In Vitro Demonstration of Synergy of Colistin with Various Antimicrobial Agents by Disc Diffusion and E Testing against Clinical Isolates of Carbapenem Resistant *Acinetobacter*

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ABSTRACT

BACKGROUND

Acinetobacter is a notoriously drug resistant pathogen with many isolates being resistant to carbapenems. Colistin is often used in combination with other antibiotics as there is a chance of clinical failure when used as monotherapy. In vitro testing for synergy is extremely important for the clinician in order to choose the appropriate drug for treatment. The present study aims to evaluate the synergy of various antibiotics in combination with colistin.

METHODS

36 isolates of carbapenem resistant *Acinetobacter* were included in the study. Two groups of freshly prepared plates of plain Mueller Hinton agar (MH) and Mueller Hinton agar with 0.5 mcg/ml colistin were taken. 0.5 McFarland suspension of the isolates was prepared, and swabbing was done on both the groups of MH agar plates. Disc diffusion testing was done by placing various antimicrobial agents like tigecycline, meropenem, cefoperazone/sulbactam rifampicin and ciprofloxacin on both the plates. Zones of inhibition were measured and differences in the zone sizes for these drugs with and without colistin combination were noted. All these isolates were further tested for synergy testing by using the E strips of these antibiotics on MH agar plates with and without colistin (0.5 mcg/ml) and comparing the minimum inhibitory concentrations (MIC) of these drugs. Synergy was considered when the difference is more than 5 mm by disc diffusion or when the MIC is decreased by 3 folds for that particular antibiotic when tested on MH agar plates with and without colistin.

RESULTS

Out of 36 isolates, highest synergy was observed for colistin in combination with rifampicin with 21 isolates (58.3%) showing synergy. This is followed by combination with cefoperazone sulbactam (19 isolates, 52.7%), meropenem (11 isolates, 30.5%). Only 5 isolates (13.8%) have shown colistin and tigecycline synergy. None of the isolates have shown synergy with ciprofloxacin. In 2 isolates synergy was observed for all the four drugs in combination with colistin. Good correlation was observed between disc diffusion and MIC testing with majority the isolates (81%) showing more than 5 mm difference in the disc diffusion test showing synergies by MIC testing.

CONCLUSIONS

Disc diffusion testing by incorporating active drug in the medium may help in empirically choosing the appropriate combination for which additional tests can be chosen which are more accurate. This methodology is cost effective and easy.

KEY WORDS

Synergy, Colistin, *Acinetobacter*, Disc Diffusion

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BACKGROUND

Emergence of multidrug resistant pathogens has become a major healthcare issue. *Acinetobacter baumannii* is a notoriously drug resistant pathogen causing nosocomial infections like ventilator associated pneumonia, bacteraemia, skin and soft tissue infections and device associated infections in health care setups. Multi-drug resistance in these organisms has become a common occurrence and many of these *Acinetobacter baumannii* isolates are also showing increased resistance to carbapenems which are considered the best drugs for treatment. There are very few alternative drugs available for treatment of carbapenem resistant strains, among which colistin is an important option. Colistin is often used in combination with other antibiotics as there is a chance of clinical failure when it is used as monotherapy.^{1,2} In vitro testing for synergy is extremely important for the clinician in order to choose the appropriate drug for treatment. Many researchers have been working to standardize the In vitro methodologies of synergy testing. Though there are different in vitro synergy testing methodologies like time kill assays, checkerboard, E test methodologies, there is no consensus regarding which method should be used. Each and every method has its own advantages and disadvantages. Though time kill assays are suggested as the best methodologies for synergy detection, they are seldom used in routine microbiology laboratories because of the laborious and time-consuming procedures and they also need expertise. Among the entire methodologies, E-test based methodologies are considered simple and user friendly and requires less expertise but there has been no standardization of these methods and its correlation with time kill studies was highly variable in different studies. Above this, the synergy effects of various combinations of antibiotics against *Acinetobacter baumannii* is strain specific. So, each and every isolate needs to be tested separately by using various combinations of antibiotics to know which combination of antibiotics work better for that particular isolate. Testing for all the different combinations of antibiotics for each isolate is extremely difficult and also highly expensive because of which routine diagnostic laboratories cannot practice this for all the isolates. As disc diffusion methodology is the most widely practiced method for susceptibility testing for bacterial isolates even in resource poor settings, we have adopted the disc diffusion-based methodology in detecting the synergy of various antibiotics in combination with colistin against the carbapenem resistant *Acinetobacter baumannii* clinical isolates.

METHODS

This prospective study was done in SVS medical college, Mahbubnagar from July to December 2015. Only carbapenem resistant *Acinetobacter baumannii* isolates obtained during the study period were further processed for synergy testing. A total of 36 non repetitive isolates of carbapenem resistant *Acinetobacter baumannii* were obtained during the study period and were included in the study. Isolation and identification were done by routinely performed methods in

our microbiology laboratory. Susceptibility testing was done by disc diffusion testing according to the CLSI Guidelines³. MICs of colistin were detected by E-strip methodology (Hi Media). Pure powder of colistin was obtained from Sigma and was incorporated into Mueller Hinton agar plates at a fixed concentration of 0.5 µg/ml.

For in vitro synergy testing, two groups of freshly prepared plates of plain Mueller Hinton agar (MH agar) and Mueller Hinton agar with 0.5 µg/ml colistin were taken. 0.5 McFarland suspension of the isolates was prepared, and swabbing was done on both the groups of MH agar plates. Disc diffusion testing was done by placing various antimicrobial agents like rifampicin, tigecycline, meropenem, cefoperazone/sulbactam and ciprofloxacin on both the plates. These antibiotics were chosen based on the data of the commonly used antibiotics in our hospital. Zones of inhibition were measured for all these antibiotics in both the plates and differences in the zone sizes for these drugs with and without colistin combination were noted. All these isolates were further tested for synergy testing by using the MIC strips of these antibiotics (HiMedia, India) on MH agar plates with and without colistin (0.5 µg/ml) and comparing the minimum inhibitory concentrations (MIC) of these drugs. Synergy was considered when the difference is more than 5 mm by disc diffusion or when the MIC is decreased by 3 folds for that particular antibiotic when tested on MH agar plates with and without colistin.⁴ *E.coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. SPSS software was used to perform statistical analysis using a paired t-test. $p < 0.05$ was considered statistically significant.

RESULTS

Out of 36 isolates, highest synergy was observed for colistin in combination with rifampicin with 21 isolates (58.3%) showing synergy by both disc diffusion and E test methods. This is followed by combination with cefoperazone/sulbactam (19 isolates, 52.7%), meropenem (11 isolates, 30.5%). only 5 isolates (13.8%) have shown colistin and tigecycline synergy. None of the isolates have shown synergy with ciprofloxacin. In 2 isolates synergy was observed for all the four drugs in combination with colistin. Good correlation was observed between disc diffusion and MIC testing with majority the isolates (81%) showing more than 5 mm difference in the disc diffusion test were showing synergies by MIC testing.

Total Number of Isolates (n=36)	Rifampicin+ Colistin	Cefoperazone/ sulbactam + Colistin	Meropenem + Colistin	Tigecycline + Colistin	Ciprofloxacin + Colistin
Synergy observed	21	19	11	5	0
No synergy	15	17	25	31	36

Table 1. Synergy of Various Antimicrobials in Combination with Colistin

Figure 1. Disc Diffusion Testing on Muller Hinton Agar Plates with (0.5 mcg/ml) and without Colistin. Enhanced Zone of Inhibition was Observed for some Antibiotics on Colistin Containing Agar (Left) Plate

Figure 2. E Test/Agar Dilution Testing on Muller Hinton Agar Plates Plain and with Colistin (0.5 mcg/ml) Incorporated. Observe the Decrease in the MICs for Cefoperazone Sulbactam and Meropenem on Colistin Containing Agar Plate (Right Side Figure)

DISCUSSION

Acinetobacter is one of the most important nosocomial pathogens in hospitals which causes serious hospital acquired infections. Carbapenems have long been used as the drugs of choice for the treatment of these nosocomial infections. With the emergence of MDR *Acinetobacter* which is resistant to carbapenems too, the old and forgotten polymyxin group of drugs have gained importance once again with colistin being used extensively for the treatment of carbapenem resistant *Acinetobacter*. However, monotherapy with colistin is problematic as hetero resistance to colistin has been observed both in vitro and also during the therapy which raises concern about its monotherapy. Therefore, use of combination therapy has drawn attention of the clinicians for the treatment of MDR pathogens. Combination therapy helps in potentiating the effect of the other drugs and increases the eradication efficacy and also prevents the emergence of resistant population.

Synergy of colistin has been tested with various antibiotics like carbapenems, rifampicin, tigecycline, doxycycline, azithromycin etc by different researchers.^{4,5,6} In the present study colistin has shown maximum synergy with rifampicin (58.3%). Many studies have evaluated colistin plus

rifampicin synergy and they found it to be synergistic. In Tan et al⁷ study 56% of the drug resistant *Acinetobacter baumannii* isolates showed polymyxin B plus rifampicin synergy by time kill assays. In Wareham et al⁸ study all the strains showed reduced MICs with the polymyxin plus rifampicin combination by E test agar dilution methodology. Many in vivo studies have also demonstrated the effectiveness of colistin and rifampicin synergy in the treatment of drug resistant gram-negative bacteria.^{9,10,11} Next to rifampicin, cefoperazone sulbactam has shown good in vitro synergistic activity against *Acinetobacter* in our study. Though there are no CLSI guidelines regarding cefoperazone sulbactam, many clinicians use this drug in health care set up. The effectiveness of this drug against *Acinetobacter* is probably due to sulbactam which is known to have inherent activity against *Acinetobacter*. Other antibiotics like meropenem and tigecycline also showed synergistic effect to some extent in our study. Addition of colistin to meropenem might have potentiated the effect of meropenem by the action of the colistin on the outer membrane which allows meropenem to interfere with the cell wall synthesis. Carbapenems have long been considered as the most important and potent drugs against *Acinetobacter* species. But with the emergence of carbapenem resistance by various mechanisms, treatment of these infections become complicated. Colistin in combination with various carbapenems have been used for the treatment of nosocomial infections especially *Acinetobacter* species. Various researchers have also done invitro synergy testing of carbapenems in combination with colistin and have observed varying results.^{12,13} In a meta-analysis by Oren Zusman et al,¹² synergy was observed for colistin and carbapenems in 77% of the *Acinetobacter* isolates by time kill assays and they have also observed antagonism in few isolates. In contrast in another study by David W Wareham et al marked synergy was not observed between polymyxin and imipenem when the synergy was done by E test methods against OXA 23 carbapenemase producing *Acinetobacter* isolates. In Lim et al¹⁴ study polymyxin plus tigecycline has exhibited bactericidal activity in 9 out of 31 isolates (29%). Whereas in Sopirala et al⁴ study no synergy was demonstrated with tigecycline plus colistin combination, but synergy/additivity was observed with imipenem plus colistin combination for all the isolates. Tetracycline group of drugs have been tested in combination with colistin by various researchers.⁷ Doxycycline and minocycline have shown good invitro synergy with colistin in their studies. The new derivative tigecycline belonging to glycylcyclines has shown different results in various studies when used in synergy.^{15,16}

As colistin acts on the cell membrane, it disrupts the integrity of the membrane which makes the way for the hydrophilic antibiotics like rifampicin and vancomycin thus showing the synergistic activity with these drugs which are not having any action against *Acinetobacter* if used alone. Vancomycin, which is an antibiotic used against only gram-positive organisms has shown good synergy with colistin against *Acinetobacter* in many studies¹⁷. Clinical experience regarding colistin treatment against the multidrug resistance *Acinetobacter* has not been always satisfactory. Poor outcomes have been noticed in many clinical studies with colistin which may be related to various factors like underlying illness severity, pharmacokinetics and

pharmacodynamics of the drug, yet it is the only option available for the treatment in many cases.^{18,19,20}

Multiple factors hinder the synergy testing including the tested strains, mechanisms of resistance, type of the methodology used and also the quality of the working environment. Each and every method of synergy testing has its own advantages and disadvantages. As of now, none of the synergistic methods have been standardized and there is an urgent need of standard and easy method for rapid identification of synergy between various antimicrobials which guides the clinicians in real time so that appropriate treatment can be given, which further decreases the mortality and morbidity and the huge economic burden associated with these resistant infections in the hospitals.

CONCLUSIONS

Disc diffusion testing by incorporating active drug in the medium may help in empirically choosing the appropriate combination for which additional tests like time kill studies, checkerboard assays or E-test based methodologies can be chosen which are more accurate. This methodology is quick, cost effective and helps in decreasing the laborious work. Timely identification of the multidrug resistant organisms and determining the appropriate treatment regimens by available synergy testing in association with proper infection control practices will not only decrease the morbidity and mortality associated with serious nosocomial infections but also help in preventing the spread of these infections which further adds to economic burden on the health care system.

Disclosure

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