Theraputic Effects of Bactriophage alone and in combination With Meropenem on Multi drug resistanace Escherichia coli in vitro study

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Abstract

Back ground: One of the most deadly infectious disease pathogens, Escherichia coli (E.coli) is a gram-negative (Gr-ve), rod-shaped, facultative anaerobe bacterium. E.coli phages are bacterial viruses that infect bacteria and can replicate in one of two ways: the lytic life cycle or the lysogenic life cycle. The majority of E.coli isolates are multi-drug resistant (MDR), making it difficult to treat their infections. Combination of antibiotics & phages, is a good option for addressing this issue.

Aims of the study: This study aims to assess the efficacy of bacteriophage activity as an antibacterial agent alone and in combination with antibiotics against MDR E. coli and to identify antibacterial combinations with synergistic effects for the treatment of MDR.

Materials and Methods: From April to June 2022, 39 isolates were collected from patients with urinary tract infections who were treated at the Medical City of Al-Imamain Al-Khadimain in Baghdad .They were identified with the help of morphological characteristics and the VITEK2 compact system. Phages were isolated from different regions in Baghdad city including (soil, sewage, irrigation channels).

Results: In this study, E.coli were isolated from urinary tract infection patients. E.coli isolates were the most prevalent (36.8%), followed by Enterococcus feacalis (24.5%).Distribution by gender reveals a higher percentage of females than males. The percentages of resistant isolates to the antibiotics tested were high resistant rate to Trimethoprim/sulphamathaxozole , ceftriaxone, while low resistant to carbapenem group & nitrofurantion. From 39 isolates, 32 were MDR, 6 were XDR, and 1 was PDR. The combination of phage with 1/4 or 1/2 MIC of each of meropenen, nitrofurantion and trimethoprim/sulphamethoxazole for 1,11,36 isolates resulted in synergist effects to the antibiotics against MDR E. coli strains obtained in this study.

Conclusions: Isolates of E. coli are highly susceptible to Imipenem, Meropenem, Ertapenem, and nitrofurantion antibiotics, but highly resistant to trimethoprim/sulfamethoxazole and cephalosporins. Most combinations of meropenem, with phage exhibited highly synergistic effects against MDR E. coli strains and decreased resistance.

Keywords: *Escherichia coli, Multidrug resistant, Bacteriophage, phage therapy.*

INTRODUCTION

Escherichia coli:

In 1885, Escherichia coli was first isolated. It is a Gram-negative, rod-shaped bacterium found in the gastrointestinal tract as normal flora, and as pathogenic E. coli that causes various bacterial infections including urinary tract infection, diarrheal infection, and other clinical infections including neonatal meningitis, pneumonia, and bacteremia (1).It is a genus of the Enterobacteriaceae Phylum of Gammaproteobacteria. Under optimal conditions, E. coli can replicate in about 20 minute (2).

The occurrence of antimicrobial resistance is one of most important global medical crisis. Once a new antibiotic is discovered, it is followed by the detection of resistance among those bacteria that are usually susceptible to it. Nowadays, the increase of multi resistance in bacteria is becoming a major public health problem, especially in hospital settings with increased dissemination of resistant strains due to the globalized world. The most important bacteria in this regard is multi drug resistant E.coli so the find alternative therapies, such as phage therapy, trying to eliminate these MDR pathogens and avoid a global medical crisis.

Bacteriophage

Bacteriophages are viruses that can infect and multiply inside bacteria. (specific to the bacteria that they infect and developed to bind to unique and essential bacterial cell wall targets) (3). they were discovered by Felix d'Herelle in 1917. However, the first suspicions of the existence of microbes antagonistic to some bacteria were made by the British bacteriologist Frederick Twort , After the discovery of bacteriophages at the beginning of the 20th century, numerous studies considered their potential to eliminate bacteria, which would undoubtedly make them promising therapeutic agents. The discovery of antibiotics during World War II, however, meant that this natural potential therapeutic agent was largely ignored and only used as a research tool for a brief period of time afterwards (4).

Phages are classified as virulent or temperate depending on the biological cycle they perform, lytic or lysogenic, respectively. Lysins are enzymes encoded by phages responsible for the bacterial cell wall lysis at the end of the lytic cycle and are interesting for their ability to disrupt biofilms , Virulent phages are the most desirable for therapeutic use against bacterial infections. Lysogenic bacteriophages persist quiescent as pro-bacteriophages, only replicating together with the bacterial host genome or exist as plasmids with their host cell (5). The first investigations were carried out analyzing the possible role of these viruses in medicine . Bacteriophages are the most numerous entities on earth

The advantages & disadvantages of phage

Bacteriophage is natural and has a number of advantages over chemical techniques, including protection, cost-effectiveness, and the availability of a number of bioactive substances that function as natural antibiotics, as opposed to only one antibiotic, thereby preventing potential bacterial resistance as a result of adaptation (6). Other advantage include: phage is not likely cause dysbactriosis or secondary infections in the body. also there has been no major reports of toxicity or negative impacts from phage on mammalian cells (7). On the other hand phage has some key disadvantage include a lack of common established and validated protocols for the routes of administration, dose, frequency, and duration of phage treatment, which hampers inter-study comparison (8). Often, the purity and stability of phage preparations used for clinical trials are also uncertain, with insufficient quality control data presented. The concentration of phages may be reduced significantly during therapy by the reticuloendothelial system or be neutralized by

antibodies, thus inhibiting their antimicrobial activity (9).

Phage Pharmacology: This field, known as phage pharmacology, investigates how phages affect both microorganisms and human cells (10). There are two type of phage–host interactions pharmacodynamics and pharmacokinetics (11).

Resistant

The development of antimicrobial resistance, and emergence of multidrug resistance (MDR)has become a global health concern (12). this resulted from continuous administration of antibiotics to animals, either for treatment or prophylaxis and growth promotion purposes, that are able to disseminate to humans through the food chain (13).

Factors that contribute to resistance include the increased use of all antimicrobial drugs and improper antimicrobial prescribing. Many of the less expensive drugs that have fewer side effects have been used too commonly. Improper prescribing may be choosing broad spectrum or ineffective antibiotics (14). In particular E. coli can be resistant to most commonly used antimicrobials, which makes the infections they cause extremely difficult to treat, leading to increased morbidity and mortality(15

Antibiotics:

Some forms of bacterial infections are treated or prevented with antibiotics. They function by eliminating bacteria or preventing their spread (16). However, in this study Meropenem used to combine with phage to treat multi drug resistant E.coli.

1.Meropenem

Meropenem is a broad-spectrum antibacterial agent of the carbapenem family. Meropenem is one of the most important members of the carbapenem class carbapenems are considered the most effective class with the most extensive spectrum of antimicrobial activity and excellent safety and tolerability profiles Carbapenems approved and available for clinical use include imipenem, meropenem, ertapenem and doripenem (17).

Overall, this carbapenem is marginally more potent against Gram-positive bacteria compared to other agents. In addition, since it is vulnerable to dehydropeptidase I (DHP-I), a renal tubular dipeptidase enzyme which causes its degradation, imipenem is usually co-administered with cilastatin or betamipron. Cilastatin is a competitive antagonist that also works to protect the kidneys from harmful damage generated by higher doses of imipenem (18).

A. Mechanism of action:

Meropenem interferes with the synthesis of the bacterial cell wall, thus inhibiting growth and resulting in cell death. It is bactericidal antibiotic except against Listeria monocytogenes, where it is bacteriostatic.

The drug readily penetrates bacterial cell walls and exert its action by:

Binding with high affinity to specific penicillin-binding proteins (PBP) rendering them inactive. Carbapenem then exhibits bactericidal activity by binding to PBPs such as enzymes (19). unlike imipenem/cilastatin, is relatively stable to human dehydropeptidase-1 (DHP-1). Meropenem has a high level of stability to hydrolysis by all serine β-lactamases.

B. Mechanism of resistance

Similarly to beta-lactams, there are four types of mechanism of resistance to carbapenems: production of zinc-dependent metallo-β-lactamases; decreased permeability of the external membrane of Gram-negative bacilli; presence of efflux pumps; modification of

molecular target (PBP). Resistance of Gram-negative bacilli is most often an enzyme resistance due to the production of carbapenemases, and to the reduced permeability of the external membrane (20).

Materials and methods

Sample Collection: Samples of bacteria were collected in Al-Imamein Al-kadhimein Medical City Hospital in Alkadymiya, Baghdad. Bacterial sampling was carried out from March 2022 to May 2022. A total of 153 urine samples for patients with urinary tract infections were obtained from the hospital's central laboratory; the samples were cultured by the conventional method of analysis. One hundred six (106) isolates showed bacterial growth, while 47 samples showed other causes.

Laboratory Diagnosis Identification of the isolated E.coli was performed by the morphological characterization of the isolated UPEC colonies on the culture media according to their colony morphology on selected media (MacConkey and blood agar).The isolated colonies were tested by gram stain according to the protocol supplied For E. coli preservation . Using the VITEK2 system, 39E.coli isolates were obtained, for further identification. The isolates were then transported on the same day to the laboratory of the Medical Microbiology Department in the College of Medicine, Al-Nahrain University, to sub-culture bacteria on nutrient agar or MacConkey to be stored in the refrigerator at 4oC for 24 hours.

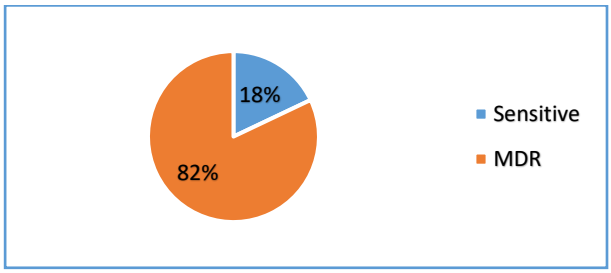
Antibiotic susceptibility test: The antibacterial susceptibility testing of the isolates was done according to Clinical and Laboratory Standards Institute (CLSI, 2019), The MIC for antibiotics used were Amoxicillin/clavulanicacid ⩾ 32µg,piperacillin/tazobactam ⩾ 128µg,cefazolin ⩾ 64µg,cefuroxime ⩾ 64µg,ceftazidime ⩾ 64µg,ceftriaxone ⩾ 64µg,cefepime ⩾ 32µg,Ertepenem ⩾ 8µg,Imipenem ⩾ 16µg,meropenem ⩾ 16µg,Amikacin ⩾ 64µg,Gentamicin ⩾ 16µg,Ciprofloxacin ⩾ 4ug,Fosfomycin ⩾ 64µg,Nitrofurantion ⩾ 128µg,Trimethoprim/sulphamethoxazole ⩾ 320µg. Isolates were classified as either resistant or sensitive based on the definition of the Clinical and Laboratory Standard Institute (CLSI, 2019) . Resistant isolates were classified into three groups depending on resistant to antibiotic groups . isolates were considered multi-drug resistant if it was resistant to at least one member in three different groups of antibiotics, while isolates resistant to at least one member in five different groups of antibiotics consider XDR, and considered PDR when resistant to almost antibiotics (21).

Results

Bacterial susceptibility rate

The results showed that different E.coli isolates had different antibiotic sensitivity profiles; of 39 isolates included in the current study, 32 were MDR, 6were XDR and 1PDR as shown in the figure

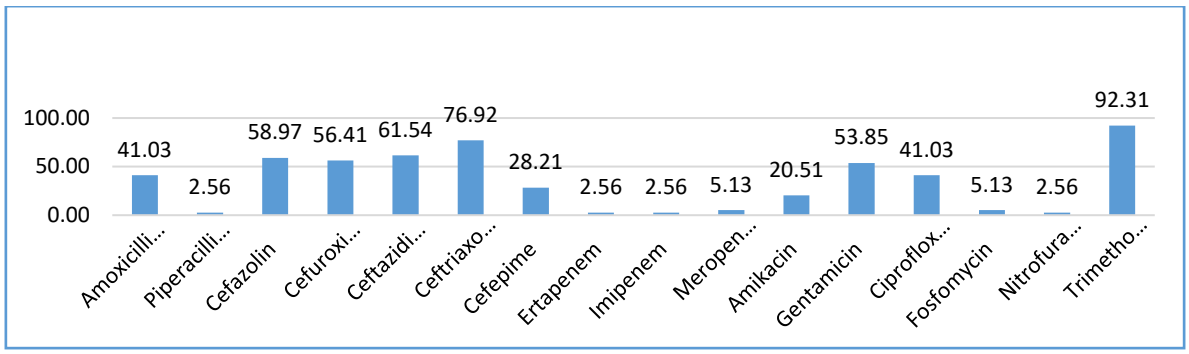
Figure 1



Antibiotic resistant rate:

The antibiotic resistant rate E.coli isolates had different antibiotic sensitivity profiles; in the current study resistant rate as shown in the figure 2

Figure 2



Bacteriophage sampling, isolation

Different crude samples for phage isolation were obtained from different regions in Baghdad city including sewage, farm soil, feces of sheep, chicken litter, and swab from surgical lounge of several hospitals in Baghdad. Overnight bacterial broth (100 µl) was mixed with 2-3 ml of crude samples and incubated overnight at 37 ºC until obtain specific lytic phage.

Determining Multiplicity of Infection (MOI):

1. A series of dilutions were performed to determine the dilution that results in confluent growth of bacteria on a 90 mm Petri dish.

2. To determine the concentration of bacteria (CFU/mL) in the previous step, serial dilution was performed.

Note: the concentration of bacteria that make confluent growth on the petri dish was 1.8 x 10^6 CFU/mL.

3. The concentration of phage suspensions was as follows,

Table (1): The concentration of phage suspensions

Bacteriophage	Concentration (PFU/ml)
ECP01	1.3 x 10^9
ECP11	2.7 x 10^10
ECP36	4.9 x 10^8

4. Different MOI was prepared to each phage to find the minimum MOI,

Table (2): Different MOI to each phage

MOI	Concentration of phage (PFU/ml)
1	1.8 x 10^6
2	3.6 x 10^6
3	5.4 x 10^
5	9.0 x 10^6
10	1.8 x 10^7
15	2.7 x 10 ^7

5. We found that 5 MOI is minimum MOI for 100% infection of bacteria.

MOI is the phage-to-bacteria ratio (22). Divide the number of phages added (ml added x PFU/ml) by the number of bacteria added (ml added x cells/ml) to get the MOI(23).

A serial dilution of bacteria was prepared (one day before infection), then spread on a 90 mm Petri dish to find the concentration that produced confluent growth (CFU/ml); then, a serial dilution was prepared again to find the concentration that produced confluent growth (CFU/ml), and specific MOI of phage were added (infection overnight) (22) .

According to the dose curve chosen, 1 MOI should contain one phage particle for every bacterial cell. In the population, the atypical dose of MOI is 0,1,2,3,10,15, and 30 MOI. It is critical to have 0 MOI as a negative control

to monitor bacterial growth. When the MOI is one, there is one phage particle for every bacterial cell (22).

We combined the necessary amount of virus and bacteria. The plates were then labeled with the appropriate MOI, and virus-infected bacteria were placed in a 37°C incubator until the next day (24 hr.). The minimum MOI (for 100% infection) revealed no bacterial growth (22).

The synergistic effect of the Meropenem-resistant isolates in combination with 1, 2, 3, 5 MOI.

Table (3)

Combination (µg/mL)		Antibacterial agents alone (µg/mL)		FIC	FIC index	Outcome
ECP01 (1, 2, 3 MOI) + MEM (16 µg/mL)	2.00	ECP01	5.00	0.40	0.65	Additive
MEM (¼ and ½ MIC) + ECP01 (3 MOI)	4.00	MEM	16.00	0.25		
ECP11 (1, 2, 3 MOI) + MEM (16 µg/mL)	2.00	ECP01	5.00	0.40	0.65	Additive
MEM (¼ and ½ MIC) + ECP11 (3 MOI)	4.00	MEM	16.00	0.25		
ECP36 (1, 2, 3 MOI) + MEM (16 µg/mL)	2.00	ECP36	5.00	0.40	0.65	Additive
MEM (¼ and ½ MIC) + ECP36 (3 MOI)	4.00	MEM	16.00	0.25		

E.coli Bacteriophage ECP01
E.coli Bacteriophage ECP11
E.coli Bacteriophage ECP36
Fractional inhibitory concentration=FIC
Minimum inhibitory concentration=MIC
Multiplicity of Infection=MOI
Meropenem=MEM

DISSCUSION:

Urinary tract infections are one of the most prevalent diseases seen in medical practice, affecting patients of all ages, from infants to the elderly. The purpose of this study was to identify and evaluate the antimicrobial susceptibility patterns of the most prevalent bacterial strains isolated from the urine of patients with urinary tract infections.

Escherichia coli was selectively isolated and identified using selective agar Separation of lactose-fermenting and lactose-non fermenting enteric microbes is also encouraged for this purpose (24),(25). MacConkey agar is selective for enteric bacteria due to the toxicity of crystal violet to gram-positive bacteria.

This study examined the prevalence of uropathogenic Escherichia coli (UPEC) among outpatients with urinary tract infections (UTI) who were treated at Al Kadhimya Teaching Hospital. One hundred six urine samples were collected from patients of UTI of both genders and different ages . All urine samples were examined for the presence of Escherichia coli using a conventional bacteriological technique (E. coli). 39 out of 106 samples tested positive for E.coli; the rate was higher in females (63.2% vs. 36.8%) than in males .

The prevalence of UPEC was the subject of numerous studies. the study conducted in three major hospitals in Zakho city the results were high rate in females than males (90.78% &9.22%), respectively (26).

Other studies were conducted at Azadi teaching hospital, Kirkuk hospital and children hospital in Kirkuk city, Iraq these studies showed that females isolates were (76.4%) while males isolates were (23.5%) (27). The higher incidence of urinary tract infections in females is due to distinctive anatomical features of the female genitourinary tract, which include a shorter urethra and the more proximal location of the urethral meatus to the anus makes it easy for bacteria to descend in the urinary tract.

Multidrug-resistant E. coli is considered a public health threat. It is primarily attributable to Extended-Spectrum Beta-Lactamases (ESBLs), which are capable of degrading a variety of -lactam antimicrobial agents, such as different generations of cephalosporins, carbapenems, and penicillins(28). depending on criteria ,isolate can be considered MDR when resist to at least one member in three different groups of antibiotics, in this study from thirty nine isolates, thirty two isolates (32/39) were MDR,six isolates(6/39) were XDR which resist to at least one member five different groups of antibiotics, from thirty nine isolates one isolate(1/39) can be PDR, which mean resistant to almost all antibiotics (21). The current study nearly agreed with (29).(in which bacterial isolates were resistant to multiple drugs (MDR) and the percentage of resistant was 87.5%. (MDR).

MIC values are used to determine the susceptibilities of bacteria to drugs and to evaluate the efficacy of new antimicrobial agents(30). In the current study, Escherichia coli strains were tested against 17 antimicrobial agents. These antimicrobials include (amoxicillin/clavulaniccid(AMC), Piperacillin/tazobactam (TZP), Cefazolin (CZ), Cefuroxime (CXM), Ceftazidime (CAZ), Ceftriaxzone (CRO), and Cefepime (FEP), Ertapenem(IR), Meropenem (MEM), imipenem (IPM), Amikacin (AM), Gentamicin (CN), ciprofloxacin (CFP), fosfomycin (FO), nitrofurantion (F), and Trimethoprim/sulfamethoxazole (TSM) according to CLSI, 2019 method.

The percentage of resistant individuals was as follows: 41.03% are resistant to amoxicillin/clavulanic acid, 2.56% are resistant to piperacillin/tazobactam, 58.97% are resistant to cefazollin, 56.41 % are resistant to cefuroxime, 61.54% are resistant to ceftazidime, and 76.92% are resistant to ceftriaxone. 28.21% are resistant to cefepime 2.56%resist to ertapenem, 2.56%resist to imipenem,5.13%resist to meropenem, 20.51% of bacteria are resistant to amikacine, 53.85% to gentamicin, and 20.51% to amikacine. 41.03% of bacteria are resistant to Ciprofloxacin, 5.13% to Fosfomycin, 2.56% to nitrofurantio, and

Trimethoprim/sulfamethoxazole (92.31%) and ceftriaxone (76.92%) had the highest rates of resistance in the current study, while nitrofurantion (2.56%) had the lowest rates .

Mechanisms of resistant:

In the current study, various E.coli isolates were resistant to a variety of antibiotics; this resistance was attributed to bacterial resistance mechanisms.

In the past 60 years, -Lactams have been among the most effective medications for treating bacterial infections caused by numerous species (31). and more than half of all antibiotics commercially available are used (32).In order to resist B-lactams, bacterial cells produce enzymes that deactivate the drugs by hydrolyzing the -lactam ring (33).

B-lactamases affect all B-lactam antibiotics with the exception of cephamycin and carbapenems. This mechanism is the most important mechanism of bacterial resistance to Blactam antibiotics and is typically inhibited by B-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (34). The Enterobacteriaceae bacteria that produce the

most ESBLs are Escherichia coli and Klebsiella pneumonia (35).

Aminoglycosides have bactericidal activity by binding to the ribosomal 30S subunit of bacteria. It is believed that they bind to the A-site (aminoacyl) on the ribosomal 30S subunit's 16S rRNA. At this Complete binding, the genetic code is misread and translation is interrupted, preventing the bacteria from carrying out protein synthesis (36), (37).

Gram-negative bacteria's resistance to aminoglycosides is primarily due to the production of aminoglycoside-modifying enzymes (AMEs) (Ramirez& Tolmasky 2010; LeClercq et al.,2010). Or through ribosome modification by acquired 16S rRNA methyltransferases (RMTases) (38).

The most prevalent resistance mechanism in E. coli is AME production. According to the reaction they catalyze, AMEs are classified into three classes: I aminoglycoside N-acetyltransferases (AAC), (ii) aminoglycoside O-phosphotransferases (APH), and (iii) aminoglycoside O-nucleotidyltransferases (ANT) (39).

Quinolones are a class of synthetic antimicrobial agents with broad antibacterial activity that are frequently prescribed to patients with urinary tract infections (UTIs) (40).

Currently, the quinolone antibiotics are the most effective class of topoisomerase inhibitors (41).

Based on their antimicrobial properties, this family has been divided into four generations. As members of the first, second, and third generations, nalidixic acid, ciprofloxacin, and levofloxacin are the most well-known quinolone antibiotics. Quinolones prevent bacterial DNA synthesis by inhibiting the enzymes DNA gyrase and topoisomerase IV, thereby causing cell death (42). The most effective antimicrobials for treating infections caused by the most resistant bacteria are carbapenem antibiotics. They are classified as -lactams alongside cephalosporins, monobactams, carbapenems, and penicillins. This class of antibiotics is the most effective against Gram-positive and Gram-negative bacteria and has a broader spectrum of activity than most other beta-lactams(43).

Enterobacteriaceae use three main types of resistance to carbapenems. Carbapenemases, enzymes that break down carbapenems (such as carbapenemase encoding genes), and efflux pumps, which actively remove carbapenems from the bacterial cell, are two examples of mechanisms by which bacteria develop resistance to carbapenems. and (c) changes to the bacterial cell membrane, including the production of beta-lactamases, which reduce the permeability of the outer membrane,(44),(45),(46). By acting as competitive inhibitors of dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) respectively, TMP and SMX inhibit the synthesis and conversion of tetra hydrofolate (THF), an essential cofactor involved in nucleotide synthesis. TMP and SMX resistance are mediated by distinct mechanisms. This consists of horizontally acquired modified target enzymes, alterations in the regulation of target enzymes, mutation of target enzymes, and alterations in permeability and efflux (47),(48).

Fosfomycin can eradicate more than 90 percent of Escherichia coli and 80 percent of Klebsiella pneumonia (49). Fosfomycin inhibits the initial step of peptidoglycan synthesis (50). The most frequently documented mechanism of fosfomycin resistance is impaired transport of fosfomycin into the cytoplasm, caused by mutations in structural or regulatory genes of the nutrient transport systems, such as in E. coli (51).

Regarding the mechanism of action, nitrofurantoin is converted by bacterial

flavoproteins into reactive intermediates that inactivate or alter bacterial ribosomal proteins and other related macromolecules. Several proposed mechanisms of nitrofurantoin resistance include mutations in the nfsA and nfsB genes and the presence of the oqxAB multidrug efflux pump genes (52).

Antibiotic bacteriophage combination:

The rapid increase in antimicrobial resistance to one or more antibiotics has become an all-encompassing public health issue (53) The discovery and use of new antibiotics have led to the emergence of antibiotic-resistant bacteria. Similarly, the number of mechanisms of resistance to various antibiotics has grown over time (54).

Phage antibiotic synergy (PAS) would have two additional benefits in the current study designed to prevent the emergence of E. coli resistant to an essential antibiotic used to treat uropathic E. coli. First, limiting the quantity of antibiotics used would aid in antibiotic stewardship and resistance management. Second, it would give antibiotics a second breath against MDR pathogens by combining them with phage treatments (55).

Torre et al., 2014 and Manohar et al., 2020 were discovered combinations of phage and antibiotics can control bacterial proliferation and antibiotic resistance by targeting distinct bacterial receptors. This is due to the fact that it is more difficult for microbes to develop resistance when they are infiltrated via multiple channels.

On the contrary, Antibiotics create a phage-antibiotic synergistic effect at sublethal dosages and reduce the bacterial population, but only when the bacteria are sensitive to the antibiotics.

In this study meropenem and bacteriophage combinations, synergism may result from the mode of action of these antibiotics on the bacterial cell wall, which enhances the entry of other antibiotics and overcomes the resistance mechanism (55).

CONCLUSIONS:

Based on the findings of this study, it is concluded that :

1.All E.coli isolates during this study were resistant to different groups of antibiotic, and considered MDR.

2. UTI is more diagnosed in females more than males.

3.High percent of E.coli isolates in this study were found highly resistant to trimethoprime/sulphamethoxazole but low percent resistant to carbapenem(s) group and nitrofurantion.

4.Antibiotic combination with phage (phage synrgesim therapy) result in additive or synergistic effect depending on mechanism of action of antibiotic.

5.Sub therapeutic doses (1/4,1/2 MIC) of Meropenem combination with phage was succeeded in treatment of MDR E.coli that refer to activity of phage.

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