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THE INTERACTION OF MECILLINAM AND FOSFOMYCIN INTO MULTI DRUG RESISTANT *ESCHERICHIA COLI* FROM URINE PATIENTS

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ABSTRACT

Urinary Tract Infection is one of the most common diseases that is very difficult to cure because of the presence of multi-Drug resistant pathogens. The cure is usually to prescribed antibiotics, however, there is a gap in terms of the type of antibiotic that was being prescribed, the amount of the dosage and the duration of the antibiotic treatment, which caused the development of multidrug resistant pathogens. The scope of this study is to look for an alternative method of curing the infection specifically caused by the *Escherichia Coli* bacteria. Two antimicrobials considered in this study namely, Fosfomycin and Mecillinam. There are six methods that were used in this study namely; Urothelial cell line culture, Bacterial Culture, Determination of Mecillinam and Fosfomycin Minimum Inhibitory Concentration (MIC), association assay, invasion assay and survival assay. The sample that used to perform the experiments were taken from the urine of patients that has shown symptoms of urinary tract infection. From these methods, it was observed that there were two antimicrobials were effective in disrupting the *E. Coli* Bacteria and the MIC for the pathogen to be susceptible is very minimal.

KEYWORDS: Fosfomycin, Mecillinam, *Escherichia Coli*, Urinary Tract Infection, association assay, invasion assay, survival assay.

1. INTRODUCTION

Urinary Tract Infection (UTI) is the inflammation of the organs in the human body that assists in the excretion of urine.[6] These organs include the kidneys, ureters, bladder and urethra. The infection is usually characterized by difficulty in urination and in most cases painful. This is usually caused by the action of foreign organisms such as bacteria into the renal system.

The bacteria commonly known as *Escherichia coli (E.coli)* is the most common cause of urinary tract infection.[4] These bacteria are normally found in the bowels where they are considered harmless. They only become dangerous when they started to invade and enter the urethra.

The treatment for UTI has been properly documented, however challenges in adhering to these standards in terms of choice of antibiotic, amount of dosage and duration of treatment has been prevalent, and these substandard practices has developed multi-drug resistant bacteria.[7] This is the primary reason for research on alternative treatment for UTI because the antibiotic remedy has slowly lost its efficacy.

In this article, two alternative treatments for UTI caused by *E. coli* has been proposed. The study will investigate the viability of Mecillinam and Fosfomycin in treating urinary tract infection caused by multi-drug resistant bacteria specifically *E. coli*.

Mecillinam.[17,20] is a penicillin in which the 6beta substituent is [(azepan-1-yl) methylidene] amino; an extended-spectrum penicillin antibiotic that binds specifically to penicillin binding protein 2 (PBP2) and is only considered to be active against Gram-negative bacteria. It has a role as an antibacterial drug and an anti-infective agent.[5]

Another option is Fosfomycin.[16,19] which according to the study of Gopichand, et al (2019), they concluded that Fosfomycin exhibited a good inhibitory effect on the

biofilms produced by the MDR organisms that studied. They used a total number of 326 multi-drug resistant (MDR) including *E. coli* taken from the urine samples of patients that showed symptoms of urinary tract infection.[3]

Several methods were used to study the effects of Mecillinam and Fosfomycin to these multi-drug resistant microorganisms. These methods are Urothelial Cell Line Culture, Bacterial Culture, Determination of Minimum Inhibitory Concentration (MIC), Association Assays, Invasion Assays, and Survival Assays.[2]

Urothelial Cell Line Culture is an established and easy tool to study the growth of pathogens in an organism. It used in the study of bladder cancer (8) by building cell lines from samples of human bladder cancer cells. In this study human urothelial cell lines were developed at St. James University Hospital in Leeds and were passaged to different flasks. The samples used in the experiment were from passages 4 to 10.[1]

The diagnosis of urinary tract infections is usually accomplished by developing tests by culturing bacteria in a petri dish. Some tests like the one that was performed at Loyola University in Chicago was able to detect more bacteria in a urine sample.[9] There were five *E. coli* strains that were used in the study and these were cultured in a cysteine lactose electrolyte deficient medium (CLED) agar and renewed weekly.

The minimum inhibitory concentration is the minimum amount of concentration for an antimicrobial to have an effective prevention of growth of pathogen in an overnight testing. The method is common to perform Drug Susceptibility Testing, for example to determine the MIC of six antimicrobial drugs in Mycobacterium tuberculosis (MTB).[10] In this study several investigations were made to determine the MIC of Mecillinam and Fosfomycin.

An association assay is performed to determine the interactivity of pathogens and antimicrobial in a laboratory test. An example would be to check the association of Biotin Ingestion with hormone and non-hormone assays in adults.[11] The association assay method was used in this experiment to observe the ability of the pathogen to associate with the urothelial cells.

Invasion assays were performed to determine the ability of the pathogen to invade or be taken over by the urothelial cells. One example was the study of Caspase-3 regulation of the invasion of colon cancer cells.[12]
The survival assay was performed to observe if the pathogens will remain after the antimicrobial were applied. A similar study on how Calcitriol diminished Eosinophil Necrosis in cases of asthma.[13] The dead and alive cells were observed in this method.

The aim of the Project is to study the use of Fosfomycin and Mecillinam antibiotics and its effect on Extended Spectrum Beta-Lactamase + (ESBL+) and *Escherichia Coli* strains which is multi-resistant to many antibiotics. During the treatment of UTIs, the antibiotic should be excreted through the kidneys while not losing its active condition, leading to pressure on kidneys and causing problems for kidney disease patients. It is hard to treat these organisms. Carbapenems are usually the only option. However, increased resistance to these antibiotics could occur through the rise of transferable carbapenemases. Thus, there is a need for substitutes in treating infections having increased resistant bacteria.

2. MATERIALS AND METHODS

2.1. Urothelial cell line culture

Human urothelial Cell (TRET-NHUC) line was kindly by Prof. Knowles, from Cancer Research UK Clinical Centre at St. James's University Hospital in Leeds, Chapman *et al.*[1] TRET-NHUC cell line was provided as a frozen sample. That freezing medium (growth medium with 10% DMSO and 10% fetal calf serum (FSC)) was disposed by centrifugation at (1000 rpm for 4 minute) and the medium was discarded. The cells were re-suspended in 5ml Keratinocyte Growth Medium 2 (purchased from Promo Cell) supplemented with [bovine pituitary extract (0.004 ml/ml), epidermal growth factor (0.125 ng/ml), insulin (5 mg/ml), hydrocortisone (0.33 mg/ml), epinephrine (0.39 mg/ml), transferrin, holo (10 mg/ml) and $CaCl_2$ (0.06 mM)]. Then, cells were passage into T25 flasks and incubated at 37°C in humidified 95% air and 5% CO_2. Theoretically, cells become confluent within a week ($1x10^6$ cells/well), and medium was changed 3 times per week. Next stage, the cells were ready to use or to passage into other T25 or T75 flasks. All passages, which used in experiment, were between passage numbers 4 to 10.

2.2. Bacterial Culture

Five different *E. coli* strains (*E. coli*, ESBL+, AmpC, OXA 48 and TEM) were used in the present. The strains were collected from the clinical microbiology laboratory at King Abdulaziz University Hospital by Dr. Dalia Marwan, Director of infection prevention and control. All *E. coli* strains were grown in Luria-Bertani (LB) broth overnight at 37C° in O2 incubator.[2] The optical density of bacteria in LB broth was measured by spectrophotometer with wavelength 595nm and optical density 0.22 was obtained (OD 595 0.22 = ~10^8 bacteria/ml). These strains were cultured onto cysteine lactose electrolyte deficient medium (CLED) agar and refreshed every week.

2.3. Determination of Mecillinam and Fosfomycin MIC

Several experiments were performed to determine the Minimum Inhibitory Concentration (MIC) of Mecillinam and Fosfomycin on bacteria under investigation. The bacterial strains were inoculated into LB broth medium with different concentrations (64, 32, 16, 8µg/ml) of

Fosfomycin and Mecillinam into 96-well plate overnight at $37C^0$ in O2 incubator. Then broths were cultured onto CLED plates and number of bacteria was calculated.

2.4. Association Assays

The bacterial association assays with the urothelial cell line were performed following the protocol of Alsam et al. (2). (Fig 2.1) remaining initial bacterial solution with optical density of 0.22 was used for the association assay which was carried out in 96-well plates where the urothelial cells have been grown ($2x10^5$ cells per 0.2 millilitre in each well) until became monolayer. Growth media was distributed in the plates, and then 200µl KGM2 controls were added to the first well 10-12 bacteria were added to each cell and left for one hour in 37C. wells were washed with 200µl of Phosphate buffered saline (PBS). cells were lysed with Sodium dodecyl sulfate (SDS) 0.5% final concentration. Number of bacteria associated with TRET-NHUC were calculated by this equation: number of bacteria associated with cells = (no. of bacteria / no. of TRET-NHUC).[2]

2.5. Invasion assays

Invasion assays were performed to determine the ability of bacteria to invade or be taken up by TRET-NHUC cells (2x105 cells per 0.2 milliliter in each well). cells were cultured to monolayers followed (Fig 2.2) by the addition of *E. coli*, ESBL+, AmpC, OXA48 and TEM in a 0.2 ml final volume of KGM2. After one hour, medium was discarded, and the urothelial cells were washed once with PBS and placed Imipenem antibiotic for killing extracellular bacteria in 30 Minutes at CO2 Incubator. Then, culture it into CLED agar and keep it overnight.

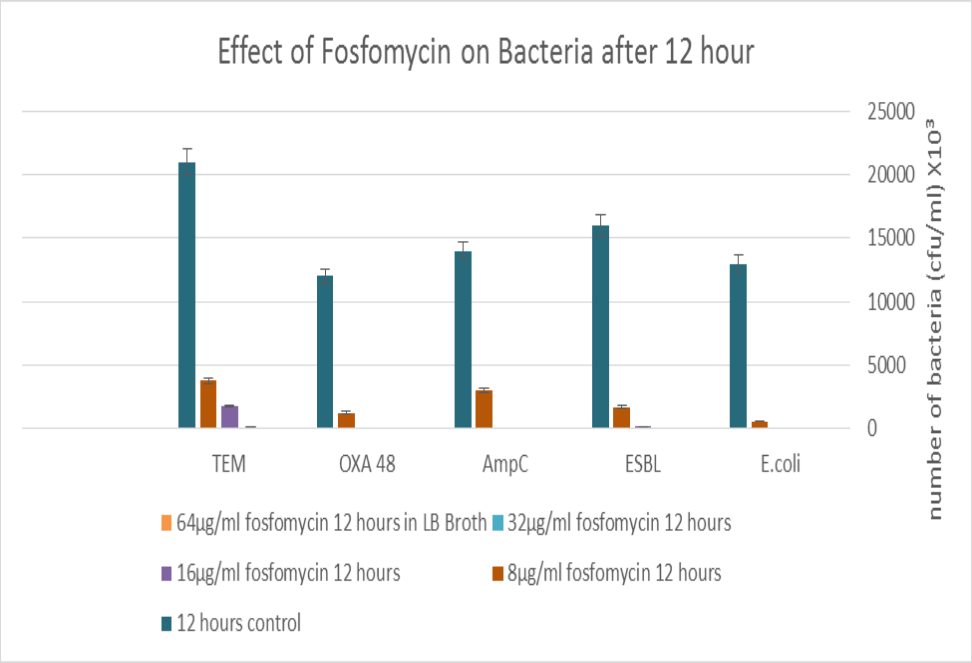
2.6. Survival assays

Survival assays were performed to determine the live and dead TRET-NHUC cells (2x105 cells per 0.2 milliliter in each well). Cells were cultured to monolayers followed (Fig 2.3) by the addition of *E. coli*, ESBL+, AmpC, OXA48 and TEM in a 0.2 ml final volume of KGM2. After one hour, medium was discarded, and the urothelial cells were washed once with PBS. Then, add Imipenem antibiotic for killing extracellular bacteria in 30 Minutes at CO2 Incubator. After that, discard the medium, wash once and placed Fosfomycin and Mecillinam overnight for killing intracellular bacteria and cultured.

3. RESULTS

3.1. The Effect of Mecillinam and Fosfomycin on survival of bacteria

The effect of Fosfomycin and Mecillinam with different concentrations were performed. At 32µg/ml concentrations of Fosfomycin and Mecillinam for 12 hours incubation, it was observed that Fosfomycin showed effects for the ESBL+ strain than Mecillinam. For 24 hours of incubation, the number of bacteria colonies were observed a more effect on the ESBL+ strain for Fosfomycin than Mecillinam. On the other hand, TEM bacteria strain was grown in concentration of Fosfomycin antibiotics in 16 and 8µg/ml but, other ESBL+ strain no grown at all. ESBL+ strain was having a less grown in concentration of Mecillinam in 8µg/ml for 12 and 24 hours (Fig 3.1 and 3.2).



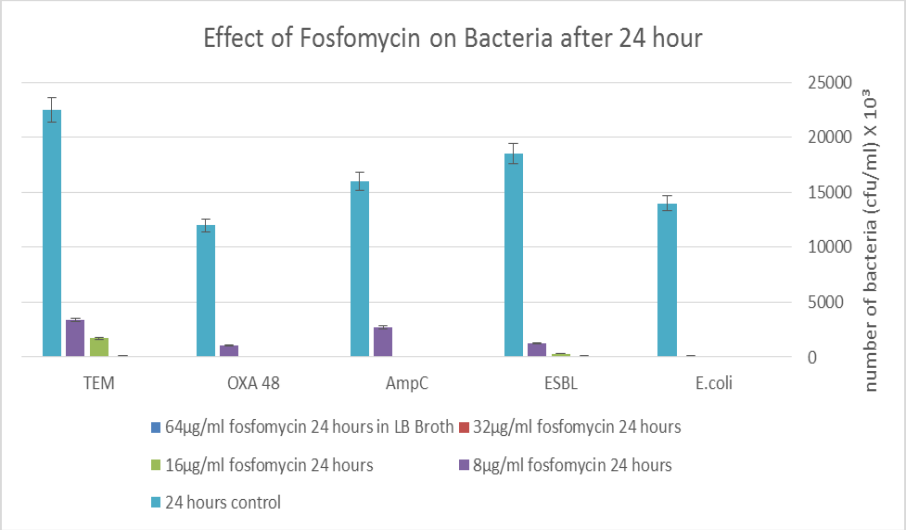


Figure 3.1: Different concentration effect of Fosfomycin on Bacteria strain in A) 12 Hour and B) 24 Hour. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

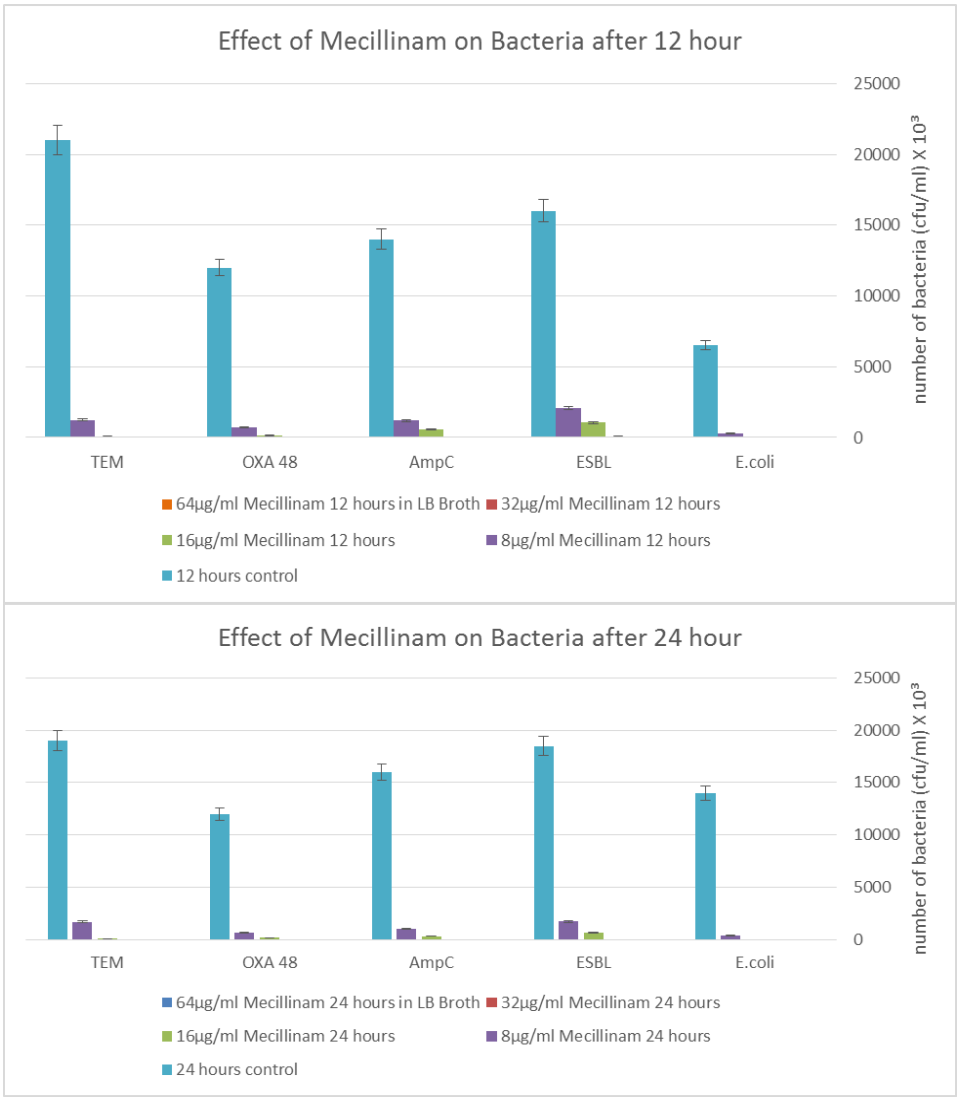


Figure 3.2: Different concentration effect of Mecillinam on Bacteria strain in A) 12 Hour and B) 24 Hour. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

3.2. Association assays

Association assays were performed to determine the ability of *E. coli*, ESBL+, AmpC, OXA 48 and TEM to interact with urothelial cells. Our findings revealed that E.coli strains exhibited significantly lesser association with urothelial cells than ESBL+ strains (Fig 3.3) Where the association percentage of *E.coli* with urothelial cells was ~0.15%, the association percentage of ESBL+ strains with urothelial cells lies between ~35% and 48%. OXA 48 and TEM were found to have a higher association rate with human urothelial cells. ESBL+ and AmpC strains have a lesser association than OXA 48 and TEM. Native *E. coli* has the least association with urothelial cells than other strains.

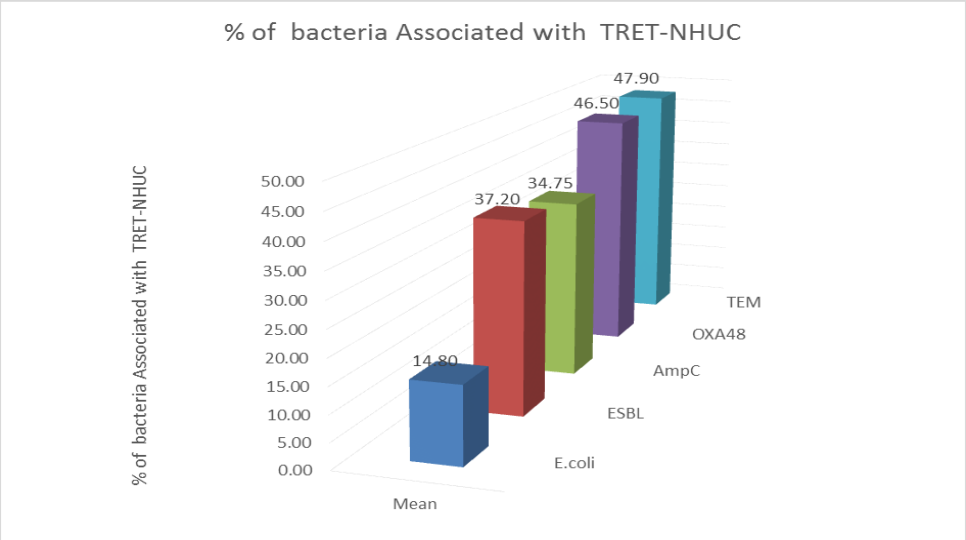


Figure 3.3: The percentage of bacteria associated with Human Urothelial Cells (TRET-NHUC). Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

3.4. Invasion assays

To determine whether *E. coli* and ESBL+ strains invade urothelial cell line, invasion assays were performed. All bacterial strains exhibited recovery from urothelial cells (Fig 3.4). E. coli exhibited significantly it should be less recovery from urothelial cells as compared with ESBL+, AmpC, OXA 48 and TEM. Result are as follows: *E. coli* showed 0.01% recovery from urothelial cells, whereas ESBL+ have a higher recovery shows 0.068%, Then AmpC 0.063%, TEM 0.06% and at least OXA 48 0.032% from urothelial cells.

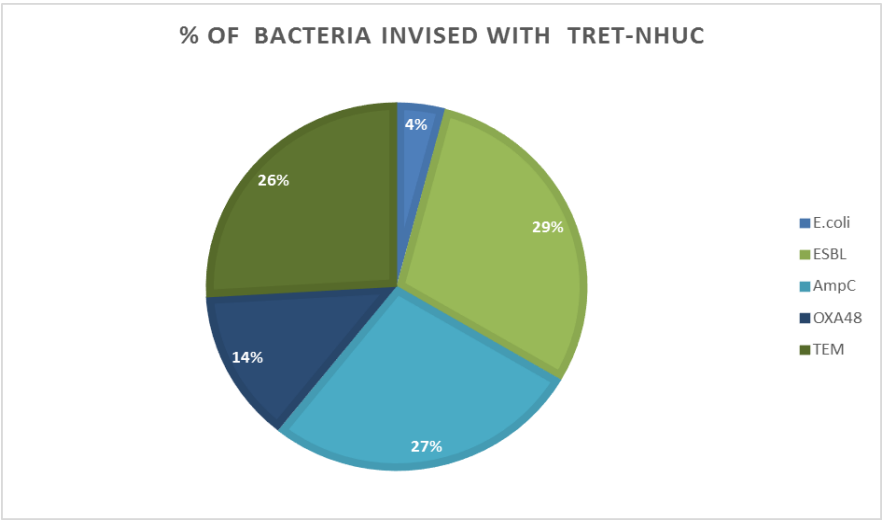


Figure 3.4: The percentage of bacteria invaded TRET-NHUC. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

3.5. The survival assays

To determine the bacteria strains still survived after placed Fosfomycin and Mecillinam, survival assays were performed. Most TRET-NHUC lines were treated from infected bacteria strains (Fig 3.5). *E. coli* was significantly it should be less survived from urothelial cell lines as compared with ESBL+, AmpC, OXA 48 and TEM. Result are as follows: *E. coli* showed 0.06% survived after used Fosfomycin and 0.032 % after used Mecillinam antibiotics Overnight, whereas ESBL+ have

a higher survived show 0.13%, Then TEM 0.11%, AmpC 0.082% and at least OXA 48 0.074% with Fosfomycin treated urothelial cell lines. On the other hand, AmpC have a higher survived show 0.11%, Then ESBL+, TEM and OXA 48 with Mecillinam treated urothelial cell lines.

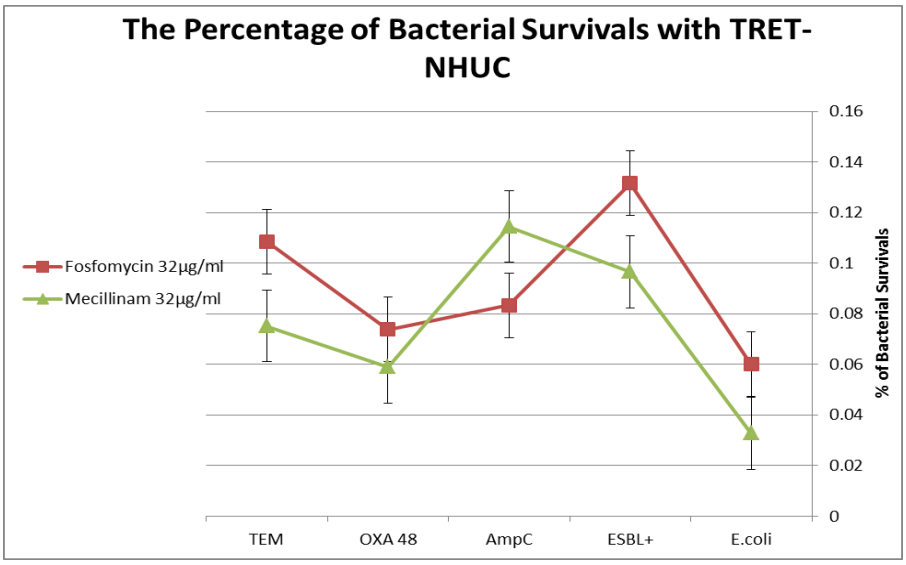


Figure 3.5: The percentage of bacteria survived TRET-NHUC. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

4. DISCUSSION

Six methods were used in this study to determine the effects of the application of Fosfomycin and Mecillinam on ESBL+ and *E. Coli* strains for Urinary Tract Infection pathogens that were taken out from the patients that have exhibited the symptoms. Three other pathogens were included namely, AmpC, OXA48 and TEM. Other studies such as the Ugandan study on the prevalence of Bacterial Urinary Tract infection,[18] was used as an inspiration for the study.

The effect of Mecillinam and Fosfomycin on the survival of bacteria showed that Fosfomycin has a better efficacy than Mecillinam. From the study of Hawkey et.al, it calculated that Fosfomycin has a 98% susceptibility rate for uncomplicated lower UTI for women 18-65 years of age in 10 countries while Mecillinam has 96%.[14] Fosfomycin MIC for *E. Coli* varies from 1-4 mg/L,[14] and Micillinam MIC is 16 to 64 mg/L for Mecillinam resistant pathogens and around 1-2 mg/L for strains with the highest dose of reduction.[15]

Based on the tables on Figure 3.1 and 3.2, these shows the concentration effect of different levels of Minimum Inhibitory Concentration (MIC) for Fosfomycin and Mecillinam in a 12-hour and 24-hour period for five pathogens, namely; *E. Coli*, ESBL, AmpC, OXA48 and TEM. From this table we can see that *E. Coli* has the lowest number of bacteria count after the 12-hour and 24-hour period with Fosfomycin having a minimal advantage over Mecillinam.

On the association assay method of testing, it was measured that the *E. Coli* has the lowest percentage of bacteria associated with the Human Urothelial Cells (TRET-NHUC). Ranking the pathogens from lowest to highest: *E. Coli* has an association rate of 14.8%, second is ESBL with 37.2%, third is AmpC with 37.45%, fourth is OXA48 with 46.5% and the last is TEM with 47.9%. The equation that was used to compute for the number of bacteria associated with TRET-NHUC is: Number of bacteria associated with cells = (number of bacteria/numbers of TRET-NHUC).

The invasion method used a monolayer of cultured cells added with pathogens: *E. coli*, ESBL+, AmpC, OXA48 and TEM in a 0.2 ml final volume of KGM2. The solution was discarded after one hour and urothelial cells were washed with PBS once. The antibiotics called Imipenem were placed to kill the pathogens in a thirty-minute period in a carbon dioxide incubator. The resulting culture was then kept overnight for observation. The invasion assay method demonstrated that *E. coli* has the lowest percentage of bacteria that invaded the Human Urothelial Cells (TRET-NHUC) after Fosfomycin and Mecillinam were introduced into the pathogens. *E. coli* has an invasion percentage of 4%, followed by OXA48 with 14%, then TEM with 26%, Amp C with 27% and ESBL with 29% round up the results.

For the survival assay method, the percentage of bacteria that has survived after the sample that has been applied with Fosfomycin and Mecillinam were left overnight. For Fosfomycin (32µg/ml), the overnight is survival rate is 0.06% for *E. coli*, 0.074% for OXA48, 0.082% for AmpC, 0.11% for TEM and 0.13% for ESBL+. The overnight survival rate for Mecillinam is 0.032% for *E. coli*, approximately .06% for OXA 48, approximately 0.074 for TEM, approximately 0.098% for ESBL+ and 0.11% for AmpC. These results showed that the survival

rate is generally lower in the Mecillinam applied pathogens than with the Fosfomycin applied pathogens.

5. CONCLUSION

The study has showed that Fosfomycin has a better performance in the survival of bacteria than Mecillinam. Although the difference is very slim roughly around 2% only. It also observed that the Minimum Inhibitory Concentration required the pathogens to be susceptible to Fosfomycin and Mecillinam is roughly 1-2%. In the three assay methods that were included in the study. The association assay has showed that the *E. coli* pathogen has the lowest percentage of bacteria associated with Human Urothelial Cell (TRET-NHUC). The same with the invasion assay methodology where *E. coli* has the lowest invasion percentage among the five pathogens. In the survival assay methodology, that the *E. coli* strain has the lowest survival rate for the five pathogens for both Fosfomycin and Mecillinam.

Based on the results of this study, we therefore conclude that Fosfomycin and Mecillinam are effective cure for Multi-Drug Resistant pathogens in Urinary tract infection caused by the *Escherichia coli*.

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