In vitro activity of colistin combined with meropenem against multidrug resistant Klebsiella pneumoniae

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Abstract:
Treatment of nosocomial infections caused by multidrug resistant Gram negative bacilli is the major hurdle faced by clinicians across the world. As new antimicrobial drugs are not expected in the near future, there is a compelling need to maximize the potential of the available antimicrobial agents as combination therapy. Colistin is currently being used as the reserve drug in the treatment of multidrug and pan-drug resistant gram-negative bacterial infections. Literature suggests that colistin when used as combination therapy with a synergistic drug is superior to monotherapy. This study intends to detect the in vitro synergy between colistin and meropenem thereby to determine whether the combination would be therapeutically useful. 35 isolates of multidrug resistant Klebsiella pneumoniae were included in the study. The minimum inhibitory concentration of colistin and meropenem were determined for all the study isolates using E-test strips. The presence of in vitro synergy between colistin and meropenem was detected by the fixed ratio method using E-test strips.

The drug combination was found to act synergistically in 29 of the tested isolates. Antagonism was noted in 11 while the remaining 60 neither showed synergy nor antagonism. This is a pilot study conducted with a small sample size. Though the results do not favor the drug combination, more number of samples should be tested to reiterate the findings. Also, the performance of the fixed ratio method of in vitro synergy testing should be validated with the gold standard time-kill assay.

Keyword: Colistin, in vitro synergy test, combination therapy

Introduction:
Irrational use of antimicrobials coupled with rapid emergence of drug resistance in bacteria is a major problem faced in health care settings across the world.(1) Multidrug resistant Gram negative bacilli of the Enterobacteriaceae family are among the frequent causes of nosocomial and systemic infections. Klebsiella pneumoniae, a member of the Enterobacteriaceae plays a causative role in nosocomial infections such as pneumonia.
bacteremia, urinary tract infections and intra-abdominal abscesses. These organisms are notorious for rapid evolution and dissemination of drug resistance. Until recently, gram negative nosocomial infections were effectively treated using carbapenems. However with the emergence of carbapenem resistant Enterobacteriaceae (CRE) in the past decade, increasing treatment failure is being noted. The increasing prevalence of these multi-drug and pan drug resistant nosocomial pathogens poses a definite threat to the treating physicians. As newer antimicrobials are not expected in the near future, there is renewal of interest in previously abandoned antimicrobials. Colistin is one such antimicrobial which was previously abandoned but currently used in the treatment of multi-drug and pan drug resistant gram-negative infections.

Colistin otherwise known as polymyxin E belongs to the polymyxin group of antimicrobials, which are surface-acting amphipathic agents. Polymyxins act by disrupting the integrity of the bacterial cell membrane resulting in increased permeability causing leakage of cellular contents, leading to cell death. The disruption of membrane integrity also increases the susceptibility of the organism to various other antibiotics. Although colistin appears to be an attractive therapeutic option, rapid selection of the hetero-resistant mutants is the major drawback. The phenomenon of hetero-resistance is of major concern during colistin monotherapy as resistant subpopulations often exist in a colistin susceptible isolate. On administration of colistin there occurs an initial rapid bacterial killing, but as the serum drug concentration decreases there is a re-growth of the resistant mutants. This problem can be overcome by using colistin in combination with another antimicrobial which acts synergistically. Synergy between two antimicrobials could be conveniently measured in vitro using the E-test based methods. The time kill assay and the checkerboard assay are the other methods of detecting in vitro synergy. The E-test based methods have an advantage over the other methods in that they are less laborious and easier to perform.

Materials and methods:
Research setting
This was a prospective descriptive study conducted in the microbiology laboratory of Christian Medical College and Hospital, Vellore. The institution is a tertiary care centre consisting of a 2400 bedded hospital with an outpatient turnover of around 5000 cases per day. The duration of the study was 16 months, extending from December 2009 to March 2011.

Study isolates
Consecutive isolates of Klebsiella pneumoniae isolated from blood stream infections were collected. 35 isolates which were found to be resistant to three or more classes of antimicrobials including carbapenems were included in the study.

Media:
All antimicrobial susceptibility tests were performed on Mueller Hinton agar (MHA) plates as recommended by the Clinical and laboratory standards institute. Commercial dehydrated powder medium (HIMEDIA, Mumbai) was obtained and prepared according to manufacturer’s instructions. Sterilization was by autoclaving at 121°C for 15 minutes at 15 psi. The medium was allowed to cool to about 40 - 50°C and then poured into sterile plastic petri dishes of 85 mm diameter (Tarsons, Bengaluru) to a depth of 4 ±1 mm. The plated media were allowed to cool for 30 minutes in room temperature for solidification after which they were dried in a 37°C incubator for 10 minutes to remove the water of condensation.
The media thus prepared were passed for quality control and stored at 2-8°C and were used within 5 days.

**MIC determination:** Minimum inhibitory concentrations of colistin and meropenem for the study isolates were determined using E-test strips (AB Biodisk, Sweden). 0.5 Mac Farland standard, pure broth culture of each test organism was prepared in nutrient broth from an overnight sub culture. The inoculum was streaked onto MHA plates using sterile cotton swabs by the rotary plating method to disperse the organisms as an even lawn. The inoculated plates were allowed to stand for 10 minutes after which the E-test strips were placed using forceps. Since one E-test strip could be placed per plate, two MHA plates were inoculated for each test organism; one for colistin and another for meropenem. The plates were incubated in a 37°C ambient air incubator and read after 16-18 hours. The point of intersection of the elliptical zone of inhibition with the E-test strip denoted the MIC of the antimicrobial for the corresponding organism on the graduation printed on the strip. Interpretation of the MIC of meropenem was according to the 2011 guidelines issued by the CLSI. As no interpretation was available for colistin MIC for the Enterobacteriaceae, the interpretation issued by the CLSI for Pseudomonas aeruginosa was extrapolated.(13)

**Synergy testing:** The fixed drug ratio method was used to determine the in vitro synergy between the colistin-meropenem combination (Figure 1).

**Figure 1:** In vitro synergy testing between meropenem and colistin for carbapenem resistant Klebsiella pneumoniae using the fixed ratio E-test method

The MIC of each drug alone was estimated previously. E-test strip containing meropenem was placed on MHA streaked with the test organism. The point corresponding to its MIC was marked on the agar surface using a sterile needle. This was incubated for one hour to allow the diffusion of meropenem from the strip into the medium. The strip was then removed and the strip containing colistin was placed exactly over the place of the previous strip, in such a way that the prior determined MIC value of colistin coincided with the mark on the agar surface. That is, MICs of both meropenem and colistin lied at the same point. The plates were incubated for 18-20 hours after which the readings were taken. This represented the MIC of the colistin-meropenem combination.

**Figure 1: Legend**
(a), (b) Determine MICs of meropenem and colistin individually (6µg/ml and 2µg/ml)
(c) Place meropenem E-test strip on inoculated lawn of test organism
(d) Mark on the agar surface coinciding with the MIC value of meropenem (6µg/ml) and incubate for one hour

(e) Place colistin E-test strip after removing meropenem strip such that the MIC value of colistin (2µg/ml) coincides with the mark

(f) Read MIC after 18 hours, 3 dilution reduction (0.38µg/ml) compared to colistin alone suggests synergy

The *in vitro* interaction between the two drugs was interpreted based on the difference in the MIC of the combination over the MIC of colistin alone. The combination was interpreted to be synergistic when the MIC of the combination was 3 dilutions lower than the MIC of colistin alone. Antagonism was interpreted when the MIC of the combination was 3 dilutions higher than the MIC of colistin alone. Decrease or increase in the MIC of the combination by one or two dilutions was considered as indifference and additive respectively.(14)

**Results:**

All the 35 isolates tested were resistant to meropenem. Four isolates were resistant to colistin. Synergy was observed in 10 isolates (29%) while antagonism was observed 4 isolates (11%). In the remaining isolates the combination was either additive or indifferent (60%). (Figure 2) All the four isolates showing antagonism were susceptible to colistin.

![Figure 2: In vitro interaction of colistin-meropenem combination on MDR Klebsiella pneumoniae](image)

**Discussion:**

Colistin plays a crucial role in the treatment of MDR gram-negative bacterial infections as in most cases it remains the only therapeutic option. Studies have shown colistin to be highly effective against the carbapenem resistant Enterobacteriaceae producing the New Delhi metallo lactamase - 1 (NDM-1).(15) As NDM-1 happens to be the commonest mechanism of the carbapenem resistant Enterobacteriaceae in the Indian subcontinent, use of colistin seems more relevant in our country.(16) Also, colistin in combination with certain drugs like meropenem, rifampicin, teicoplanin has found to be highly effective against the common MDR non fermenting gram-negative bacteria such as Acinetobacter spp. and Pseudomonas aeruginosa.(17)(18)(19) It has been well documented by various in vitro studies that colistin combination with a synergistic drug performs superior to colistin alone.(11) Most of the published studies have evaluated the efficacy of colistin combination on MDR Acinetobacter spp. or Pseudomonas aeruginosa and have demonstrated promising results. However, the findings of Souli et al. reveal a poor synergy between colistin and fosfomycin against KPC producing Klebsiella pneumoniae.(20) Likewise, Elemam et al. did not observe synergy on combination of polymyxin B with cephalosporins or imipenem.(21) But neither of the studies mentioned afore demonstrated antagonism. In the present study, meropenem was selected as the combination drug for colistin because it is a smaller molecule and can enter into the target organism.
in sufficient amounts even with a little increase in membrane permeability caused by colistin. Also, it was found to be highly synergistic to colistin against MDR Acinetobacter spp. In this study the colistin-meropenem combination showed synergy in only 29% of the tested isolates. Antagonism was also observed in a minority of the isolates which was hardly noted in other studies. This could be due to the drawback of the diffusion method used for testing colistin. Colistin being a large molecule diffuses inadequately into the medium. However the E-test method was employed in the study because it was stated to perform equivalent to dilution techniques by other studies.

Conclusions:
Although the results of this study do not favor the use of colistin-meropenem combination against MDR Enterobacteriaceae, it would be unjust to conclude so. This is due to the small sample size tested and also the controversy in the usage of diffusion methods for testing colistin. The time-kill assay which is considered the gold standard test for in vitro synergy testing should be performed on more number of isolates to justify these findings.

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