution. The same procedure was applied to the other tubes, from number 3 onwards. The 4 mL of the final solution remaining in the last tube were discarded. All tubes in the antibiotic dilution series had a final volume of 4 mL. The tubes with ampicillin were named A through F, whereas those with gentamicin were numbered 1 through 6.

In the checkerboard method assay, a series containing 36 tubes was used. Into the tubes 1 through 6, 500 μL of culture medium containing 1.5×10^8 UFC/mL *Enterococcus sp.* were poured. To each tube, 250 μL antibiotic ampicillin or gentamicin were added. The final volume in each tube was 1 mL. After 24 and 48 h at $37 \pm 2^\circ\text{C}$ in an incubator, the contents of each tube were homogenized and turbidity was read, which represents microorganism growth. Turbidity was classified according to its intensity, which ranged from 0 to 4+. At this stage, the MICs of antibiotics were observed.

Following bacterial growth assessment, subcultures on Müller-Hinton agar plates were incubated at $37 \pm 2^\circ\text{C}$ for 24 h in order to evaluate the MBC.

A 500 μL inoculum of culture medium containing 1.5×10^8 CFU/mL *Enterococcus sp.* was added to all tubes. To each tube labeled A through F, 250 μL of diluted ampicillin were added, whereas to tubes labeled 1 through 6 250 μL of gentamicin were added. The final volume in each tube was 1 mL.

In the checkerboard method assay, a series containing 6 tubes was used. Into tubes 2 through 6, 500 μL of culture medium

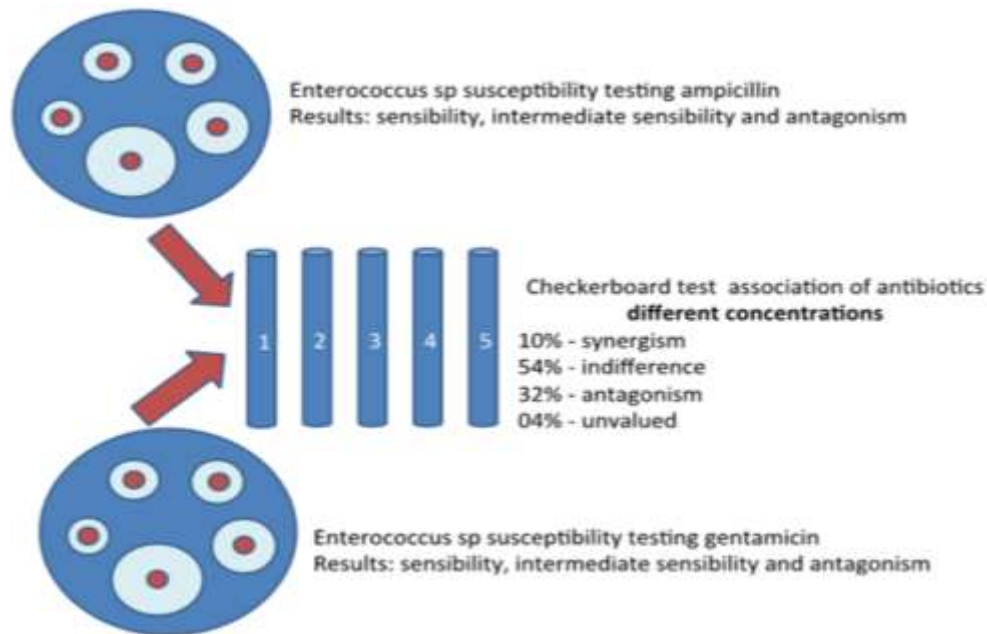


Figure 2. Checkerboard Test results - association of gentamicin and ampicillin in different concentrations "in vitro": a) synergism in 10% of the samples; b) indifference in 54% of the samples; and c) antagonism in 32% of the samples, which might indicate a potential therapeutic failure in 32% of the associations between these agents.

containing 1.5×10^8 CFU/mL *Enterococcus sp.* were placed. Into tubes labeled 1, 1 ml of antibiotics was placed, with these tubes being considered the positive control. From tube 1, 500 μ L were pipetted up and transferred to tube 2, which was homogenized – this procedure was subsequently repeated from tube 3 through tube 5, from which, in turn, 500 μ L were discarded. All tubes in the series had a final volume of 500 μ L. After 24 and 48 h at $37 \pm 2^\circ\text{C}$ in an incubator, the contents of each tube were homogenized and turbidity was read, which represents microorganism growth. Turbidity was classified according to its intensity, which ranged from 0 to 4+. The initial concentration of ampicillin and gentamicin used in the study was 0.4 mg/mL.

The dilution of the antimicrobial agents used in the antimicrobial association test was made as serial decreasing doses. Into each tube, 500 μ L of culture medium containing 1.5×10^8 UFC/mL *Enterococcus sp.* were poured. We used the isolated antibiotics ampicillin (A) and gentamicin (G) at an initial 0.4 mg/mL concentration, an A + G association (simultaneous addition of both antibiotics) and A5G (addition of gentamicin 5 min after that of ampicillin). The following observations need to be considered:

- Negative control (NC): tube number 1, which contained the antibiotic alone and
- Positive control (PC): tube number 6, which contained only bacteria.

To obtain the results, the reading of all tubes was made with the lowest concentrations of drugs that inhibited bacterial growth (absence of turbidity) being recorded. The readings were made after 24 and 48 h, and the turbidity was observed for growing further subcultures on Müller-Hinton agar plates.

Statistical analysis

Data were expressed as mean and standard deviation and

compared by using simple one-factor analysis of variance (ANOVA) followed by the Bonferroni test. The obtained values for which $p < 0.05$ were considered significant. The software of choice used was *Statistica*, version 10, 1010 series. For greater robustness of the results, a nonparametric test – the Chi-squared test – was also used for comparing variables in each experiment.

RESULTS AND DISCUSSION

An important alert triggered by this study relates to the fact that the clinical practice indicates classic synergism between aminoglycosides and beta-lactams; nevertheless, "in vitro", we have observed the following results: a) synergism in 10% of the samples; b) indifference in 54% of the samples; and c) antagonism in 32% of the samples, which might indicate a potential therapeutic failure in 32% of the associations between these agents (Figure 2).

In the experiment involving the use of ampicillin alone in cells cultured TSB medium with decreasing concentrations of ampicillin (0.2; 0.1; 0.05; and 0.025 mg/mL), it was demonstrated that the lowest antibiotic concentrations: 0.1; 0.05; and 0.025 mg/mL showed inhibition of bacterial growth in 38, 35 and 34 tubes, respectively. While the highest concentration, 0.2 mg/mL, showed bacterial inhibition in tubes 47, that is, there was 94% inhibition, showing a statistically significance of $p < 0.001$ (Figure 3). If we extrapolate this result "in vitro" to "in vivo" conditions, it would imply that more diluted concentrations of ampicillin would not be indicated, since

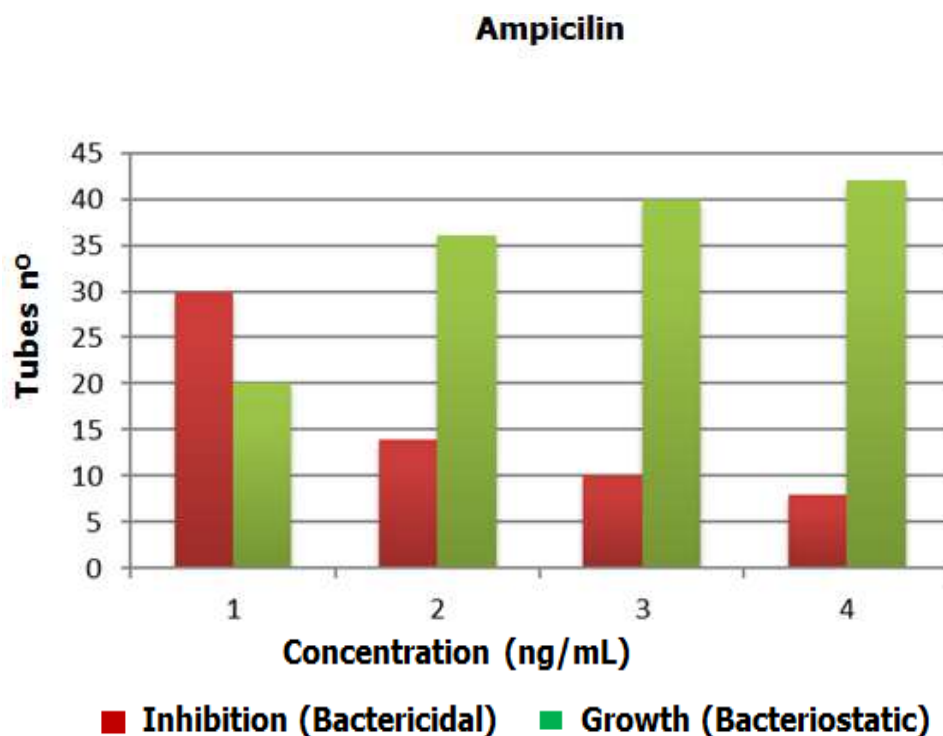


Figure 3. Assessment of bactericidal action and bacteriostatic action of ampicillin at the different concentrations tested (mg/mL) in 48 strains of *Enterococcus sp.* Statistical analysis of the effectiveness of the different concentrations showed $p < 0.001$.

they could lead to a risk of bacterial resistance, therefore some susceptibility to ampicillin was observed at a higher concentration. Thus, as has been reported by Horner et al. (2005), just a 10% resistance of enterococci to ampicillin was found.

After seeding the bacterial cultures into Müller-Hinton medium, in the culture performed after 48 h, ampicillin was observed to have a more effective action at higher concentrations. It was also observed that the longer the time, the lower the action of ampicillin as compared to the results after 24 h with 94% inhibition of the growth of enterococci, whereas the inhibition was observed to be equal to 60% after 48 h.

The experiment with isolated gentamicin in culture with TSB medium had a similar behavior to that observed with ampicillin, showing 68% inhibition, that shows a statistical significance $p < 0.001$, a less effective action compared to that of the ampicillin, which showed a 94% inhibition at this stage (Figure 4).

After seeding the bacterial cultures into Müller-Hinton medium, inhibition was also observed to decrease in the 48-h culture when compared to that observed after 24 h, with an effectiveness of 58%.

In the evaluation related to the association between ampicillin and gentamycin, at the same ratios and decreasing doses (0.1, 0.05, 0.025 and 0.0125 mg/mL),

the activity was observed to improve when compared to that assessed in the presence of ampicillin alone, with growth inhibition having reached 92% after 24 h, with a $p < 0.001$, lower than the inhibition observed with ampicillin alone (Figure 5). However, one must bear in mind that, at the highest concentration, better results were obtained compared to those with the lower concentrations used in the experiment.

After seeding the cultures into Müller-Hinton, effectiveness was observed to be best at higher concentrations when compared to that obtained at lower concentrations, with an inhibition of 62%. When compared to the reading made after 24 h, it becomes clear that over time effectiveness decreases considering that the response was 92% after 24 h, thus suggesting that it is very important that treatment be started as soon as possible. Another fact deserving attention is that only at the highest concentration was observed greater response in inhibiting bacterial growth, whereas in the following concentrations, growth predominated over inhibition. This condition corresponds to the one found by Martinbiancho et al. (2007), who states that the pharmaceutical incompatibility responsible for inactivating aminoglycosides by beta-lactam “*in vitro*” (Paiva and Moura, 2012).

The association of gentamicin and ampicillin (added

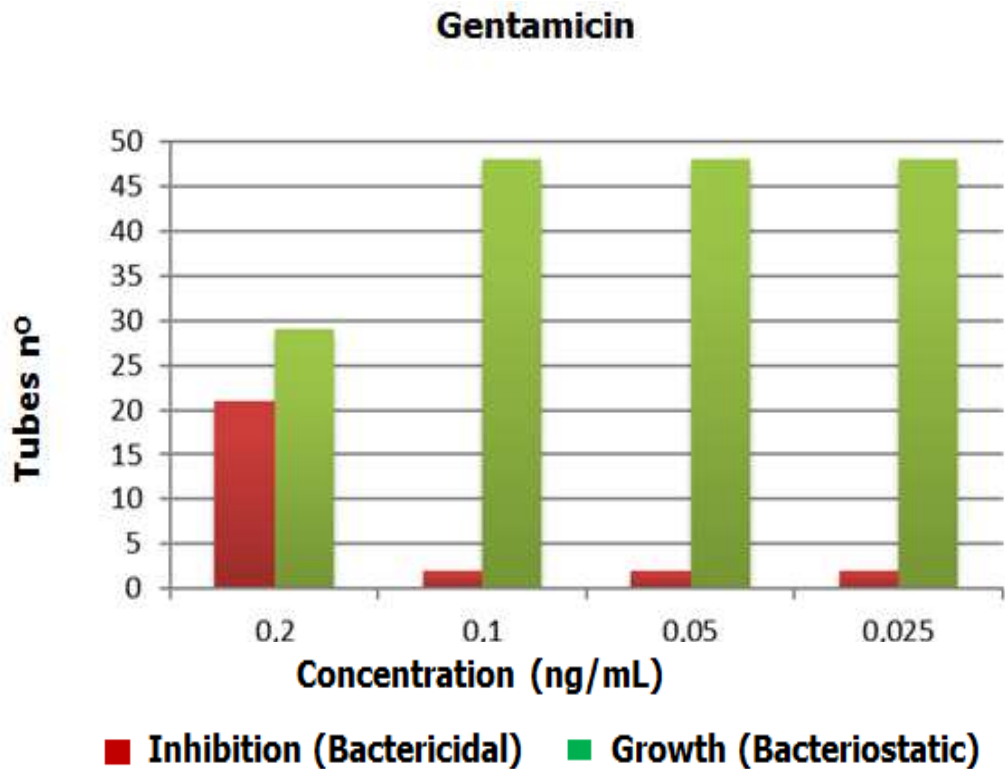


Figure 4. Assessment of bactericidal action and bacteriostatic action of gentamicin at the different concentrations tested (mg/mL) in 48 strains of *Enterococcus sp.* Statistical analysis of the effectiveness of the different concentrations showed $p < 0.001$.

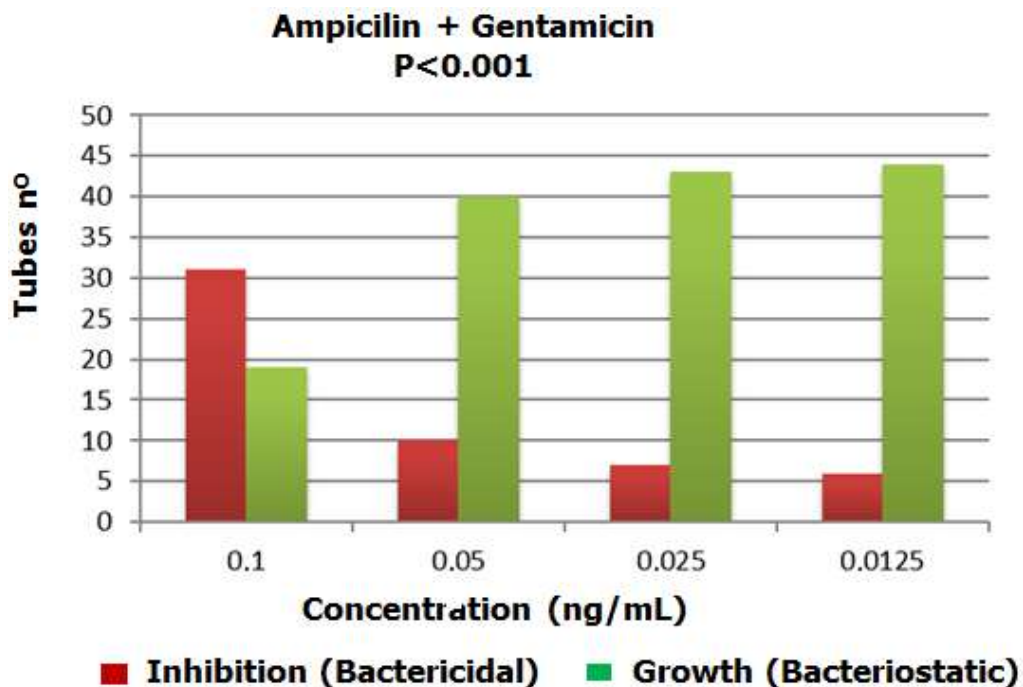


Figure 5. Assessment of bactericidal action and bacteriostatic action of ampicillin + gentamicin combination at the different concentrations tested (mg/mL) in 48 strains of *Enterococcus sp.* Statistical analysis of the effectiveness of the different concentrations showed $p < 0.001$.

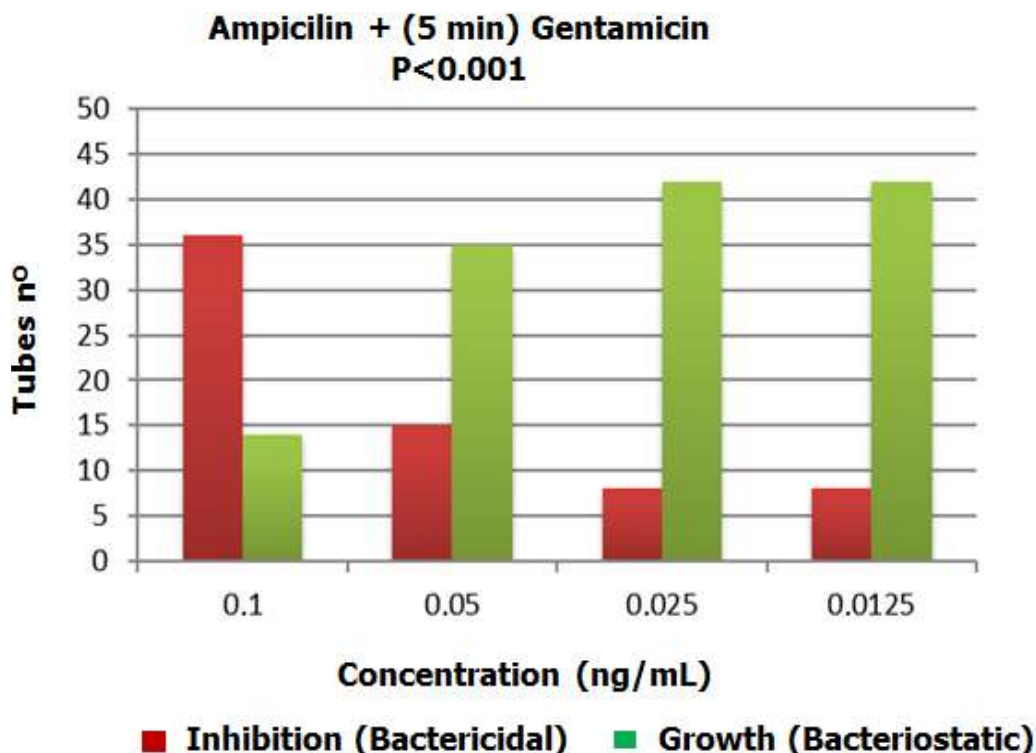


Figure 6. Assessment of bactericidal action and bacteriostatic action of ampicillin + gentamicin combination, after 5-minute interval, at the different concentrations tested (mg/mL) in 48 strains of *Enterococcus sp.* Statistical analysis of the effectiveness of the different concentrations showed $p < 0.001$.

after a 5-min interval) at decreasing concentrations (0.1, 0.05, 0.025 and 0.0125 mg/mL) and at the same ratios, there was observed greater inhibition at all concentrations tested, but the greatest inhibition was obtained at the highest concentration with a $p < 0.001$ (Figure 6).

Following that, the bactericidal activity of ampicillin in association with gentamicin (added after a 5-min interval) was observed after culturing in Müller-Hinton medium, demonstrating that an effective action only took place at the highest concentration used, whereas at the other concentrations no bactericidal action was observed. This action dwindled with the dilution of the association between ampicillin and gentamicin. Such result draws our attention to that described by Martinbiancho et al. (2007) who reported pharmacological synergism between beta-lactam and aminoglycoside antibiotics, even though ampicillin and gentamicin are incompatible when administered simultaneously.

The results can find support in the article by the authors Paiva and Moura (2012) and Gawryszewska et al. (2012) who state that the most frequently found drug interaction in their work was that between ampicillin and gentamicin, with the “*in vitro*” inactivation of aminoglycosides by beta-lactam. The occurrence of this interaction can prevent the

antimicrobial activity of the aminoglycoside, which would imply therapeutic failure in the treatment of infections caused by microorganisms sensitive to it. The interaction also occurs with other drugs of the two antimicrobials classes. It is important to emphasize the fact that, although ampicillin and gentamicin are incompatible when administered simultaneously, there occurs pharmacological synergism between the two classes of beta-lactam antibiotics and aminoglycosides (Paiva and Moura, 2012; Gawryszewska et al., 2012).

Hence, in evaluating the behavior of the actions verified under the conditions tested, the response is time-dependent as described by Paiva and Moura (2012) who recommend that the administration of such antibiotics be made intravenously with an interval of 1-2 h, thus avoiding incompatibilities between aminoglycosides and other drugs. Therefore, it is of the utmost importance to evaluate a laboratory method for determining the association of antibiotics, aiming at a more detailed and safer marketing program for patients.

The determination of the optimal concentration of antibiotics, especially when associated, has the purpose of obtaining the best clinical response.

It is extremely important that a faster and more practical method for determining the exact concentrations of

antibiotics to be administered be employed, especially when an association of such antibiotics is used, aiming at obtaining a better clinical response with the lowest possible renal and hepatic impairment.

Conclusion

It was found that under all the *"in vitro"* conditions tested, the response to antibiotics is time-dependent, stressing the need for intervention as soon as possible to permit the best response when using antibiotic therapy. The determination of the MIC, MBC and FIC can be a powerful tool for guiding strategies aimed at preventing bacterial resistance through the rational use of antibiotics, thus averting and avoiding therapeutic failures.

Considering that pharmaceutical care aims at promoting measures to improve the quality of patients' health and the results obtained, we suggest that a better strategy be devised for the use of antibiotics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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