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An In Vitro Analysis of Combination Therapy on Colistin, With Minocyline, Rifampicin, Teicoplanin, and Trimethoprim/Sulfa, Against Extensive Drug Resistant Strains Of *Acinetobacter Baumanii* (Xrdab) Using E-Test/Gradient Testing

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Abstract*: The general objective of this study was to determine the bacteriological effect of drug combinations with colistin to Extensive Drug resistant Acinetonacter baumanii strains (XDRAB). The antimicrobial combinations tested for Acinetobacter baumanii were colistin and teicoplanin, colistin and tigecycline, colistin and minocycline, colistin and trimethoprim/sulfa, colistin and rifampicin (n = 57). The methods used in this study are pairs of E-test strips which is easy and inexpensive to perform and using the determined MICs of single antimicroabial and the in combination with colistin. The degree of agreement between FIC results calculated by E-test method varies in the literature depending on the type of bacteria tested. For example, 86% agreement was found between the results of the two tests when used with Acineto baumanii. A limitation of the E-test method is that it does not provide information about the bactericidal activity of the combination. We have shown that this method particularly useful in screening a large number of isolates against several combinations of antimicrobials.*

The results revealed that The combination of colistin and teicoplanin although shows synergy but in the lowest mean 10.5% while combinations of colistin with trimethoprim/sulfa gave the highest percentage of 86% followed by colistin and tigecycline 80.7% and colistin and rifampicin with 61% which means that if you combined this antimicrobial agents it gives greater results than using them separately effects against XDRAB.

The other antimicrobial agent gives less than 50% demonstrating either an additive affect or synergy. Other combinations tested were predominantly indifferent. We did not find a combination of antimicrobials that was consistently antagonistic when used against Acinetobacter baumanii.

There are significant differences in the effect of single utilization of antibiotic (colistin) and combined drugs such as colistin and teicoplanin although it shows synergy but in lowest percentage of 10.5%, while 86% gives the highest percentage of combination with colistin and trimethoprim/sulfa then followed by colistin and tigecycline 80.7% and colistin and rifampicin with 61% which means that if you combined this antimicrobial agents it gives greater results than monotherapy against Extensive Drug resistant Acinetonacter baumanii.

In conclusion, although the in vitro activity of an antimicrobial does not necessarily compare with the in vivo biological activity, results in previous work suggest a relationship between the MIC of an antimicrobial and clinical outcome of Acinetobacter baumanii against a single antimicrobial. In particular, a lower MIC was associated with a faster healing response. It is reasonable to assume that the lower the MIC of an antimicrobial for a given isolate, the more likely it is that the infection will respond to treatment and that the MIC of the antimicrobial can be used to evaluate the potential efficacy of a given agent for the treatment. It is not known whether the distributions of antimicrobial sensitivities of bacterial isolates tested in this study were affected by prior antimicrobial treatment before isolation of the bacteria. However, the isolates were selected from a national collection with a distribution of isolates similar to that in previous studies, and we think they are therefore representative of the bacteria.

Based on IN VITRO analysis antimicrobial combination that demonstrates a synergistic or additive effect as determined by MIC, this combination may prove more effective than monotherapy with the individual agent. It should be noted that the definitions of effect, from synergy through to indifferent, are definitions that relate to interaction in vitro, and it is unknown whether they translate into an improved outcome for topical combination therapy. If the extrapolation to an in vivo effect is valid, a synergistic or additive antimicrobial combination offers a broader spectrum of activity that may reduce selective pressures and the emergence of resistance.

Keywords: *Antimicrobial Combination therapy, Gradient testing, Minimal Inhibitory Concentration (MIC), Extensive Drug resistant Acinetonacter baumanii ((XDRAB), Fractional Inhibitory Concentration (FIC).*

I. Introduction

Acinetobacter baumanii is a genus of aerobic, non-fermentative gram negative *coccobacillary organism* has emerged and recognized worldwide as nosocomial threat due to multiple resistance mechanisms affecting mainly critical or immune-compromised patients. Several factors becomes a burden to infection control practitioners, hospital epidemiologists, clinicians, and hospital administrators are struggling to control due to the ability of this organism to chronically colonize patients and cause high outbreaks which is usually hard to eradicate. Intrinsic resistance to commonly used antibiotics (CLSI, 2016, p.216) which yield failure in treatment drug resistant strains of *Acinetobacter baumanii* have been associated with higher mortality and prolonged hospital stay are considerable infection control issues. Indeed, the World Health Organization has identified antimicrobial resistance as one of the three greatest threats to human health. Extensive drug resistant *A. baumannii* (XDRAB) is fast becoming a global threat, having developed resistance to major classes of antibiotics and have increasingly been reported worldwide as a cause of nosocomial outbreaks. Despite intensive efforts, nosocomial acquisition of XDRAB is still a problem due to the organism's great ability to colonize human and environmental reservoirs.

Acinetobacter baumanii is commonly found in soil, water and skin of healthy people, especially in health care settings it can survive in the environment for several days and may colonize or live in a patient without causing infection or symptoms, especially in tracheostomy sites or open wounds (Fournier PE, Vallenet D, Barbe V, 2006, p.2) Clinical illness associated with *Acinetobacter baumanii* includes pneumonia, meningitis, endocarditis, peritonitis, skin and soft tissue infections, urinary tract and blood stream infections. Unfortunately, this organism has developed one of the most impressive patterns of antibiotic resistance ever observed.

In the age of increasingly resistant organisms, the likelihood that empiric antimicrobial therapy will provide adequate coverage for potential pathogens causing an infection is increased with the use of two antimicrobial agents compared to a single agent

Prompt institution of antimicrobial therapy active against the causative pathogen is crucial in the treatment of severely ill patients suspected of having a bacterial infection. (Alvarez-Lerma F. 1996).

The wisdom of continued combination therapy after an organism is isolated and antimicrobial susceptibility data are known, however, is more controversial. One area where the approach to antibiotic use needs to be readdressed is the use of combination antibiotic therapy, which generally consists of a β-lactam, carbapenems, aminoglycoside or fluoroquinolone, for the treatment of infections with Gram-negative bacteria. There is evidence supporting the initial use of combination therapy for severe infections such as sepsis or ventilator-associated pneumonia (VAP), in the existing environment of XDRABthe broad empiric coverage provided by two antimicrobial agents with different spectra of activity (Pranita D. Tamma, et al, 2012).

II. Methods

This study utilized the experimental design wherein the researcher employed an investigator-controlled manipulation of the independent variable, and control of the study situation by the researcher, including the use of control variable.

The study was conducted using experimental design that helped determine the effect of **colistin** as the main antibiotic against extensive drug resistant Acinetobacter baumanii, in relation to each antimicrobial combination such as: colistin+teicoplanin, colistin+tigecycline, colistin+ minocycline, colistin+ rifampicin, and colistin+ trimethoprim/sulfa and classified as synergistic, additive, indifferent, or antagonistic, against extensive drug resistant strains of *acinetobacter baumanii* (xrdab) using e-test/gradient testing. Colistin as the main component of each antimicrobial combination sets as the independent variable which will be utilized along with other antibiotics known to have significant reaction to extensive drug resistant strains of acinetobacter baumanii, bacteriological outcomes such specifically zone of inhibition using several microbiological culture/plates is the dependent variable, the effect of colistin as a single drug will be used as the control variable to highlight effects and or reactions of each antimicrobial combinations against extensive drug resistant acinetobacter baumanii.

1. Sources of Data

A total of 57strains of Extensive drug resistant *Acinetobacter baumanii* were collected into the study from different patients comprising of 22 female and 35 male patients, majority of the patients studied were admitted into the ICU while the others are from the different wards. The ages of the patients range from below

20 to over 80 years, majority of the patient are 60-103 years age group (54%) while the 20-60 years age group (33%) and below 20 years age group (12.2%). The specimen sources were taken from respiratory specimens like tracheal aspirate and sputum 30 (52.6%), wound swab 10 (17.5%), body fluid 3 (5.26%), urine 9 (15.3%), blood culture 2 (3.5%), ear swab 2 (3.5%) and eye swab 1 (1.7%). Majority of the specimens were collected from elderly patients from respiratory samples.

2. Bacterial isolates:

Over 57 isolates of Extensive Resistant *Acinetobacter baumanii* were collected from this study. Majority of the samples were collected from respiratory specimens of ICUs elderly patients in a period of 6 months. The isolates were identified on the basis of colony morphology, motility, staining, VITEK GN-identification and oxidase test. Colonies of *Acinetobacter baumanii* are smooth, opaque, and slightly smaller that grew colorless or slightly non-lactose fermenters in Mac Conkey agar (SPML, Riyadh, KSA) under aerobic condition within 24-48 hours incubated at 36°C.

3. Instrumentation and Validation

The quality control was checked base on the performance of E test reagents, quality of media, inoculum and procedure used. Appropriate quality control strains such as E. coli ATCC # and *Acinetobacter baumanii* BAA. The reagent and test procedure are considered satisfactory if MIC values obtained fall within the quality control specification.

The procedure for identification of *Acinetobacter baumanii* and its susceptibility testing have been made to the Quality Control (QC) testing recommendations for the VITEK® 2 Identification (ID) cards. With the use of CLSI document which provides guidance to improve the flexibility of assessing the quality of commercial microbial identification systems (MIS).

The VITEK 2 system is an automated test methodology based on the minimum Inhibitory Concentration (MIC) technique reported by MacLowry and Marsh and Gerlach. The AST card is essentially a miniaturized and abbreviated version of the doubling dilution technique for MICs determined by the micro dilution method.

Each test contains 64 micro wells. A control well, that contains only microbiological culture medium, is resident on all cards, with the remaining wells containing pre-measured amounts of specific antimicrobial combined with culture medium.

The isolate to be tested must be diluted to a standardized concentration in 0.45% saline before being used to rehydrate the card. The card is then filled, sealed and placed into the instrument incubator/reader. The instrument monitors the growth of each well in the card over a defined period of time (up to 18- 24 hours incubation). At the completion of incubation, MICs are determined for each antimicrobial on the card.

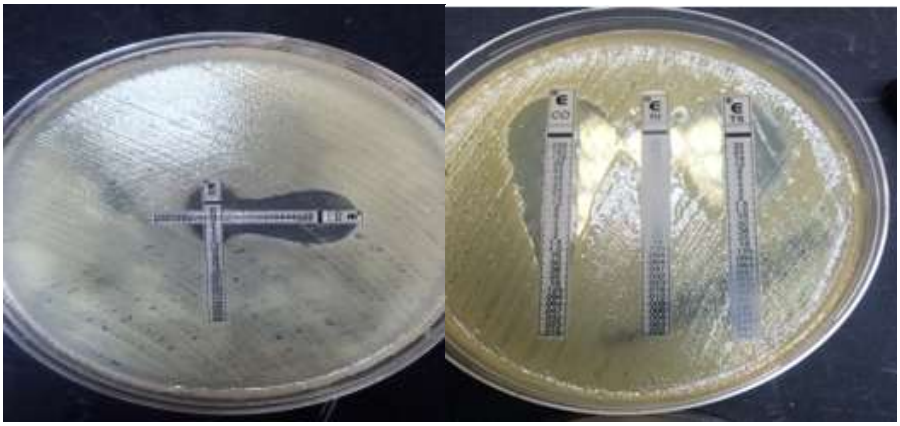
III. Results and Discussions

Micro broth dilution (MIC) were performed using 2-3 well isolated colonies of *Acinetobacter baumanii* from Mac Conkey agar plate, emulsified into Tryptic Soya Broth medium to achieve the specified inoculum turbidity of 0.5 Mc Farland standard. Inoculate the bacterial suspension using Mueller Hinton agar medium with predetermined battery of antimicrobial E-test strips (Colistin, tigecycline, trimethoprim/sulfa, minocycline, rifampicin). Read the precise MIC value by the presence or absence of elliptical zone of inhibition or growth around the strips.

The quality controlled strains of Escherichia coli ATCC 25922 for antimicrobial susceptibility testing and *Acinetobacter baumanii* ATCC BAA-747 for identification of isolates were tested in the individual E-tests to ensure that the expected values were obtained, as previously described.

For testing, the combination of E test strips were placed on the same culture medium in a cross formation, with a 90°C angle at the intersection between the scales at the respective MICs for *Acinetobacter baumanii*, and the plates were incubated at 35°C for 24-48 hrs. Determination of the MIC by E test was interpreted at the point of intersection between the zone of inhibition zone and the E test strips. Each bacterial isolate was tested with each antimicrobial combination such as: colistin+teicoplanin, colistin+tigecycline, colistin+ minocycline, colistin+ rifampicin, and colistin+ trimethoprim/sulfa and classified as synergistic, additive, indifferent, or antagonistic, according to their fractional inhibitory concentration (FIC).

Fig 1: Combination of antimicrobial strips



The antimicrobial combinations tested for *Acinetobacter baumanii* were colistin and teicoplanin, colistin and tigecycline, colistin and minocycline, colistin and trimethoprim/sulfa, colistin and rifampicin (n = 57). Using the results of MICs determined with the antimicrobial alone and in combination, the fractional inhibitory concentration (FIC) was calculated for each antimicrobial combination according to the following formulas: (Lorian, 1996).

$$\text{FIC of drug A} = \frac{\text{MIC drug A when tested in combination with drug B}}{\text{MIC of drug A alone}}$$

$$\text{FIC of drug B} = \frac{\text{MIC drug B when tested in combination with drug A}}{\text{MIC of drug B alone}}$$

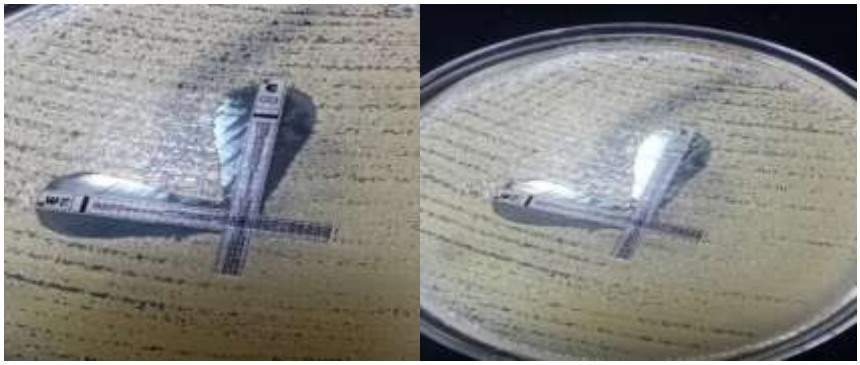
$$\text{FIC} = \text{FIC}_A + \text{FIC}_B$$

Our interpretations of the FIC results, according to accepted criteria, were as follows:

Synergy	≤0.5
Additive	0.5 to 1.0
Indifference	1.0 to 4.0
Antagonism	>4

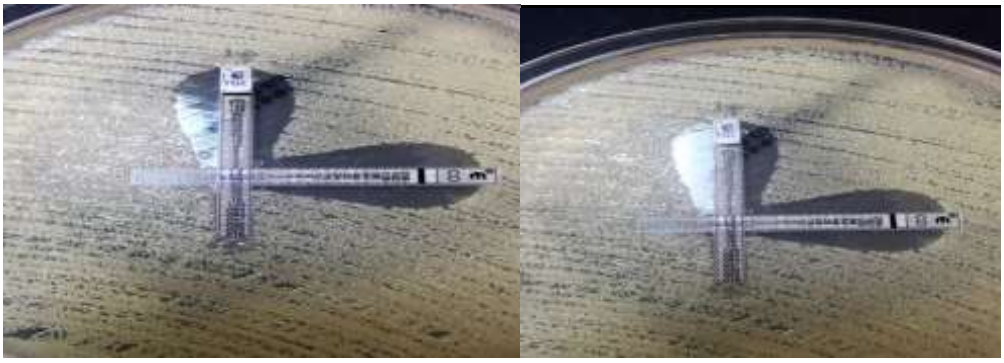
Examples are demonstrated in Figures 2 (Colistin – drug A and Rifampicin drug B) The MICs of A and B were 0.38 and 8.0 mg/L and decreased to 0.125 and 2.0 mg/L when measured in combination (FIC = 0.125/0.38 + 2.0/8.0 = 0.58). Figure 2b shows additivity: The MICs of A and B were 0.38 and 8.0 mg/L and decreased to 0.125 and 2.0 mg/L, respectively, when measured in combination (FIC = 0.125/0.38 + 2.0/8.0 = 0.58). It is apparent that a synergistic or additive effect can occur for the combination only if both FIC_A and FIC_B are each less than 1.0

Fig. 2- Combination of Colistin and Trimethoprim/sulfa:



Photograph of an E-test (Fig. 2) combination experiment between Colistin and Trimethoprim/sulfa against *Acinetobacter baumanii*, demonstrating additivity. The MICs of FICAColistin and Trimethoprim/sulfa alone were 0.5 and 1.0 mg/L, respectively, and when measured in FICBcombination were 0.19 and 0.32 mg/L, respectively (FIC = 0.70).

Fig 3: Combination of Colistin and Tigecycline:



Another E-test combination (Fig.3) experiment between Colistin and Tigecycline against *Acinetobacter baumanii*, demonstrating synergy. The MICs of Colistin and Tigecycline on FIC-A were 0.75 and 1.0 mg/L, and when measured the combination in FIC-B were 0.19 and 0.38 mg/L, respectively (FIC = 0.44).

The mean FIC for each antimicrobial combination for a particular isolate was then used to determine whether the combination would demonstrate a synergistic, additive, indifferent, or antagonistic effect on that bacterium. The mean, standard deviation, minimum and maximum, FIC was calculated for each antimicrobial combination against all Extensive drug resistant *Acinetobacter baumanii* (Lorian, 1996).

S- Synergy - in the synergistic response, the applied antibiotics work together to produce an effect more potent than if each antibiotic were applied singly.

A- Additive - additive effect, where the potency of an antibiotic combination is roughly equal to the combined potencies of each antibiotic singly**.**

I- Indifferent - A situation that arises when you combined two drugs , and the combination of two will not yield a result greater than the concentration and effectiveness of the most active drug in the combination.

Table C:- Synergy: (<0.5 value)		
Antimicrobial agent	**Results**	**Percentage**
1. Trimethoprim/sulfa	49/57	86%
2. Tigecycline	46/57	80.7 %
3. Rifampicin	35/57	61 %
4. Minocycline	27/57	47.4 %
5. Teicoplanin	6/57	10.5 %

Table D:- Indifference (1.0-4.0 value)		
Antimicrobial agent	**Results**	**Percentage**
1. Tigecycline	11/57	19.3%
2. Teicoplanin	8/57	14.0 %
3. Minocycline	7/57	12.3 %
4. Rifampicin	3/57	5.3 %
5. Trimethoprim/sulfa	0/57	0 %

Table E:- Additive (0.5- 1.0 value)		
Antimicrobial agent	**Results**	**Percentage**
1.Teicoplanin	43/57	75.4%
2.Minocycline	23/57	40.4%
3.Rifampicin	19/57	33.3%
4.Tigecycline	11/57	19.3%
5.Trimethoprim/sulfa	8/57	14 %

Sample computation for Fractional Inhibitory Concentration (FIC)

Drug A = Colistin

MIC value of colistin in combination with rifampicin = 0.094

MIC value of colistin (based on E-test result) = 0.5

(Combination of two MIC results of colistin and rifampicin)

Computation for Drug A: $\frac{0.094}{0.5}$ **= 0.188 or 0.19**

Drug B = Rifampicin

MIC value of Rifampicin in combination with colistin = 4

MIC value of Rifampicin (based on E-test result) = 8

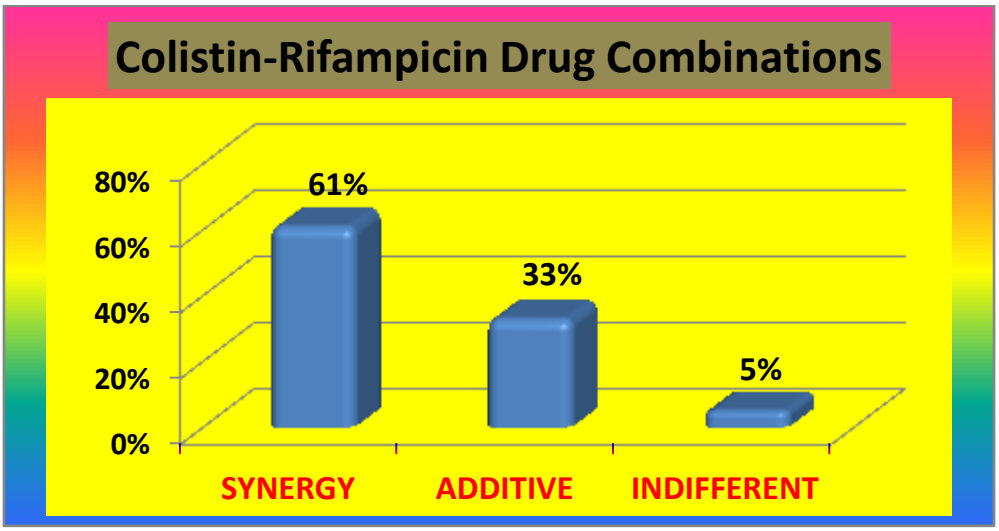
(Combination of two MIC results of colistin and rifampicin)

Computation of Drug B:

$\frac{4}{8}$ **= 0.5**

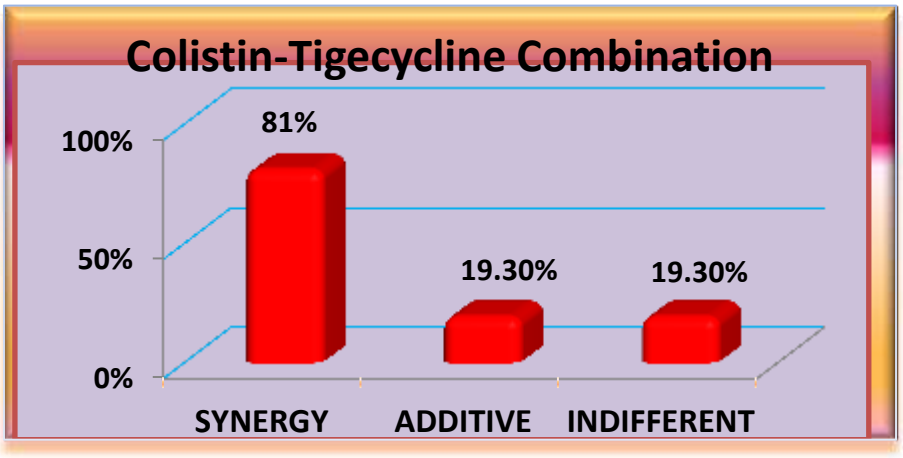
Then add drug A and drug B results: 0.19 + 0.5 = 0.69 Additive

Fig 4 – Combination therapy of Colistin-Rifampicin



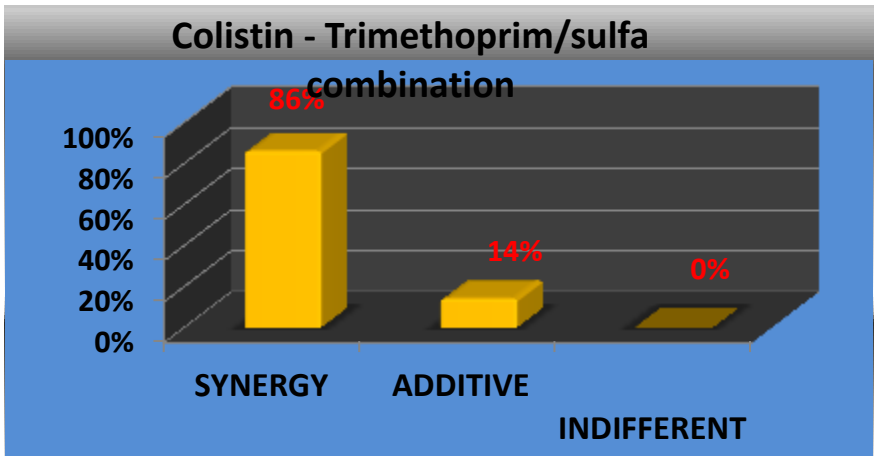
In this invitro antimicrobial reaction (Fig 4) of colistin-rifampicin shows an increased synergistic outcome (61%) it will yield greater effect and will produce more potency than if each antibiotic were applied singly or monotherapy against multidrug resistant Acinetobacter baumanii, as compared to the additive effect, 33% and indifferent %% (monotherapy) where the antibiotic is roughly equal to the combined potencies of each antibiotic.

Fig.5 Combination therapy of Colistin - Tigecyline



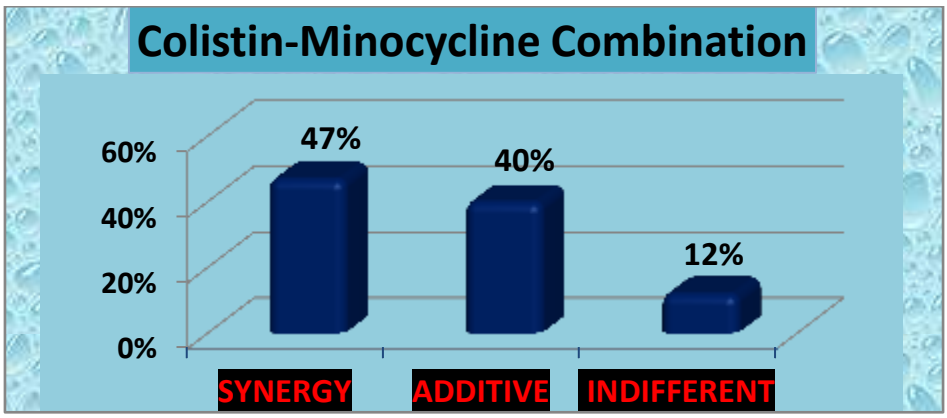
In this combination (Fig 5) a substantial increased of synergistic reaction 81% shows in combination of Colistin and Tigecycline whereas same 19.3% additive and indifferent reaction. This presume that when you combine the colistin-tigecycline this will have greater effect than using it alone.

Fig. 6 Combination therapy of Colistin-Trimetho/Sulfa



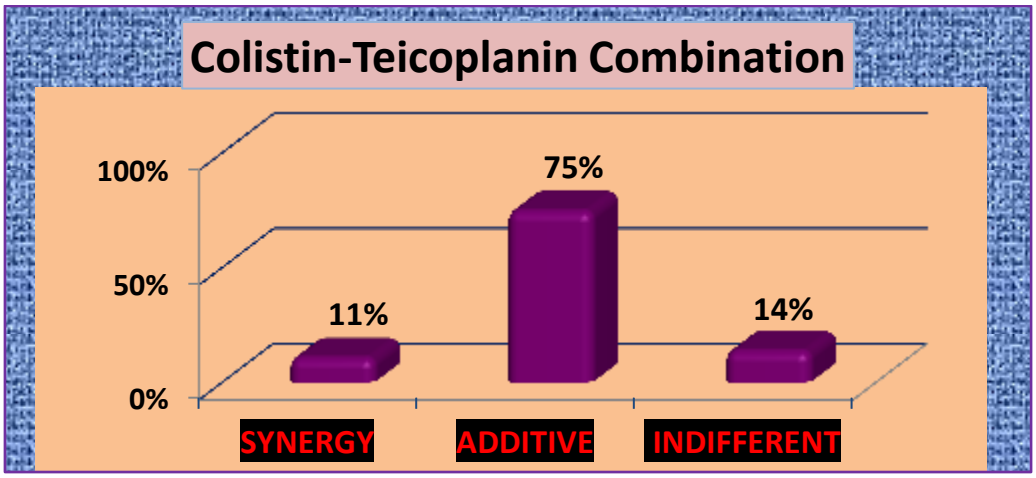
In combination(Fig 6) of colistin-trimethoprim drug yield a remarkable increase in synergy shows 86% while the additive 14% and 0% for indifferent. Synergism produce a greater effect than using this drug separately or in monotherapy.

Fig. 7 Combination therapy of Colistin-Minocycline



In this combination (Fig.7) a similar the effect of synergism and additive 47-40% found which means when you combined this two drugs it might have a similar of equal reaction as to the effectiveness of drugs. However, when you use this drug separately only 12% of chances of its effectiveness.

Fig 8 Combination therapy of Colistin-Teicoplanin



In the combination (Fig.8) of colistin-teicoplanin it shows synergistic reaction but in minimal effect (11%) and indifferent (14%) whereas the additive increase at 75%. The reaction resulting to combination of colistin-teicoplanin drug the effectiveness is less while if this drug will be use separately it will have an independent reaction and the most active drug concentration like colistin will have a greater effect than using it in combination.

Summary of findings

The results of drug combinations against Extensive drug resistant-*Acinetobacter baumanii* isolates are presented in Table-A & B Tabulation of Data: Trimethoprim/sulfa and colistin combination show the highest rate of synergism 86% , followed by tigecycline 80.7%, rifampicin 61% and Minocycline 47.4% these drug combinations shows good synergistic reaction while Teicoplanin demonstrated the lowest results with synergy observed in 10.5%.The additivity results for combination of drugs Teicoplanin shows the highest results 75.4%, Minocycline 40.4%, Rifampicin 33.3%, Tigecycline 19.3% and lowest result with 14% Trimethoprim/Sulfa. The Trimethoprin/sulfa were predominantly indifferent with 0%. No antagonistic effect was seen.

IV. Conclusion

The purpose of antimicrobial sensitivity testing is to provide a prediction of success or failure when a particular antimicrobial is used to treat a specific infection. The in vitro activity of an antimicrobial does not necessarily compare with the in vivo biological activity, results in previous work suggest a relationship between the MIC of an antimicrobial and clinical outcome of *Acinetobacter baumanii* against a single antimicrobial. In particular, a lower MIC was associated with a faster healing response. It is reasonable to assume that the lower the MIC of an antimicrobial for a given isolate, the more likely it is that the infection will respond to treatment and that the MIC of the antimicrobial can be used to evaluate the potential efficacy of a given agent for the treatment. It is not known whether the distributions of antimicrobial sensitivities of bacterial isolates tested in this study were affected by prior antimicrobial treatment before isolation of the bacteria. However, the isolates were selected from a national collection with a distribution of isolates similar to that in previous studies, and we think they are therefore representative of the bacteria.

The combination of colistin and teicoplanin although shows synergy but in the lowest mean 10.5% while combinations of colistin with trimethoprim/sulfa gave the highest percentage of 86% followed by colistin and tigecycline 80.7% and colistin and rifampicin with 61% which means that if you combined this antimicrobial agents it gives greater results than using them separately effects against XDRAB.

The method used in this study with pairs of E-test strips has the advantage that it is easy and inexpensive to perform. The degree of agreement between FIC results calculated by E-test method varies in the literature

depending on the type of bacteria tested. For example, 86% agreement was found between the results of the two tests when used with *Acineto baumanii.* A limitation of the E-test method does not provide information about the bactericidal activity of the combination. We have shown that this method particularly useful in screening a large number of isolates against several combinations of antimicrobials.

Clinical studies, manifestation and several types of infection are warranted to optimize colistin combination therapy against multidrug resistant Acinetobacter baumanii. This observational study could aid in the selection of the most appropriate antimicrobial agent for the empirical treatment of patients with MDRAB and, possibly, could also provide a useful background for planning further clinical trials.

V. Recommendations

In the light of the findings and conclusions, the following are offered as recommendations for possible actions and for future studies

1. A comparison with the in vivo biological activity, which results in previous work that suggest a relationship between the MIC of an antimicrobial and clinical outcome of *Acinetobacter baumanii* against a single antimicrobial.

2. Test the distributions of antimicrobial sensitivities of bacterial isolates that were tested in this study whether affected by prior antimicrobial treatment before isolation of the bacteria.

3. Utilize isolates that are selected from other sources aside from a national collection used in this study to determine if representative of bacteria is applicable.

4. Determine whether that the effects, of synergy through to indifferent, are definitions that relate to interaction in vitro, and whether they translate into an improved outcome for topical combination therapy.

5. A study on a broader spectrum of activity of synergistic or additive antimicrobial combination that may reduce selective pressures and the emergence of resistance.

6. To review the combination of colistin and teicoplanin that shows synergy but in the lowest mean 10.5% also combinations of colistin with trimethoprim/sulfa which gave the highest percentage of 86% using other methods

7. Reassess combination of antimicrobials that was consistently antagonistic when used against *Acinetobacter baumanii*.

8. Verify the advantages and disadvantages of other method, aside from the one used in this study with pairs of E-test strips. The degree of agreement between FIC results.

9. Explore limitation of the E-test method does not provide about the bactericidal activity of the combination.

10. Further observational study that could aid in the selection of the most appropriate antimicrobial agent for the empirical treatment of patients with MDRAB and, possibly, to also provide a useful background for planning further clinical trials.

11. A study on other multidrug resistant or extensive drug resistant organisms/on other population/ethnicity and or other cities of the same objectives to further strengthen the legitimacy of the study.

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