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RESEARCH ARTICLE

EVALUATION OF IN VITRO SYNERGY BETWEEN AMIPICILLIN AND KANAMYCIN AGAINST *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

The emergence of antibiotic resistance in bacteria is threatening as in case of *Staphylococcus aureus* and is well known for causing nosocomial infections in humans. The main objective of this study was to find ways to inhibit the growth of nosocomial infection causing *S.aureus* using antibiotic combinations. In this study a total of 50 isolates of *S.aureus* were isolated from clinical samples collected from wound, boils, abscesses, ulcers and post operative infections (POI). The isolate exhibiting multi-antibiotic resistance was subjected for test of synergism. Synergism can be best achieved by checkerboard or Fractional Inhibitory Concentration (FIC). A combination of ampicillin and kanamycin were used for the checkerboard method. From the checkerboard, the MIC of ampicillin and kanamycin alone were 160µg/ml and 80µg/ml respectively. However a combination of both antibiotics required were just 10µg/ml and 20µg/ml of ampicillin and kanamycin with a FIC index of 0.562 indicating their synergistic effect. Thus from this study it can be concluded that antibiotic combination therapy is the best way to achieve synergy when the causal organism is a multi-antibiotic resistant one.

Key words: Antibiotic resistance, *Staphylococcus aureus*, Checker board and Synergy test.

INTRODUCTION

Among the various infections caused by bacteria, antibiotic resistant ones are of major concern because of their non-responsiveness to treatment with a single drug regimen thus resulting in therapeutic failure [1]. The use of antibiotic combinations has been known since a long time and is often applied when several mechanisms of action and toxicity profiles of the agents involved can be brought to halt at once [2]. The biocidal (bactericidal, fungicidal or virucidal) activity could be best achieved by the combination of two different antibiotics rather than the effect obtained by an individual antibiotic [3]. Combination of antibiotics is one such methodology and is applied only under certain circumstances. Although the combination of two or more antibiotics may not provide accurate control as observed in few cases, it can atleast delay the emergence of bacterial resistance [4]. Synergistic combinations are often applied in situations of resistance development and when ineffectiveness of a single antibiotic are prevalent, in treatment of life threatening infections based completely on experimental terms, prior to the identification of the causal organism, prevention of bacterial resistance development and in the treatment of infections caused by a combination of microbes (bacteria) [3]. *Staphylococcus spp* being well known for causing nosocomial infections, it could also lead to severe clinical manifestations like pneumonia, deep abscesses, osteomyelitis, endocarditis, phlebitis, arthritis, mastitis and meningitis, thus stressing the need for combination therapy. It can also produce beta-lactamase enzyme which is capable of penicillin resistance [5,6]. Thus, there is a need to find new ways to control evolving of drug resistant *S. aureus* infections and embark on the need for a continued search of new methods for treatment with the application of combination therapy [7].

Among the techniques employed in the evaluation of the combination of two antimicrobials potentially exhibiting synergism, is the checkerboard or fractional inhibitory concentration (FIC) technique. FIC employs a methodology similar to that utilized for the determination of the minimum inhibitory concentration (MIC). The combination is said to have synergistic effect if there is a 4-fold reduction in the MIC of each antimicrobial agent tested alone [2]. Kanamycins are members of the aminoglycoside family having a broad spectrum of activity against many species of bacteria. Their mechanism of action is specific to aerobic, Gram negative bacteria however it can act synergistically against some Gram positive bacteria. Kanamycin mainly targets the proteins synthesis by irreversibly binding to the 16s rRNA of 30s ribosomal subunit. Binding of kanamycin to the decoding region of 3' end of rRNA prevents the tRNA from binding to the ribosomal A-site thus bringing about translational errors [8]. Ampicillin is a commonly used broad-spectrum aminopenicillin with known activity against *E. coli* and *Staphylococcus aureus* [4]. These aminopenicillins inhibit the final stage of synthesis of cross- links of peptidoglycan which occurs outside the cell and is catalyzed by a transpeptidase [1]. In this study, the interaction between ampicillin and kanamycin is investigated using the checkerboard method. The result of the study could provide rational basis for clinical use of these combination of antibiotics against infections caused by these drug resistant organisms.

METHODS

Materials:

The culture media used in the study included Nutrient agar, Mueller-Hinton agar, Mueller-Hinton broth and Mannitol salt agar (all of them from Hi-Media). Ampicillin and Kanamycin were obtained from Hi-Media. Clinical isolates of microorganisms were isolated from clinical samples.

Preparation of culture media:

The culture media were prepared according to the manufacturers' specifications. Initially they were weighed, dissolved in required amount of water and then sterilized by autoclaving at 121°C for 15 min.

Isolation and confirmation of *S.aureus*:

The clinical samples (50 samples) for the isolation of *S.aureus* were collected from various laboratories of Bangalore during the period October 2011 to December 2011. These isolates were initially grown on nutrient agar. The colonies on nutrient agar were identified based on colony characteristics and Gram staining. Further characterization was carried out by streaking the inoculum on selective media i.e., Mannitol salt agar. The organism was confirmed by performing biochemical tests, most important of them are catalase and coagulase tests both of which gave positive results. The isolated and confirmed isolates were subcultured weekly on fresh nutrient agar slants and stored at 4°C [3]. 24 hr fresh culture was obtained prior to each study.

Antibiotic susceptibility test:

Antimicrobial susceptibility was determined by Kirby-Bauer disc diffusion technique. Antibiotic discs used in this investigation are standardized discs: Kanamycin (30mcg/disc), Chloramphenicol (30mcg/disc), Ampicillin (30mcg/disc), Vancomycin (30mcg/disc), Erythromycin (15mcg/disc) and Penicillin (10units/disc). Actively growing 16hr old culture was surface spread using sterile cotton swabs onto the Mueller-Hinton agar surface. The plates were kept aside for absorption for 15 min. The antibiotic discs were then placed onto the agar surface and the plates were incubated at 37°C for 16-18 hr. The plates were examined for the zone of inhibition around the antibiotic discs that would indicate the sensitivity of the organism [1].

Preparation of antibiotic stock solution:

Standard powder forms of Ampicillin and Kanamycin were stored at 2 to 8°C until use. The stock solution of Ampicillin and Kanamycin was prepared by weighing and subsequently dissolving appropriate quantities of the antibiotic in 50ml of Mueller- Hinton broth to obtain a concentration of 1000µg/ml of each antibiotic.

Preparation of inoculum:

The inoculum was obtained by transferring a small volume of stored culture to fresh media which was incubated overnight containing actively dividing cells. This was used for synergism test.

Test for Synergism:

Serial two fold dilutions of each antibiotic stock to at least double the MIC were prepared. The first antibiotic of the combination was serially diluted along the ordinate while the second antibiotic was diluted along the abscissa [9]. Varying concentrations of 2.5, 5.0, 10, 20, 40, 80, 160 and 250µg/ml of each antibiotic was used for combination. A total of 2000µl of Mueller -Hinton broth was distributed into each 2.5ml screw cap tubes which also contained 100µl of the bacterial culture. The tubes were incubated at 37°C for 24 hr. The resulting checker board contains a combination of two antibiotics, with the tubes that contain the highest concentration of each antibiotic at opposite corners [9].

According to the NCCLS guidelines for broth microdilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as observed detected with the naked eye. Synergy is more likely to be expressed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was same for all components of the mixture. Interaction was assessed algebraically by determining the fractional inhibitory concentration (FIC) indices. The FIC (Fractional Inhibitory Concentration) were calculated as follows: FIC= FIC A+ FIC B, where FIC A is the MIC of antibiotics in combination/ MIC OF antibiotic A alone and FIC B is the MIC of antibiotics in combination/ MIC OF antibiotic B alone.

RESULTS

The clinical samples were collected for the study from different laboratories [6, 10]. A maximum of 36% clinical samples were obtained from wound and abbesses and post operative infections (POI) showed 21% followed by ulcer 13% and boils 9%. Results show incidence of *S. aureus* infections (Fig 1).

The disk diffusion test plates were examined for zone of inhibition around the antibiotic disks. Thus disk diffusion results indicate the multi drug resistance pattern of *Staphylococcus aureus*. The development of resistance by *S. aureus* is an ever increasing problem. Hence a combination of antibiotics using checker board method gave better control against *S.aureus* [11,12].

In the Checkerboard technique, the interaction between combination of ampicillin and kanamycin against *Staphylococcus aureus* were predominantly synergistic with FIC index of 0.5625, although there were few variations. Thus no growth or turbidity clearly illustrated the extensive activity of aminoglycoside which was enforced by the second drug, ampicillin resulting in an antibacterial effect (Fig.2). FIC-index values less than 1 were considered as synergy and the degree of synergy increases as the value tends towards zero. FIC-index value greater than one but less than two indicates indifference while FIC value greater than two represents antagonism.

DISCUSSION

Goals of combination therapy are to obtain broad spectrum coverage, enhance antimicrobial activity through synergistic interaction and minimize resistance development for most infections. A combination therapy may be needed for treatment of mixed infections, when not all organisms are susceptible to the same antibiotic; prevention or delay of development of bacterial resistance to an antibiotic; ability to use non-toxic amounts of two antibiotics when toxic doses of a single antibiotic would be required and when combination therapy may be more effective against a single organism than the use of single antimicrobial agent.

In this study the FIC-index for combination of ampicillin and kanamycin against S.aureus was found to be 0.5625 indicating the synergistic effect of the combination, the FIC value being in agreement with the report of Nworu et al report (2006) the combination being ampicillin and ciprofloxacin. This report is also in contradiction with that

of Ibezim et al (2006) using ampicillin and cotrimoxazole combination. This is because the combination used could vary depending upon the strain of S.aureus, the environment in which they exist and several other conditions. The combination of two antibiotics exhibited significant synergism, the mechanism involving the penetration of ampicillin into the peptidoglycan layer leading to cross-links and inhibition of cell wall synthesis and there by increases the permeability of the bacterium to kanamycin that binds to the 30s ribosome inhibiting the protein synthesis [13,14]. On the other hand the organism exhibited a higher MIC for one of the antibiotic alone and this is in support with the report of Sato et al (2004) [15, 16]. It is hoped that these approaches, if well standardized and adopted, will not only provide useful alternatives to pre-existing time-kill and checkerboard titration method, but will also circumvent problems and methodological limitations inherent in their use [17,18].

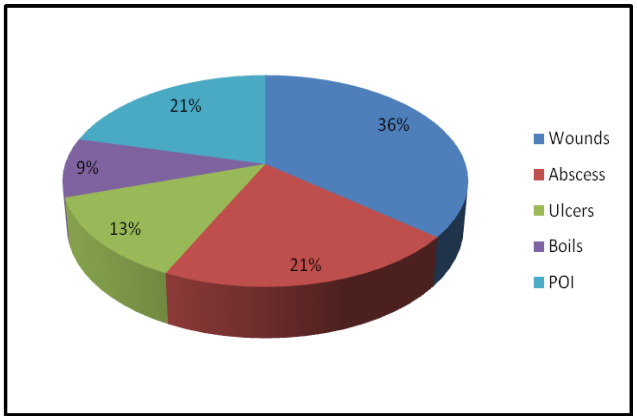

Figure 1: Incidence of infection by *S.aureus* in clinical samples

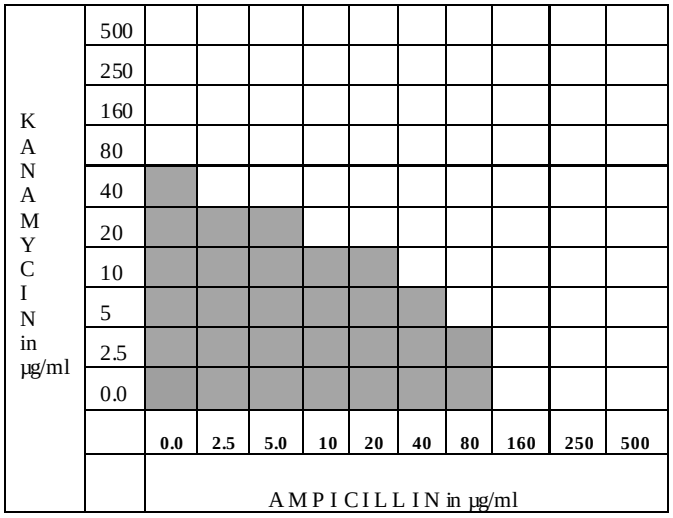

Figure 2: The synergistic effect of the two antibiotics against *S. aureus*

CONCLUSION

Staphylococcus aureus, the 'king of nosocomial infections' is of major concern all over the globe because of the multi-antibiotic resistance pattern exhibited by the organism. Although researchers are finding newer antibiotics for the control of 'the nosocomial king', its multi-antibiotic resistance pattern still exists and hence synergistic effect of known antibiotics is the best tool that could be employed to control nosocomial infections caused by *S. aureus*.

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