Therapeutic Effect of Bacteriophage alone and in combination with Meropenem on multidrug resistance enterococcus faecalis in vitro study

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Abstract

Background: Enterococcus faecalis is a Gram-positive bacterium which can be found in soil, water, and as a commensal organism in animals and humans GIT. E. faecalis phages are bacterial viruses which infect bacteria and can reproduce in 2 possible ways: lysogenic life cycle and lytic life cycle. Almost all E. faecalis isolates are multi-drug resistant (MDR) that lead to difficulty in their treatment. So antibiotics &phage combination are feasible choices to get rid of that problem.

Aims of the study: The aim of this study is to appraise the benefit of the activity of bacteriophage as antibacterial agent alone and in combination(s) with antibiotic(s) against MDR E. faecalis and to get the proper antibacterial combination(s) for MDR treatment with synergistic effects.

Materials and Methods:Twenty six isolates were picked up from patients with urinary tract infections attended to the Medical City of Al-Imamain Al-Khadimain (peace being upon them) from April 2022 to June 2022.They were specified using morphological characteristics,VITEK2 compact system.

Results: In this study, E.faecalis was obtained from patients with urinary tract infection. The result include different types of bacteria causing UTI, E.coli isolates were higher percent (36.8%) followed by Enterococcus faecalis (24.5%). Distribution according to gender was show high percent in female than in male. The percentages of resistance of the isolates to the tested antibiotics were as follows: the higher is Tetracycline 84.62% and the lowest is Nitrofurantoin 3.85%. From 26 isolates, 12 isolates were MDR, 4 Isolates were XDR and 0 isolate was PDR combination of phage with ¼ or ½ MIC of each of meropenen for 11,20,22 isolates which were resulted in synergist effects to the antibiotics against MDR E. faecalis strains obtained in this study.

Conclusions: During this study all E.faecalis isolates were resistant to different groups of antibiotic, and considered MDR.

Keywords: *Bacteriophage, E.faecalis, multidrug resistant, bacteriophage therapy.*

INTRODUCTION

Enterococcus faecalis:

Enterococcus faecalis is a Gram-positive, non-motile, facultative anaerobe found in the environment and gastrointestinal tracts of people and animals as a commensal bacteria [1]. It can resist fluctuations in PH, temperature, and osmotic pressure, allowing it to colonize and flourish in various habitats [2]. E.faecalis has naturally high levels of antibiotic resistance. It is presently one of the world's most frequent multidrug-resistant hospital infections [3].

All illnesses induced by enterococci are urinary tract infections (UTIs), wound infections (mainly surgical, decubitus ulcers, and burn wounds), and bacteremia [4]. They are frequently related to endocarditis, intra-abdominal, and pelvic infections [5].

Biofilm:

Biofilm is a complex and organized community of microbes that cling to abiotic or biotic surfaces and are surrounded by an extra polymeric matrix [6]. Antibiotics, phagocytosis, and antimicrobial agents are more resistant to bacteria in biofilms [7]. Biofilm formation is thought to be an essential virulence factor in many E. faecalis syndromes [8]. Enterococcus is one of the most common bacteria that causes biofilms. The most common biofilm infections caused by E. faecalis are endocarditis and urinary tract infections.

Bacteriophage:

Virulent bacteriophages (phages) are obligate intracellular viruses that infect and reproduce only within bacterial cells without invading other cells and have been used for therapeutic purposes to control a bacterial infection, particularly with multidrug-resistant (MDR) bacteria, since their discovery [9]. 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 [10].

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, [11]. The first investigations were carried out analyzing the possible role of these viruses in medicine . Bacteriophages are the most numerous entities on earth .

A. Phage therapy, advantages and disadvantages:

Phage treatment is a novel therapeutic method. A lack of trustworthy evidence on its effectiveness, as well as regulatory obstacles, impedes widespread implementation. A bacteriophage, sometimes known as a phage, is a virus that infects bacteria. Because of their selectivity and effectiveness in generating deadly effects in the host bacteria via cell lysis, they have therapeutic promise in medicine to treat MDR infections [12]. Some advantages of using phage therapy instead of antibiotics include lower development costs, a 100% bactericidal nature, high specificity,

which prevents secondary infections, and the need for only a single dose or phage multiplication at the infection site, as opposed to antibiotics, which require multiple doses. Moreover, phage treatment can be used with standard antibiotics or a mixture of several phages to broaden its antibacterial spectrum [13].

Using an entire phage to treat infection may have certain drawbacks since the genetic material in temperate phage may boost the virulence of some bacteria through virulence gene transmission [14].

B. Phage Pharmacology:

Gelman [15] demonstrated that a mouse model that combines a phage cocktail and antibiotics had the best clinical impact on severe septic peritonitis induced by E. faecalis. As a result, combining phage and antibiotic therapy broadens the options for combating resistant infections.

Gelman tested a combination of bacteriophages and antibiotics against VRE Enterococcus faecalis in a mouse model and concluded that this combination has an additional beneficial effect on treatment success, as a single injection of the bacteriophage cocktail was enough to reverse the VRE-caused 100% mortality trend completely.

Resistant

The development of antimicrobial resistance, and emergence of multidrug resistance (MDR)has become a global health concern [16]. 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 [17].

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 [18].

Antibiotics:

Antibiotics are drugs used to treat or prevent certain types of bacterial infections. They work by either killing germs or inhibiting their spread. However, in this study antibiotic (Meropenem), was used to combine with phage to treat multi drug resistant E.faecalis.

A. Carbapenems (Meropenem):

Meropenem is a carbapenem-class broad-spectrum antibacterial agent. (carbapenems are regarded as the most effective class, having the broadest spectrum of antibacterial action and good safety and tolerability profiles.) Meropenem is a vital member of the carbapenem class. Ipenem, meropenem, ertapenem, and doripenem are carbapenems authorized for clinical usage [19].

Generally, this carbapenem is modestly more effective against Gram-positive bacteria than other medicines. Also, because imipenem is susceptible to dehydropeptidase I (DHP-I), a renal tubular dipeptidase enzyme that causes its breakdown, it is generally co-administered with cilastatin or betamipron. Cilastatin is a competitive antagonist that protects the kidneys from the adverse effects of greater imipenem dosages [20].

B. Mechanism of action:

Meropenem inhibits bacterial cell wall formation, slowing growth and resulting in cell death. Except against Listeria monocytogenes, where it is bacteriostatic, it is a bactericidal antibiotic. The medicine quickly penetrates bacterial cell walls and works by attaching to specific penicillin-binding proteins (PBP) with great affinity, making them inactive. Carbapenem then exerts

bactericidal action by binding to PBPs with high molecular weight, such as PBP1a, 1b, 2, and 3 [21].

C. Mechanism of resistance:

Similarly to beta-lactams, there are four forms of carbapenem resistance mechanisms: development of zinc-dependent metallo—lactamases; the presence of efflux pumps; and change of molecular target (PBP) [22].

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 :

Using the VITEK2 system, 26 E.fecalis isolates were obtained, after cultured on blood agar, MacFarland agar, and gram staining for primary 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 and MacFarland agar or to be stored in the refrigerator at 4oC for 24 hours.

Antibiotic susceptibility test:

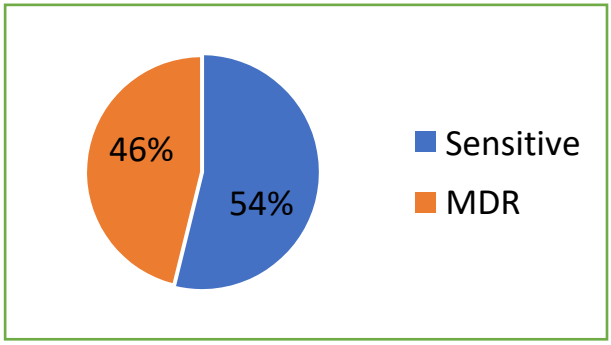
The antibacterial susceptibility testing of the isolates was done according to Clinical and Laboratory Standards Institute (CLSI, 2019), The MIC for antibiotics used were: Ampicillin ⩾ 32, Ciprofloxacin ⩾ 8, Erythromycin ⩾8, Linezolid ⩾8, Teicoplanin ⩾32, Vancomycin ⩾32, Tigecycline ⩾64, Gentamicin High level ⩾1024, Imipenem ⩾ 16, Meropenem ⩾ 16, Tetracycline ⩾ 16, Nitrofurantoin ⩾128. . 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 . isolate 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 considered XDR, and considered PDR when resistant to almost Antibiotics [23].

Result

Bacterial susceptibility rate

The results showed that different E.faecalis isolates had different antibiotic sensitivity profiles; of 26 isolates included in the current study, 12 were MDR, 4 were XDR and 0 PDR as shown in figure1:

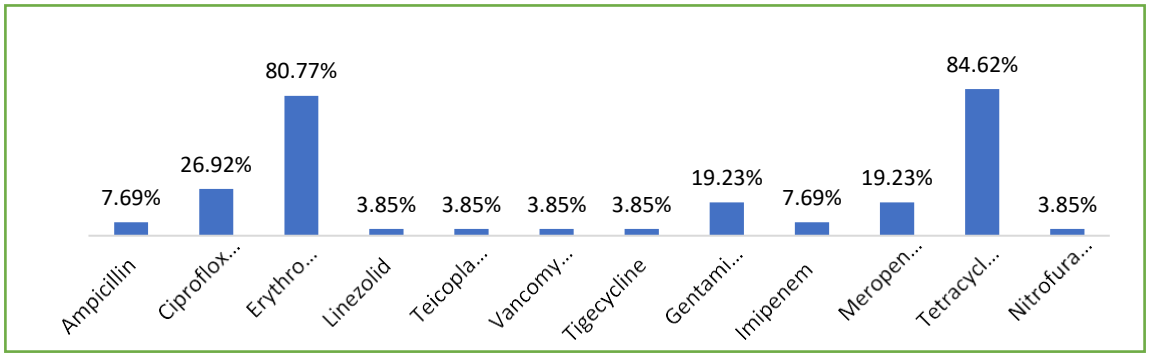
Figure 1



Antibiotic resistant rate

E.faecalis isolates had different antibiotic sensitivity profiles; in the current study resistant rate as shown in figure2:

Figure 2



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.

Determination MOI

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 (2-6):

Table (1): The concentration of phage suspensions

Bacteriophage	Concentration (PFU/mL)
EF11	7.9 x 10^10
EF20	5.2 x 10^9
EF22	4.4 x 10^12

4. Different MOI was prepared to each phage to find the minimum MOI, Table (2-7):

Table (2): Different MOI to each phage

MOI	Concentration of phage (PFU/mL)
1	2.1 x 10^8
2	4.2 x 10^8
3	6.3 x 10^8
5	1.1 x 10^9
10	2.1 x 10^9
15	3.2 x 10^9

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

MOI is the phage-to-bacteria ratio [24]. 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 [25].

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) [26].

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 [27].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 [28].

Table (3): The synergistic effect of the Meropenem-resistant isolates in combination with 1, 2, 3, 5 MOI

Combination (µg/mL)		Antibacterial agents alone (µg/mL)		FIC	FIC index	Outcome
EF11 (1, 2, 3, 5 MOI) + MEM (16 µg/mL)	5.00	EF11	10.00	0.50	0.75	Additive
MEM (¼ and ½ MIC) + EF11 (½ mMOI "5 MOI")	4.00	MEM	16.00	0.25		
EF20 (1, 2, 3, 5 MOI) + MEM (16 µg/mL)	5.00	EF20	10.00	0.50	0.75	Additive
MEM (¼ and ½ MIC) + EF20 (½ mMOI "5 MOI")	4.00	MEM	16.00	0.25		
EF22 (1, 2, 3, 5 MOI) + MEM (16 µg/mL)	5.00	EF22	10.00	0.50	0.75	Additive
MEM (¼ and ½ MIC) + EF22 (½ mMOI "5 MOI")	4.00	MEM	16.00	0.25		

E.fecalis Bacteriophage ECP11
E.fecalis Bacteriophage ECP20
E.fecalis Bacteriophage ECP22
Fractional inhibitory concentration=FIC
Minimum inhibitory concentration=MIC
Multiplicity of Infection=MOI
Meropenem =MEM

Discussion

Antibiotics provide clear benefits to patients. Their overuse and misuse, on the other hand, have contributed to the growing problem of uropathogenic bacteria resistance, a serious threat to public health [29].

The exponentially increasing number of studies on phage therapy over the last decade highlights the need for alternative antibiotic therapies due to multi-resistance. Although there are some concerns, phage therapy is a viable alternative to antibiotics for humans [30]. Combinations of phages and antibiotics were successfully used in Soviet medicine in the 1950s and 1960s [31].

Phages and antibiotics synergy (PAS) was demonstrated in vitro and in vivo [32], and even when no benefits were obtained, the emergence of antibiotic- or phage-resistant phenotypes was greatly reduced. As concluded by Torres-Barceló [33] and Tagliaferri [34], the combination of phage therapy and antibiotics would be beneficial due to improved bacterial clearance and reduced bacterial capacity to develop resistance to one or both therapies.

Bacterial growth and Gender distribution:

The current study found that out of 153 cultured samples, 106 (69%) showed positive bacterial growth during culture. More than half, 63.2% of all participants were females;

this finding was in keeping with the results of the study conducted in the United States by Hayakawa [35] and in Turkey by Asgin [36], which revealed that 53.3%, and 55.3%, respectively, of participants, were females. This could be due to anatomic and physical factors that favor increased UTI in females [37].

Gram stain count:

Current work observed that 34% of isolated bacteria were gram-positive, which is inconsistent with the study in China by Gu [38] recorded that only 20% of isolated bacteria were gram-positive. This is related to more than 90% of UTIs being due to enteric Gram-negative organisms [39].

Bacterial isolated types:

This study showed that Escherichia coli and Enterococcus faecalis were the main bacterial isolates found in the urine samples (36.8% and 24.5%). These findings were agreed with a study carried out in China by Klein [40], which found that Escherichia coli and Enterococcus faecalis were isolated in (40.8% and 15.4%), respectively.

Another study in China by Gu [41] found that Escherichia coli 41% and Enterococcus faecalis 8.3%. Because these bacteria are common flora in the gastrointestinal tract, colonization of gastrointestinal pathogens may explain it around the periurethral.

Susceptibility of the isolated bacteria:

Regarding the culture susceptibility, this study showed that from the total 26 bacterial isolates, 53% were sensitive and resistant to at least one member in 3 different groups of antibiotics (MDR) 46%. These findings were not similar to the results in China by Klein [42], which observed that MDR was 31.5%; it could be due to the high empiric use of antibiotics for the treatment of UTI in our country.

Comparison between antimicrobial groups according to resistance rate in the urine sample:

Antibiotic resistance is becoming a major global health issue [43], and current surveillance of antimicrobial susceptibility to a specific type of infection is critical for initial empirical therapy [44].

A current study showed tetracycline and erythromycin represent the higher resistance rate (84.62% and 80.77%), respectively, which somewhat differ from the study in Iran by Ghalavand [45] that found the highest resistance rates were orderly observed against tetracycline and minocycline (88.9% and 87.3%) respectively. Arbitrary usage of antibiotics for the treatment of infections could explain a higher rate of resistance to this antibiotic compared with other antimicrobials.

Conclusions

1. All E.feacalis isolates during this study were resistant to different groups of antibiotics and considered MDR.

2. Antibiotic combination with phage (phage synergism therapy) results in additive or synergism effect depending on the antibiotic mechanism of action.

3. Antibiotic combination with phage (phage synergism therapy) results in additive or synergism effect depending on the antibiotic mechanism of action.

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

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