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ORIGINAL ARTICLE **OPEN ACCESS**

Investigating the ability of Synergistic Antibacterial Drugs against Uropathogens using *in vitro* Anti-biofilm assays and Anti-bacterial activity

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ABSTRACT
To reduce the multidrug resistant properties of certain urinary tract associated organisms, combination of two different groups of antibiotics were used with the aim of increasing the synergistic antibacterial activity. Ofloxacin+Ornidazole and Cefixime+Ciprofloxacin are the two drug combinations selected for the aim of study. Biofilm forming ability of the test organisms using Exit-site test and Microtitre plate assay was investigated. Synergistic antibacterial activity of selected drug combinations was evaluated. Exit site test revealed that among the test organisms Staphylococcus epidermidis and Staphylococcus aureus colonized with in 24h; Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae and Enterobacter sp colonized the catheter surface after 48 hours. Microtitre plate assay exhibited that, test organisms, Escherichia coli (0.286), Staphylococcus aureus (0.268), Klebsiella pneumoniae (0.276) and Staphylococcus epidermidis (0.264) considered as strong biofilm producers. Synergistic antibacterial test showed that all the five test organisms had complete synergistic effect for ofloxacin-ornidazole combination; most significantly, E. coli, Klebsiella pneumoniae and Staphylococcus epidermidis showed complete synergy with best Fractional inhibitory concentration (FIC) of 0.24. Perhaps varied results were evident for Cefixime+Ciprofloxacin against test organisms with E. coli alone showing synergism with FIC value 0.24. The findings revealed that Ofloxacin + Ornidazole combination will restrict urinary tract causing pathogen to become multi-drug resistant in near future.
Keywords: *Uropathogen, Synergism, Biofilm, Exit-site, Ofloxacin, Cefixime*

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INTRODUCTION

Urinary tract infections (UTIs) due to multidrug resistant (MDR) uropathogens have increased concern globally since the last 2 to 3 decades (17). Escherichia coli has been reported to be a common uropathogen, accounting for 75 to 90% of the UTI isolates (7). Urinary tract infection (UTI) is a common bacterial infection that frequently contributes to morbidity in the hospitalized and outpatients. Microorganisms have developed resistance to the newer and more potent antimicrobial agents thereby limiting the therapeutic options. Therefore, the situation of prevailing MDR uropathogens is crucial for deciding the proper use of antimicrobial drugs in order to fight against MDR UTIs (2). Hence, lot of research on combination therapy using two or more antibiotics were used to treat UTIs and other types of bacterial infections. Combination antibiotic therapy is used in critically ill patients due to widespread emergence of multidrug resistance organisms (MDR). Multidrug resistance is defined as lack of susceptibility to at least one agent in three or more antibiotic categories (11). Using dual coverage for organisms producing enzymes (beta lactamase, carbapenemase etc) is intuitively thought to be better with combination therapy when compared with monotherapy in sepsis patients (14). As per literature survey, combination therapy is mostly practiced because of one or more of the following reasons. (13) reported that using more than one antibacteirla agent broadens the antibacterial spectrum of the empirical therapy and thus ensures that at least one agent will cover the infecting organism. This is mainly due to Chances of emergence of resistance against two drugs are lower as compared with a single drug (10),(15) highlighted the significance of synergistic antimicrobial therapy. Antibiotic combinations are also used for their synergistic action. Synergy is defined as combined effect of two agents together being greater than the sum of their individual activities, e.g. certain beta-lactams and aminoglycoside combinations. Among the recent antibiotics, some of the researchers highlighted Cefixime as a first line antibiotic in community-acquired URTI (8). (5) reported that Cefixime is a potent broad-spectrum antibiotic with excellent efficacy in community acquired infections resistant to

macrolides. Another classic antibiotic ofloxacin is considered as the drug of choice for the empirical treatment of UTI. It belongs to a new generation of fluorinated quinolones and is active against Gram-Negative and Gram-Positive bacteria (7). (9) earlier stated that Ofloxacin has shown to be effective against acute and chronic UTIs, is well tolerated and has a unique feature of being exempted from plasmid-borne bacterial resistance. Based on these concepts, two different drug combinations were tested to prove their synergistic antibacterial activity against different bacteria associated with urinary tract infections. Ofloxacin+Ornidazole and Cefixime+Ciprofloxacin are the two drug combinations evaluated in the study. Following objectives were framed and the findings were reported.

- To determine the biofilm forming ability of the test organisms using Exit-site test and Microtitre plate assay.
- To evaluate the synergistic antibacterial activity of selected drug combinations (Ofloxacin+Ornidazole and Cefixime+Ciprofloxacin) against test bacteria.

MATERIAL AND METHODS

Collection of test bacteria associated with urinary tract infections

Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Enterobacter sp and Klebsiella pneumoniae cultures were procured from a diagnostic laboratory, Coimbatore, India. All cultures were processed, sub-cultured in Microbiology Department, Nehru College of Arts and Science, Coimbatore, India. All cultures were stored as pure cultures under refrigeration condition.

Determining the biofilm forming abilities test organism

Exit-site challenge test (3)

Exit-site challenge test was performed as the preliminary test. This test was used to identify the ability of specific test organism to grow on a type of biomedical materials used in the study. In this method, three-quarter strength of Iso-sensitest semi solid Agar was poured into a sterile boiling tube and allowed to solidify. The surface of the agar was then inoculated with 10µl of 18h test bacteria. The pre-measured size (length - 5mm) of catheter sample was cut, sterilized and partially inserted into the Iso-sensitest semi-solid medium through the inoculated area and incubated at 37°C. Migrating ability of the test bacteria from the exit site down the material track i.e., outside of the materials were assessed visually up to 24-48 hours.

2.2.2 Microtitre plate assay (6)

Bacterial attachment to an abiotic surface is assessed by measuring the stain taken up by adherent biomass in a 96-well plate format by means of microtitre biofilm assay. The test organisms were grown in 96-well microtitre plate for 48h. Each of the test organisms was inoculated in a 5ml culture broth and grown to stationary phase. Cultures were diluted at 1:100. Following this, 100µl of each diluted cultures was pipetted into eight wells in a fresh microtitre plate. The plate was covered and incubated at optimal growth temperature for 24 to 48h. About 125µl of 0.1% crystal violet solution was added to each well. Staining was done for 10min at room temperature. The crystal violet solution was removed by shaking each microtitre dish out over the waste tray. The plates were allowed to air-dry. Added 200µl of 95% ethanol to each stained well. The plates were covered to allow solubilization by incubating for 10 to 15min at room temperature. The contents of each well were briefly mixed by pipetting. Following this, 125µl of the crystal violet-ethanol solution was transferred from each well to a separate well in an optically clear flat-bottom 96-well plate. The optical density (OD) of each of these 125µl samples was measured at a wavelength of 500 to 600nm. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader. The OD values were considered as an index of bacteria adhering to surface and forming biofilms. Based on the OD value the adherence of organism in the plate can be classified as below (Table-1).

Table1: Classification of biofilm formation

Mean OD values	Biofilm formation	Biofilm index
<0.120	Nil	Non / weak
0.120-0.240	Moderately	Moderate
>0.240	Strong	High

Selection of Synergistic antibacterial drugs

As per literature surveys (5) (9), the oral antibiotic drugs (Cefixime and Ofloxacin) were selected in the present study. To investigate its synergistic activity two fluoroquinolone drugs (Ornidazole and Ciprofloxacin) was selected. Ornidazole was used as combinational therapy by adding with Ofloxacin and Ciprofloxacin was added with Cefixime. Synergistic characteristics of the two combination was analyzed in the next step.

Selection of synergistic drug from two different combinations using Checker Board titration method (16)

The synergistic activity of Ofloxacin-Ornidazole on all the test bacteria was determined by the standard checker board titration method. To determine the inhibitory concentrations of each drug separately and in

combinations, the minimal inhibitory concentrations (MIC) was simultaneously identified in this method. The fractional inhibitory concentrations (FIC) of the drugs were calculated from MIC values to determine the synergism between the two drug combinations (Ofloxacin-Ornidazole). To assess antimicrobial combinations *in vitro* the checkerboard method was selected. In this technique by using agar dilution method, the concentrations tested for each antimicrobial agent were typically ranged from four or five below the expected MIC to twice the anticipated MIC as in the 45-degree line (each square represents one plate). The predetermined concentrations (µg/ml) used for this method were 0.06, 0.12, 0.25 and 0.5. According to Fig.1, the plates in the left hand column were used for the predetermined concentrations of Drug-A (Ofloxacin), the plates in bottom row were used for Drug-B (Ornidazole) and the plates in the 45 degree line were used for mixed drug combinations. In all the arranged plates, 1ml of predetermined dilutions of the antimicrobial agents was added with sterile and molten Muller-Hinton agar. Then the surface of each plate was inoculated with 1 X 10^4 CFU/spot of bacteria. After 16-20 h incubation at 37 ºC, the plates were examined for evidence of visible growth. Experimental set up was made for all the drug combinations in triplicate. Similar experimental set up was carried out for Cefixime + Ciprofloxacin.

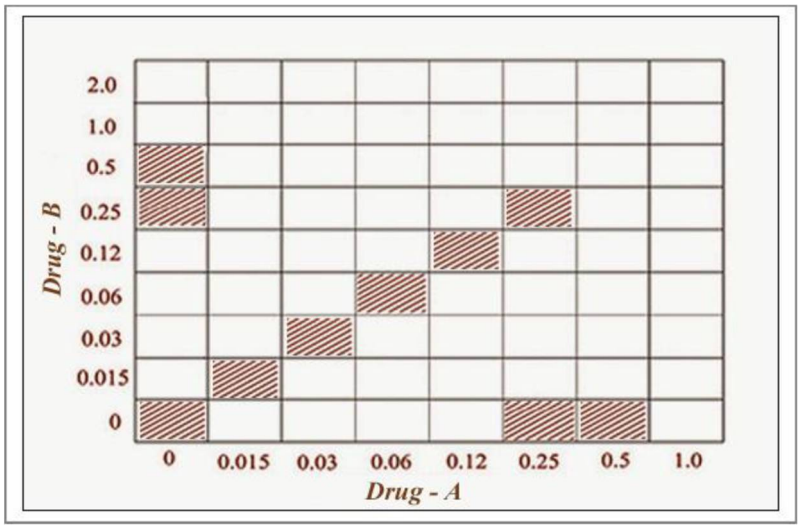


Fig. 1: Checkerboard model to determine synergism of two drugs

(The picture was adapted from (16)
Bottom row: To determine MIC of [Drug-A: Ofloxacin or Cefixime],
Left column: to determine MIC of [Drug-B: Ornidazole of Ciprofloxacin],
Diagnol: MIC of (Drugs) - Ofloxacin-Ornidazole OR Cefixime-Ciprofloxacin

Evaluating the synergism between antibiotic drug combinations (4)

Fractional inhibitory concentration index (FICI) was calculated by using the following equation.

$$FIC\ index = FIC_A + FIC_B$$

'A' was the minimal inhibitory concentration of Drug-A in a plate that was the lowest inhibitory concentration in its row, and 'B' was the MIC of Drug-B in a plate that was the lowest inhibitory concentration in its column. MIC_{AB} was the lowest inhibitory concentration of Drug 'A' and 'B' in combination in the 45 degree line. With this method, synergism has traditionally been defined as an FIC index of 0.5 or less and partial synergy as a FIC index of >0.5 - ≤1.0; antagonism has been defined as a FIC index of ≥2.0.

Interpretation:
Mean FICI ≤ 0.5 → Synergy, (p< 0.5),
Mean FICI >0.5 - ≤1.0→ Partial synergy, (p> 0.5)
Mean FICI ≥ 2.0→ Antagonism

RESULT AND DISCUSSION

Biofilm assay

Exit-site challenge test

(3) reported that the most frequent routes of catheter associated infection are from the skin exit site, the tissue tunnel associated with the catheter and the catheter lumen. Similar exit-site challenge model under *in vitro* condition used by (3) showed surface colonization of methicillin resistant *Staphylococcus aureus* (MRSA) on the CSF silicone shunts surface. In this present study the surface colonizing ability of test

bacteria on the urinary catheter sample materials was investigated using exit-site challenge test. Migration or growth of the test organism around the materials after incubation was indicated by tracking of bacteria along the abluminal surface. All the test organisms used in the research colonized the material surfaces between 24h to 48h. Among the test organisms *Staphylococcus epidermidis* and *Staphylococcus aureus* colonized with in 24h; *Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Enterobacter* sp colonized the catheter surface after 48 hours. According to Bayston.s concept, the inoculated site was considered to be as skin exit-site and migration and growth of the organisms along the media surface was considered to be as the tissue tunnel and tissue surroundings. So the obtained results were considered as the preliminary test to determine the surface colonizing ability of the test organisms.

Microtitre plate assay

The optical density (OD) values and biofilm index of the test organisms were tabulated based on the biofilm classification described(6). The test organisms considered as strong biofilm producers in MTP assay were, *Escherichia coli* (0.286), *Staphylococcus aureus* (0.268), *Klebsiella pneumoniae* (0.276) and *Staphylococcus epidermidis* (0.264). These organisms showed OD values >0.240. Moderate biofilm formation was observed during the MTP assay for *Enterobacter* sp (0.186). The differences in OD values were due to the amount of crystal-violet (dye) absorbed by the test organisms in the microtitre well. The high and moderate biofilm producers were identified by the colour intensities formed in the microtitre plates. Appropriate control was maintained (Table-2 and Fig. 2). (12) reported similar findings as observed in the present study. The researchers described that this method was found to be most sensitive, accurate and reproducible screening method for the detection of biofilm formation. The method has the advantage of being a quantitative model to study the adherence of organism on biomedical devices. In this test, even though some of the test organisms were proved to as weak or moderate biofilm producers, still due to their clinical complications and pathogenicity in the medical sciences, it made curious to proceed for further analysis.

Table-2: Microtitre plate assay

S. No.	Samples and Bacteria	Biofilm formation (OD 570nm)	Biofilm index
1	*Escherichia coli*	0.286	Strong
2	*Staphylococcus aureus*	0.268	Strong
3	*Klebsiella pneumoniae*	0.276	Strong
4	*Enterobacter sp*	0.186	Moderate
5	*Staphylococcus epidermidis*	0.264	Strong
6	Control C_1 (Crystal violet)	0.09	Nil
7	Control C_2 (Nutrient broth)	0.09	Nil

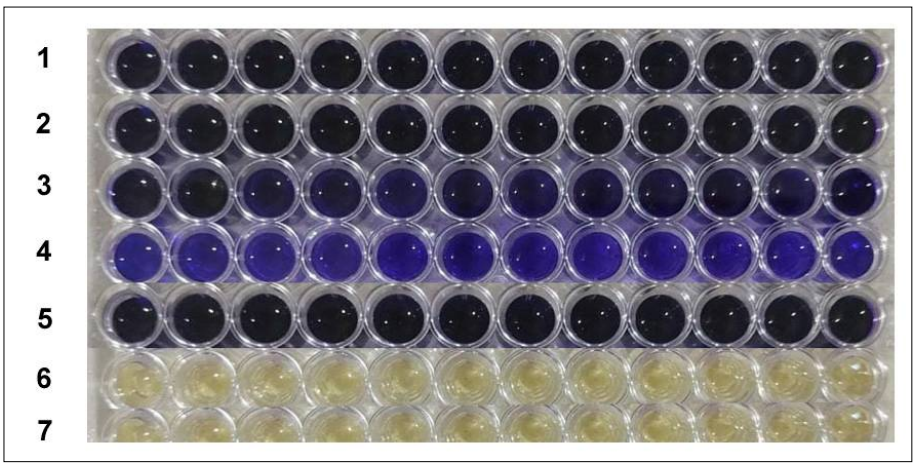


Fig. 2: Microtitre plate assay

Synergistic antibacterial activity

Effect of Ofloxacin-Ornidazole against test bacteria

All the five test organisms showed complete synergistic effect for ofloxacin-ornidazole combination (Table-3). Most significantly, E. coli, Klebsiella pneumoniae and Staphylococcus epidermidis showed complete synergy with the mean MIC value 0.03 µg/ml (Fig. 3A, 3B and 3C) with best FIC value 0.24 respectively.

Other significant organisms, Enterobacter sp and Staphylococcus aureus also showed complete synergy with the mean MIC value 0.12 µg/ml and with best FICI value 0.72.

Table-3: Effect of Ofloxacin-Ornidazole against test bacteria

Test Bacteria	MIC_A	MIC_B	MIC_{AB}	FIC_A	FIC_B	FIC_{AB}	Index
Escherichia coli	0.25	0.25	0.03	0.12	0.12	0.24	S
Staphylococcus aureus	0.25	0.5	0.12	0.48	0.24	0.72	S
Klebsiella pneumoniae	0.25	0.25	0.03	0.12	0.12	0.24	S
Enterobacter sp	0.25	0.5	0.12	0.48	0.24	0.72	S
Staphylococcus epidermidis	0.25	0.25	0.03	0.12	0.12	0.24	S

Mean value of three trials were tabulated
A – Ofloxacin, B - Ornidazole, AB- Combined concentration of Ofloxacin-Ornidazole
S – Synergy, Units for all the presented values - µg/ml

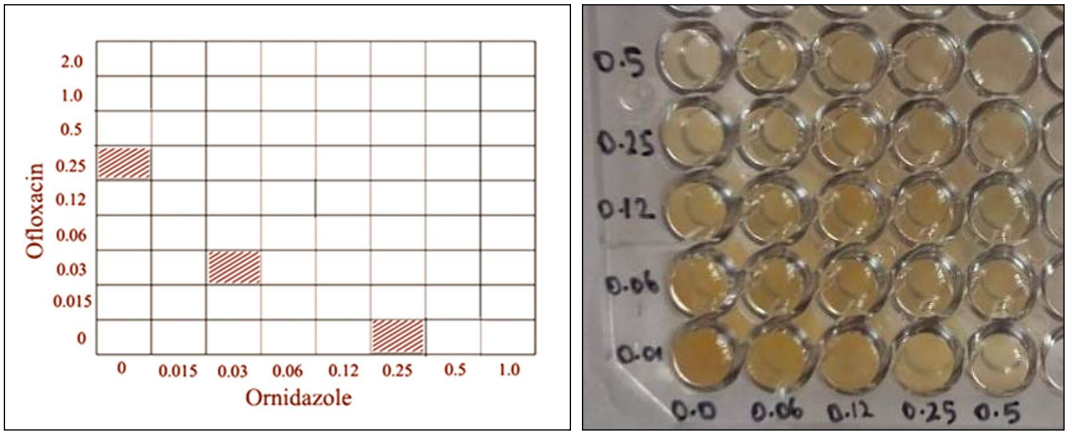
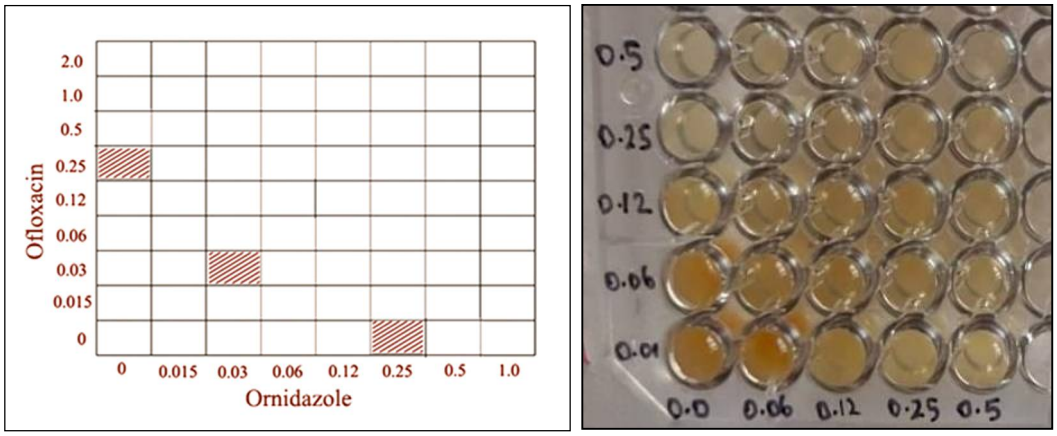
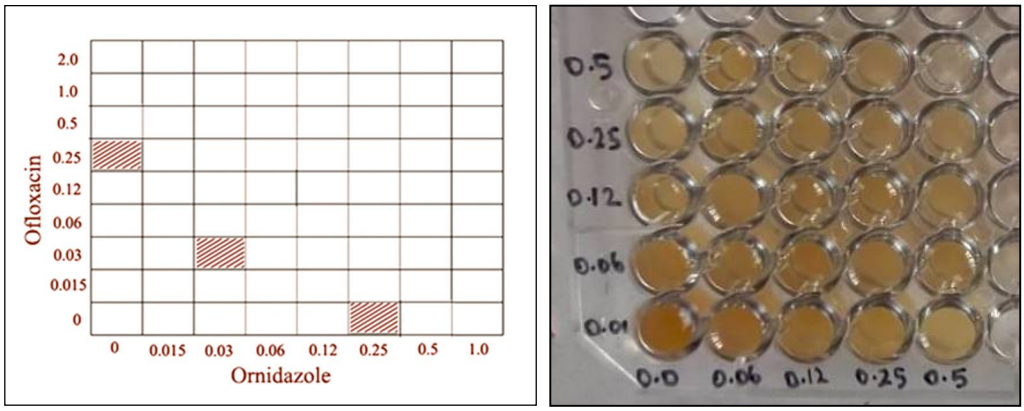


Fig. 3: Simplified Checker Board Method of Ofloxacin-Ornidazole
MICA – 0.25, MICB – 0.25, MICAB – 0.03
3A: Test Bacteria: *E. coli*



MICA – 0.25, MICB – 0.25, MICAB – 0.03
3B: Test Bacteria: *Klebsiella pneumoniae*



MICA – 0.25, MICB – 0.25, MICAB – 0.03

3C: Test Bacteria: *Staphylococcus epidermidis*

Effect of Cefixime-Ciprofloxacin against test bacteria

E. coli alone exhibited synergistic activity for Cefixime-Ciprofloxacin combination with the mean MIC value 0.03µg/ml and with best FIC value 0.24 ($p<0.5$) (Fig. 4). Among the test organisms, S. aureus and S. epidermidis showed partial synergism with MIC value of 0.06µg/ml and FIC value of 1.0 (Table-4). Klebsiella pneumoniae also exhibited partial synergistic character with MIC value of 0.25 µg/ml and FIC value of 0.75. Interestingly, Enterobacter sp expressed antagonism with MIC of 1.0 µg/ml and FIC value of 3.0. This may be due to higher concentration of drugs required to combat the species.

Table4: Effect of Cefixime-Ciprofloxacin against test bacteria

Test Bacteria	MIC_A	MIC_B	MIC_{AB}	FIC_A	FIC_B	FIC_{AB}	Index
Escherichia coli	0.25	0.25	0.03	0.12	0.12	0.24	S
Staphylococcus aureus	0.12	0.12	0.06	0.5	0.5	1.0	PS
Klebsiella pneumoniae	0.5	1.0	0.25	0.5	0.25	0.75	PS
Enterobacter sp	1.0	0.5	1.0	1.0	2.0	3.0	A
Staphylococcus epidermidis	0.12	0.12	0.06	0.5	0.5	1.0	PS

Mean value of three trials were tabulated

A – Cefixime, B - Ciprofloxacin, AB- Combined concentration of Cefixime-Ciprofloxacin

S – Synergy, PS – Partial synergy, Units for all the presented values - µg/ml

Fig. 4: Simplified Checker Board Method of Cefixime-Ciprofloxacin

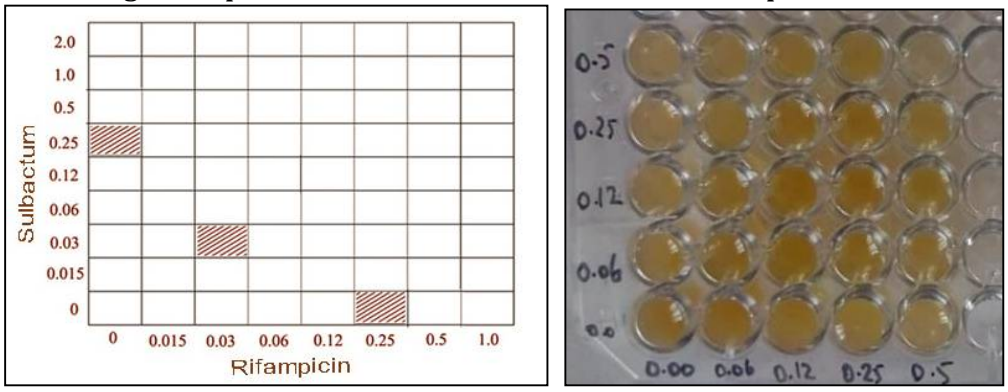


Fig. 4: Simplified Checker Board Method of Cefixime-Ciprofloxacin

MICA – 0.25, MICB – 0.25, MICAB – 0.03

Test Bacteria: E. coli

From literature survey, it was depicted that, many researchers reasoned the synergism between Ofloxacin and ornidazole respectively, reported that ofloxacin and ornidazole have a common target action on the bacterial DNA even though the drugs are the derivatives of different groups. They conducted and experiment to explore the possibility of an in vitro synergistic effect of the drugs by checking their efficiency to inhibit DNA synthesis. Their results revealed that the interference rendered by synergistic drugs on the mechanism of DNA synthesis in the pathogens at time of drug exposure. (18) reported that mode of action

of Ofloxacin, a synthetic floroquinolone inhibits the enzyme bacterial DNA gyrase, which in-turn nicks the double stranded DNA that leads to negative super-coiling of it and then again reseals the nicked end. Damaged DNA transmits signals to produce exonucleases that result in digestion of DNA depicting the rapid bactericidal action of Ofloxacin. (18) stated that Ornidazole, a nitro group of drug reduced by redox protein to reactive nitro radicals, which produces cytocidal action by destabilizing DNA helix and possess high volume distribution. Ofloxacin, a rapidly and completely absorbed after oral ingestion, widely distributed in the body due to its high volume distribution and acts as bactericidal by acting on DNA (DNA gyrase and topoisomerase II & IV), prevent DNA transcription to RNA and subsequent protein synthesis. While in other two combination (Cefixime and ciprofloxacin) antimicrobial absorbs very slowly and possess low volume distribution thus remain longer in the gastro intestinal tract, facilitate longer action on intestinal pathogen ensuring early recovery and cure without any untoward effects.

CONCLUSION

As uropathogens are considered as significant epidemiological characteristic in the field of Medical and allied sciences, reducing its multidrug resistant property are equally challenging in the current drug formulation industries. To combat this feature, a combination therapy was studied as preliminary step in the present research. Hence, combination of two different groups of antibiotics (Ofloxacin+Ornidazole and Cefixime+Ciprofloxacin) were used with the aim of increasing the synergistic antibacterial activity against uropathogens. Biofilm forming ability of the test organisms using Exit-site test and Microtitre plate assay revealed that *Staphylococcus epidermidis* and *Staphylococcus aureus* colonized with in 24h; microtitre plate assay exhibited that, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Staphylococcus epidermidis* reported as strong biofilm producers. Synergistic antibacterial test showed that all the five test organisms had complete synergistic effect for ofloxacin-ornidazole combination; whereas, Cefixime+Ciprofloxacin showed varied results with E. coli alone showing synergism of FIC value 0.24. The findings revealed that Ofloxacin + Ornidazole combination will restrict urinary tract causing pathogen to become multi-drug resistant in near future. Further research must be carried out comparing different concentrations of the antibiotics, both alone and together, in influencing bacterial densities. Since ofloxacin, ornidazole were both easily studied, most of other commonly known antimicrobial compounds having the similar mode of action on bacterial cell components shall be used in a similar manner, and future research should be expanded to include testing a wide spectrum of antimicrobial compounds.

CONFLICT OF INTEREST

Authors declare no conflict of interest in the present study.

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