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Estimation of the In-Vitro Synergistic Effect of Vancomycin with Bacteriophage and its Endolysin on Iraqi Local Isolates of *Enterococcus faecalis*

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Abstract

Enterococcus faecalis is known to cause many serious infections with an upsurge in the development of antibiotic resistant strains, especially vancomycin. Lytic bacteriophage and its endolysin have the potential to affect and lyse specific bacterial cells making them a possible alternative therapy to antibiotic resistant strains. The study aimed to investigate the synergistic effect of phage/ phage cocktail/ endolysin with vancomycin on *E. faecalis* isolated from urine samples. The antibioresistant profile 17 *E. faecalis* was done with an emphasis on the sensitivity to vancomycin. The lytic phages were isolated from the local environment. The infectivity and the coverage rate for the phage and phage cocktail were calculated. The enzyme of the lytic phage was isolated by chromatography. The synergistic effect was tested by turbidity reduction assay to evaluate the growth reduction level. It had been found that 6/17 *E. faecalis* isolates were resistant to vancomycin, 7/17 isolates were intermediate, and 4/17 were susceptible. Three lytic phages were identified with an infectivity rate of 100%, 76.4%, and 88.2%. The phage cocktail yielded 100% rate of infectivity and 91.6 % coverage rate. The reduction in the growth levels of the 17 *E.faecalis* after treatment with phage / phage cocktail/ vancomycin/ mixture of vancomycin and phage cocktail was effective. The synergistic effect of vancomcyin together with the phage cocktail significantly reduced the growth of 70.6% of the isolates. The antibacterial activity of vancomycin plus endolysin treatment was revealed in a time dependent manner. Thus, the synergistic effect of vancomycin with phage/ endoloysin illustrated an effective method to treat the antibiotic-resistant strains of *E. faecalis* to overcome this emerging clinical problem.

Keywords: Bacteriophages, *Enterococcus faecalis*, Endolysin, Multidrug resistance bacteria, Phage therapy, Phage cocktail.

Introduction

The Gram-positive non-spore-forming bacterium; *Enterococcus faecalis*, is an inhabitant of the gastrointestinal tract. It is known to be an essential commensal member of the human gut. The

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emergence of Multi-Drug Resistant (MDR) variants is due to a remarkable ability of the bacterium to adapt to different environments and a tendency to acquire antibiotic resistance gene [1]. In susceptible hosts, *E. faecalis* can act as an opportunistic pathogen producing severe and life-threatening infections, including bacteremia, endocarditis, urinary tract infections (UTI), catheter-related infections, intra-abdominal and pelvic infections, and wound infections [2].

Antimicrobial resistance poses an increasingly important public health challenge as it causes thousands of deaths every year that can be increased to millions of deaths worldwide [3]. Scientists worldwide are inspired to search for viable alternatives due to the high incidence of MDR bacteria and the inadequacy of existing antibiotics to overwhelm infectious diseases, particularly, urinary tract infections [4-6].

Bacteriophages or phages, also known as viral predators of bacteria, are viruses that infect and replicate only in bacterial cells. They are widely distributed in the environment. They are different in size, morphology, and genomic content [7]. Similar to all viruses, bacteriophages are very species-specific to their hosts. They have specific interactions with the bacterial host. They infect a single bacterial species or even specific strains within a species [8]. Phages can be mixed with other phages having different host ranges. This phage–phage mixture can be described as a phage cocktail and it is proven to be active against many MDR bacteria [9].

Bacteriophages or phages, with their related cell-wall lysing enzymes (endolysins), have the ability to be useful tools to tackle MDR bacteria [10]. Many studies indicate the potential of phages and their endolysins as suitable alternatives to treat *E. faecalis* [11, 12]. A recent study has come to the fact that a combination of a phage and an antibiotic has better killing efficiency against *E. faecalis* [13]. In addition, *E. faecalis* vancomycin-resistant strains have been used for screening to understand the potential of phages as novel therapeutic agents [14].

Dependently, the current study tried to investigate the synergistic effect of vancomycin with phage cocktail then vancomycin with endolysin on the reduction level of *Enterococcus faecalis* growth, isolated from urine samples. The investigated synergistic effect was also compared with the growth reduction level after treatment with phage alone, phage cocktail, and phage endolysin. Thus, unveil a new era of MDR bacteria treatment and their associated critical diseases by objects from Mother Nature.

Materials and Methods

Bacterial Samples

An experimental study was conducted to isolate 79 *E. faecalis* from urine specimens. The samples were collected from patients with UTI attending Al-Furat General Hospital and Ghazi Hariri Hospital for Specialized Surgery, Baghdad, Iraq in the period from Oct. to Dec. 2021. All the samples have undergone a series of identification and confirmatory tests to isolate and identify *E. faecalis*.

Isolation and Primary Identification of Enterococcus spp.

Azide Blood Agar Base medium

The collected urine samples were cultured on a selective blood agar medium; Azide Blood Agar Base and incubated at 37 °C overnight. This medium is recommended for the isolation and cultivation of clinical and nonclinical samples of *Enterococci*, *Streptococci*, and *Staphylococci*. The growth of gram-negative bacteria was inhibited by sodium azide while sparing the growth of *Enterococci*, *Streptococci*, and *Staphylococci* [15].

Bile Esculin Agar Medium

After overnight incubation, the bacterial growth from Azide Blood Agar Base medium was transferred to Bile Esculin Agar plates. This test is used to identify and differentiate *Enterococci* and group D *Streptococci* from non-group D Viridans group *Streptococci*, which grow poorly on bile. The identified bacteria are bile tolerant and can hydrolyze esculin to esculetin [15]. The cultured plates were incubated at 37 °C for overnight.

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Confirmatory Identification and Antibiotic resistant Profile

The identification of a species-level and the antibiotic sensitivity profile was confirmed by VITEK 2 Compact System. The instructions of the manufacturer were followed to prepare the bacterial samples. Blood agar with overnight bacterial cultures was used. The VITEK 2 Compact system uses a Gram-positive cocci identification kit (ID-GP) as a fluorogenic recognition method in less than eight hrs. While a turbidimetric susceptibility test in less than 18 hrs was used for bacterial sensitivity of all the isolates against sixteen antibiotics. The percentage of the resistant rate was measured as follows [16]:
No. of resistant isolates/ total No. of tested isolates*100

Sensitivity Test against vancomycin

Agar-Disc Diffusion Assay

The antimicrobial susceptibility for all the isolates was tested as described by [13]. The bacterial suspension was prepared by transferring of few colonies; two to four, from overnight Brain Heart Infusion agar to 2 ml normal saline. The bacterial suspension was adjusted to (0.5 McFarland) turbidity which is equal to 1.5×10^8 CFU/ml. The bacterial suspension was inoculated onto a Mueller Hinton agar plate by a sterile cotton swab and left to dry. Later, vancomycin antibiotic discs 30 µg were laid on the surface of the agar medium. The inoculated plates were incubated at 37°C overnight. The assay was achieved using Kirby-Bauer disk diffusion technique [17]. The inhibition zone diameter of the antibiotic disc was measured and the measures were interpreted referring to the Clinical and Laboratory Standards Institute criteria [18].

Minimum Inhibitory Concentration (MIC)

The broth serial dilution method was used to determine Vancomycin MIC on *E. faecalis*. The inhibition of the microorganism's visible growth after overnight incubation by the lowest concentration of vancomycin is known to be MIC [19].

The interpretation of the isolates as being Resistant (R), Intermediate (I), and Susceptible (S) was calculated according to the value breakpoints of MIC and the zone diameter R ≥ 32 µg/ml and ≤ 14mm, I ≥8-16 µg/ml and 15-19mm, and S ≤ 4 µg/ml and ≥ 20mm [18].

Isolation and Identification of the Bacteriophage

Characteristics of the Isolated Phage

Three phage crude suspensions were collected from sewage in the city of Baghdad during one week in Nov. 2020. About 3 ml of the crude sample was mixed with an overnight *E. faecalis* broth 100 µl. The mixture was incubated at 37C° overnight until obtaining the specific lytic phage. The specific characteristics of the phages' plaques such as size, clarity, morphology, and margin were determined [20]. Their titer was verified by the top layer assay [21]. The clearest and largest plaque had been selected [21, 22].

Spot Lyses Assay

The phage spotting technique was used to test the specific phage on nutrient agar plates. The presence of the lytic phages was concluded by the formation of inhibition zones [23]. A sterile swab was used to spread an overnight cultured bacterium on nutrient agar plates to form a bacterial lawn. A phage suspension of 10 µl was applied as spots after 10-15 min and the plates were incubated overnight at 37C°. The identified specific lytic phage was collected by a sterile pasture pipette after an overnight incubation. One ml of SM buffer (50 ml of SM buffer, the following materials were used: Sodium chloride 0.29gm, Magnesium sulfate 0.1gm, Gelatin 250 µl (2% w/v), and 2.5 ml of Tris-HCl (1 M, pH 7.5), all the materials were mixed on a magnetic stirrer and stored at 4 C°) was used with the collected phage then gentle vortex and shaking were done for 20 sec. The mixture was centrifuged at 1000 g for three min after bacterial lyses by Chloroform (1: 10 v/ v). The required phages supposed to be in the collected supernatants were transported to a new sterile Eppendorf tube and kept at 4C°. The saved phages were kept for further tests in this study.

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Bacteriophage infectivity rate

The Infectivity and the Coverage Rates of the Isolated Phages and the Developed phage Cocktail against *E. faecalis*

The infectivity rate of the three isolated phages was determined against 17 new samples of *E.faecalis* randomly collected from patients in the Medical City Hospital. In addition, the phage cocktail was formed by the addition of 100µl of 10^9 plaque forming unit (PFU)/ml of (BP-EF1, BP-EF2, and BP-EF3) phage stocks in 15ml tube. By applying the spot lyses assay, 10µl of the phage/ phage cocktail suspension was spotted on the bacterial lawn. After dryness, all plates were incubated at 37C˚for overnight. The next day, the susceptibility to the phage/ phage cocktail was determined by the formation of the lyses zones. Moreover, the coverage rate of the phage cocktail was determined against another 12 new samples of *E.faecalis* using the same method mentioned above [11].

The infectivity and the coverage rates were calculated according to the following formula [11]:

The infectivity = number of bacteria lysed by phage or phage cocktail/ total number of bacteria * 100%

The coverage rate = number of bacteria lysed by phage cocktail/ total number of bacteria * 100%

Isolation of Phage Lysin

Enterococcu faecalis (EF-4) in 100 ml of Nutrient broth medium was cultured and incubated overnight at 37C˚. Afterward, the bacterial growth with 250 ml of sterile Nutrient broth medium was incubated for another three hours. Ten milliliters of BP-EF2 at titer 1×10^9 PFU/ml were mixed with EF-4 then put directly on ice for 20 min. The sediment was collected after centrifugation of the mixture at 10,000 g for 20 min. Ten milliliters of 0.05 M phosphate buffer saline plus 5mg deoxy ribonuclease was used to suspend the sediment and incubated for 60 min at 37C˚. EDTA at 0.005 M was added and centrifuged at 10,000 g for 60 min then the supernatant was collected. Ammonium sulfate was supplemented to 85 % saturation then the suspension was incubated overnight at 4C˚. On the next day, centrifugation at 10,000 g for 60 min was carried out to the suspension and re-suspended in five milliliters of 0.05 M phosphate- buffered saline at (pH 7.5). Subsequently, dialysis versus 200 ml of 0.05 M phosphate buffer saline was done. Every two hours, the buffer was changed two times and left overnight at 4C˚. Sephadex G100 column chromatography (3×20 cm) was used to isolate the enzyme. The dialyzed solution was added with 20 ml of 0.05 M phosphate- buffered saline at pH 7.5 as eluent. Every five minutes the elute 1 ml was collected in 50 Eppendorf tubes [11, 12]. A spectrophotometer was used to measure the absorbance of each fraction at 280 nm. The tube with the endolysin lytic activity was recognized in each Eppendorf tube by dropping 10 µl of the elute onto *E. faecalis* lawns [12].

Bacteriophage and Phage Cocktail treatment with/without Vancomycin

Quantitative Analysis of the Bacterial Growth Reduction

The antibacterial activity against *E.faecalis* was measured by turbidity reduction assay: the overnight bacterial cells were centrifuged at 4000 g for 30 min and re-suspended with phosphate-buffered saline (pH 7.5). At room temperature, 30 µl of phage/ phage cocktail/ vancomycin/ mixture of vancomycin and phage cocktail were added to 270 µl of the bacterial culture broth. Then at 600 nm, the optical density was measured by spectrophotometer [12]. In order to measure and assess the antibacterial activity of phage cocktail/ lysin/ vancomycin/ vancomycin plus lysin the same procedure was followed except that the optical density was measured every 5 min for 35 min against only one bacterium EF-5.

The Bacterial concentration (CFU/ml) was measured according to the below formulae [11].

ΔOD = OD (before treatment) - OD (after treatment)

Δ Bacterial concentration (CFU/ml) = CFU/ml (before treatment) – CFU/ml (after treatment)

Data Analysis:

Microsoft Excel Version (14.0.6023.1000) was used to represent the plots and measure the percentages. Vitek 2 system with software version (5.01) was

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used to evaluate the bio-resistant profile of *E. faecalis*.

Results and Discussion

Bacterial isolation and Identification

The preliminary identification of *Enterococcus* species depends mainly on the appearance of these bacteria on the selective and differentiation media in addition to the microscopic characteristics. The tests demonstrated that 27 isolates from the total 79 urine samples' isolates were identified as *Enterococcus spp.* On Azide Blood Agar Base medium *Enterococcus* species appeared as a small glassing gray colony with no hemolysis (Fig. 1). While on Bile Esculin Agar Medium *Enterococcus* species colonies were surrounded by black halos. The dark brown or black color is due to the formation of a complex of esculetin and Fe^{3+} ions in the medium when *Enterococcus* spp. produce esculinase that hydrolyses esculin into esculetin (Fig. 2).

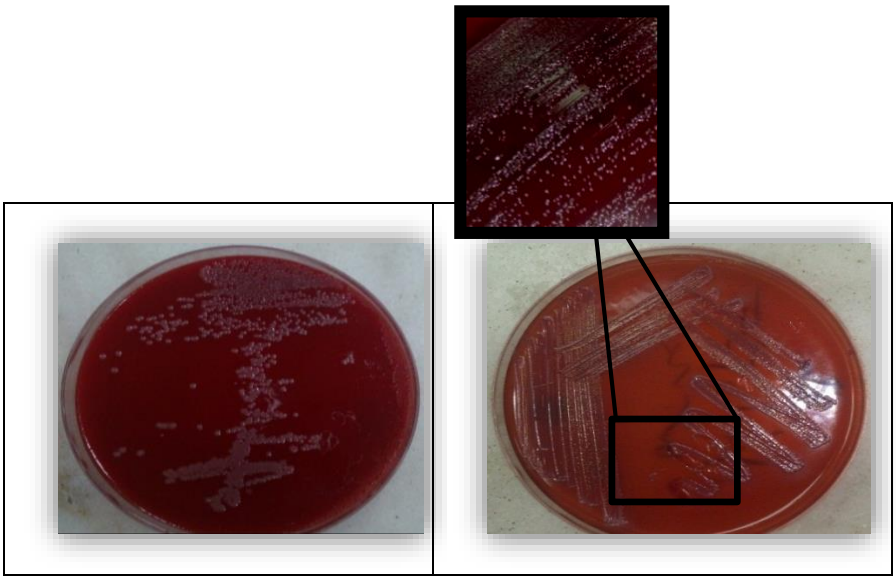

Figure 1. Azide Blood Agar Base medium *Enterococcus* species appears as small glassing gray colony with no hemolysis. Plates were incubated aerobically at 37˚C for overnight.

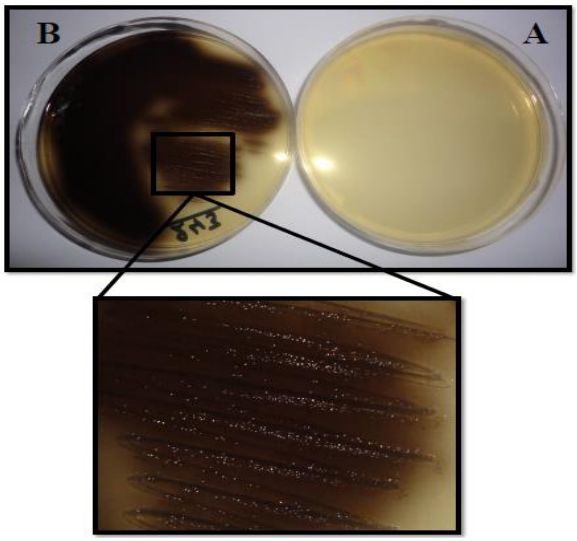

Figure 2. Bile Esculin Agar Medium (A) Medium without culture. (B) Esculin hydrolysis by *Enterococcus* species. Plates were incubated aerobically at 37˚C for overnight.

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Confirmation and Antibioresistant Profile

Enterococcus faecalis was confirmed by VITEK 2 System with 98.99% matching value. From the 27 *Enterococcus* spp., 17 isolates were confirmed as *E.faecalis* and 10 isolates of *E.faecium* (not included in this study).

The turbidimetric susceptibility test by VITEK 2 System for 17 *E.faecalis* isolates, demonstrated variable percentages of bacterial sensitivity against the sixteen antibiotics (Table 1). The test displayed that only (29%) of the isolates were resistant to vancomycin.

Table 1. The Antibiotic susceptibility of *E. faecalis* isolates.

Antibiotic	Percentage of Resistance	Antibiotic	Resistance
Erythromycin	**22 %**	**Oxacillin**	**78 %**
Ciprofloxacin	**8%**	**Inducible- clindamycin**	**0%**
Benzylpencillin	**3%**	**Vancomycin**	**35%**
Ampicillin	**16 %**	**Gentamycin**	**44%**
Tigecyclin	**0%**	**Fusidic acid**	**11%**
Levofloxacin	**33%**	**Tobramycin**	**58%**
Trimethoprim/sulphamethaxazole	**2%**	**Teicoplanin**	**9%**
Tetracyclin	**32%**		

Sensitivity Test against Vancomycin

Zone diameter and MIC breakpoints for the 17 *E. faecalis* isolates was shown in (Table 2). The acquired results found that 6/17 *E. faecalis* isolates were resistant (R), 7/17 isolates were intermediate (I), and 4/17 (S) were susceptible. The rate of *E. faecalis* resistant to vancomycin was confirmed by the results given by VITEK 2 System.

Table 2. The bioresistant profile of 17 *E. faecalis* isolates against Vancomycin.

Isolate no.	Inhibition zone diameter (mm)	MIC µg /ml	Bioresistant Profile
E. *faecalis 1*	15	16	I
E. *faecalis 2*	23	4	S
E. *faecalis 3*	10	128	R
E. *faecalis4*	33	4	R
E. *faecalis* 5	8	32	R
E. *faecalis 6*	16	8	I
E. *faecalis 7*	15	8	I
E. *faecalis 8*	15	16	I
E. *faecalis 9*	16	16	I
E. *faecalis 10*	12	128	R
E. *faecalis 11*	14	32	R
E. *faecalis 12*	8	32	R
E. *faecalis 13*	20	4	S
E. *faecalis 14*	21	4	S
E. *faecalis 15*	16	8	I
E. *faecalis 16*	16	8	I
E. *faecalis 17*	28	4	S

***R: resistant, I: intermediate, and S: susceptible.**

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Characteristics of the Isolated Phage

Three distinct lytic *E. faecalis* bacteriophages (BP-EF1, BP-EF2 and BP-EF3) were isolated and characterized by the top layer assay. The plaques were characterized as clear or turbid with a regular or irregular margin cut, sized from 1mm to 5mm, and had a circular shape. BP-EF2 demonstrated the largest plaque in size while BP-EF3 was the smallest one. All bacteriophages showed no hallow appearance. The results showed that all phages have sharp-regular plaque edges. In addition, BP-EF2 and BP-EF3 were turbid while BP-EF1 was clear (Table 3). The titer of the isolated bacteriophages was calculated ranging between 10^6 to 10^{12} PFU/ml.

Bacteriophage infectivity and coverage rates

The isolated bacteriophages were significantly lytic and formed an obvious clear zone on targeted bacteria. The three bacteriophages were obtained with different coverage rates of infectivity. The infectivity rates were found to be 100%, 76.4%, and 88.2% for BP-EF1, BP-EF2 and BP-EF3, respectively. An extensive overlapping was observed from mixing the three bacteriophages together yielding 100% rate of infectivity (Table 4).

Clear inhibition zones were formed by the phage cocktail on the most examined bacterial lawns. The coverage rate of the phage cocktail was shown to be 91.6 % in which 11 out of 12 *E. faecalis* bacteria were lysed (Fig. 3).

Table 3. Characterization of the isolated specific bacteriophages.

Phage	Size (mm)	Turbidity	Hallow	Plaque edge	Shape
BP-EF1	3.5	Clear	None	Sharp-regular	Circular
BP-EF2	5.0	Turbid	None	Sharp-regular	Circular
BP-EF3	1.0	Turbid	None	Sharp-regular	Circular

Table 4. Bacteriophage infectivity rate against 17 new *E. faecalis* isolates.

Bacteriophage	*E. faecalis* Isolates (n=17)	Infectivity rate (%)
BP-EF1	17/17	100
BP-EF2	13/17	76.4
BP-EF3	15/17	88.2
Phages cocktail	17/17	100

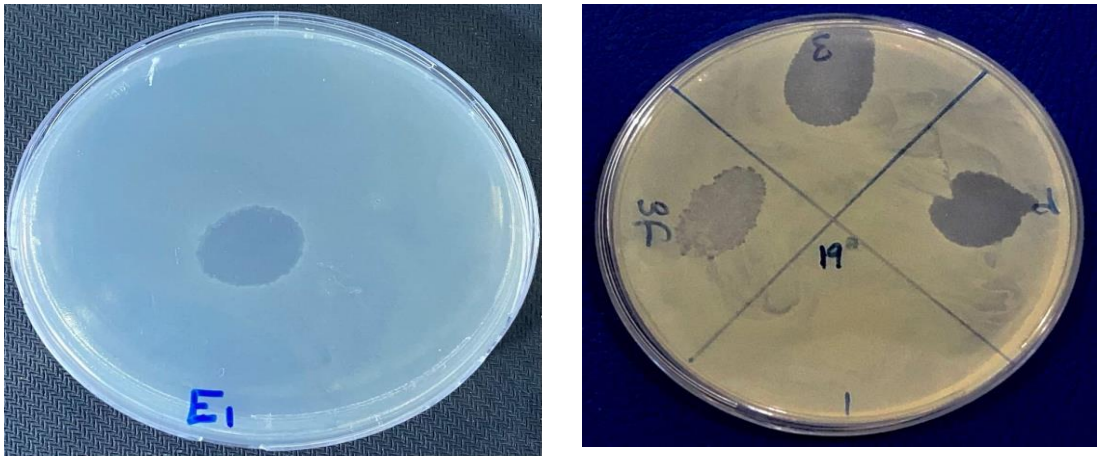

Figure 3. Clear inhibition zones on E. *faecalis* lawns by phage cocktail.

The Quantitative Growth Reduction Analysis

The growth of 11 *E.faecalis* was reduced by different levels when treated with every single phage/ phage cocktail (Table 5). BP-EF1 was able to reduce the growth to a non-detected level of EF-4 and EF-6. BP-EF3 reduced the growth of EF-4 to a non-detected level. Significant activities against EF-1, 4, 6, and 7 were clarified by phage cocktail treatment.

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The reduction in the growth levels of the 17 *E.faecalis* after treatment with phage BP-EF2/ phage cocktail/ vancomycin/ mixture of vancomycin and phage cocktail was tested. The effectiveness of the treatment was clear when the bacteria were treated with phage cocktail, vancomycin, and a mixture of vancomcyin plus phage cocktail in comparison to treatment with BP-EF2 alone or vancomycin (Table 6). The synergistic effect of vancomcyin together with the phage cocktail significantly reduced the growth of EF-1, 4, 6, 7, 9, 10, 11, 13, 14, 15, 16, and 17.

Moreover, the synergistic effect of vancomycin with the extracted endolysin was tested (Table 7 and Fig. 4). By bringing the antibacterial activity of both vancomycin and the endolysin together, a significant reduction in EF-4 growth was revealed especially after 20 min and more.

Table 5. the growth reduction analysis was investigated after treatment with phage versus phage cocktail.

#	Initial (CFU/mL)	BP-EF 1	BP-EF2	BP-EF3	Phage cocktail
EF-1	$1.3* 10^{10}$	$2.6*10^{5}$	$2.5*10^{7}$	$1.4*10^{7}$	-
EF-2	$4.5* 10^{9}$	$1.7.*10^{6}$	$1.7*10^{7}$	$1.3*10^{6}$	$0.2*10^{7}$
EF-3	$9.2 * 10^{9}$	$3.5*10^{6}$	$4.4*10^{7}$	$0.7*10^{7}$	$0.1*10^{6}$
EF-4	$3.2 * 10^{9}$	-	$1.2*10^{6}$	-	-
EF-5	$4.3* 10^{11}$	$0.7*10^{7}$	$1.4*10^{5}$	$1.2*10^{7}$	$0.5*10^{2}$
EF-6	$5.7 * 10^{8}$	-	$0.2*10^{7}$	$1.2*10^{7}$	-
EF-7	$5.6 * 10^{7}$	$1.2*10^{5}$	$3.5.*10^{6}$	$2.7*10^{6}$	-
EF-8	$1.9 * 10^{10}$	$0.5*10^{7}$	$1.1*10^{7}$	$2.4*10^{6}$	$0.3*10^{5}$
EF-9	$3.3 * 10^{9}$	$0.5*10^{5}$	$1.2.3*10^{7}$	$2.4.*10^{8}$	-
EF-10	$2.8 * 10^{9}$	$1.4*10^{6}$	$1.4*10^{7}$	$1.2*10^{7}$	-
EF-11	$5.1 * 10^{9}$	$0.2*10^{5}$	$0.3.1*10^{6}$	$1.7*10^{6}$	-

Table 6. The growth reduction analysis was investigated after treatment with phage BP-EF2, phage cocktail, vancomycin, and vancomycin plus phage cocktail.

E. faecalis	Initial (CFU/mL)	Vancomycin (CFU/mL)	Phage (BP-EF2)	Phage cocktail (CFU/mL)	Phage cocktail + vancomycin (CFU/mL)
EF-1	$2.3 * 10^{11}$	$5.6 * 10^{7}$	$1.2 * 10^{3}$	$5.7 * 10^{2}$	-
EF-2	$1.6 * 10^{10}$	$6.6 * 10^{9}$	$7.1 * 10^{4}$	$8.8 * 10^{1}$	$2.3 * 10^{1}$
EF-3	$3.9 * 10^{4}$	$8.2 * 10^{2}$	$9.1 * 10^{1}$	$8.8 * 10^{1}$	$4.8 * 10^{1}$
EF-4	$1.6 * 10^{8}$	$5.5 * 10^{5}$	$8.4 * 10^{3}$	-	-
EF-5	$2.6 * 10^{12}$	$8.4 * 10^{10}$	$1.9 * 10^{5}$	$5.4 * 10^{2}$	$2.6 * 10^{2}$
EF-6	$2.2 * 10^{8}$	$7.7 * 10^{6}$	$7.1 * 10^{4}$	$6.5 * 10^{2}$	-
EF-7	$3.2 * 10^{7}$	$9.1 * 10^{6}$	$5.4 * 10^{4}$	$6.9 * 10^{2}$	-
EF-8	$3.1 * 10^{11}$	$4.7 * 10^{8}$	$3.9 * 10^{6}$	$1.9 * 10^{4}$	$5.2 * 10^{2}$
EF-9	$4.8 * 10^{7}$	$4.8 * 10^{4}$	$4.8 * 10^{2}$	$5.5 * 10^{2}$	-
EF-10	$7.8 * 10^{10}$	$7.7 * 10^{8}$	$1.3 * 10^{5}$	$5.1 * 10^{2}$	-
EF-11	$5.7 * 10^{7}$	$3.9 * 10^{5}$	$8.6 * 10^{2}$	-	-
EF-12	$1.6 * 10^{10}$	$2.8 * 10^{9}$	$9.5 * 10^{6}$	$6.6 * 10^{3}$	$3.1 * 10^{3}$
EF-13	$8.9 * 10^{6}$	$8.9 * 1^{4}$	$8.9 * 10^{3}$	$8.9 * 10^{1}$	-
EF-14	$5.1 * 10^{8}$	$5.8 * 10^{5}$	$9.7 * 10^{3}$	$7.3 * 10^{2}$	-
EF-15	$9.4 * 10^{11}$	$2.8 * 10^{7}$	$6.9 * 10^{3}$	-	-
EF-16	$9.8 * 10^{11}$	$6.6 * 10^{9}$	$9.1 * 10^{5}$	$2.3 * 10^{2}$	-
EF-17	$4.6 * 10^{11}$	$5.5 * 10^{7}$	$1.1 * 10^{4}$	$9.7 * 10^{3}$	-

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Table 7. The growth reduction analysis was investigated after treating EF-4 with phage cocktail, lysin, vancomycin and vancomcyin plus lysin by different time intervals.

Time	Vancomycin (CFU/mL)	phage cooctail treatment (CFU/mL)	Lysin (CFU/mL)	lysin + vancomycin (CFU/mL)
0	$2.6 * 10^{12}$	$2.6 * 10^{12}$	$2.6 * 10^{12}$	$2.6 * 10^{12}$
5	$9.4 * 10^{11}$	$4.4 * 10^{8}$	$9.1 * 10^{10}$	$3.1 * 10^{4}$
10	$9.4 * 10^{11}$	$7.7 * 10^{8}$	$9.9 * 10^{4}$	$2.2 * 10^{3}$
15	$8.7 * 10^{10}$	$4.1 * 10^{7}$	$1.8 * 10^{3}$	$2.9 * 10^{3}$
20	$6.3 * 10^{8}$	$1.9 * 10^{5}$	$5.4 * 10^{2}$	0
25	$3.2 * 10^{7}$	$4.8 * 10^{3}$	0	0
30	0	0	0	0
35	0	0	0	0

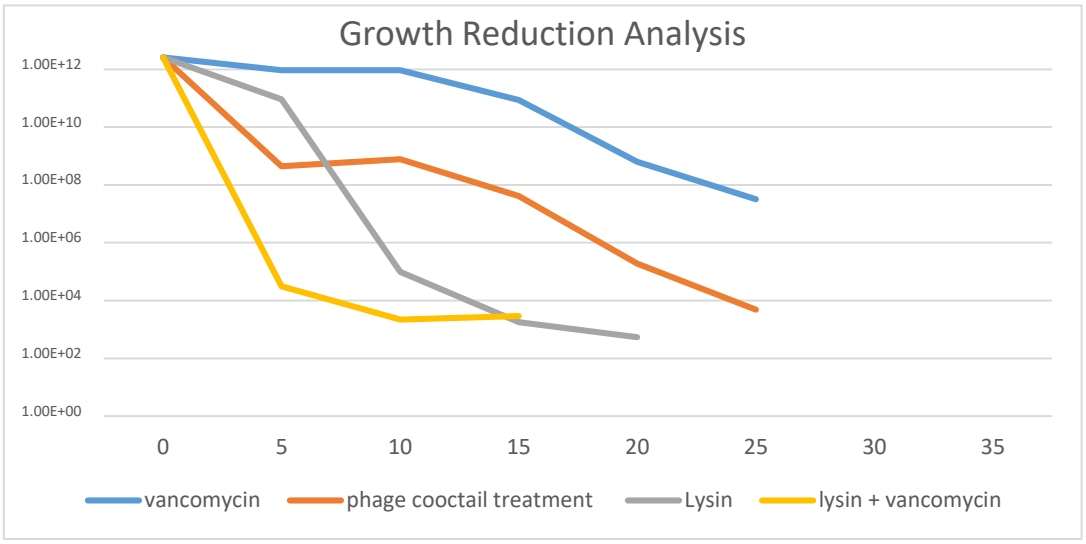

Figure 4. The flow chart shows the significant reduction by log value in EF-5 growth after treatment with phage cocktail, lysin, vancomycin and vancomcyin plus lysin starting from time zero to 35 min.

Discussion

One of the common causes of UTI is *E. faecalis* with an increasing rate of cases. It has an intrinsic resistance to a lot of antimicrobials and has the ability to develop resistance to a wide range of antibiotics causing many serious infections like UTI [24, 25].

In the present study, *E.faecalis* from urine samples was found to be resistant in about 78%, 58%, 44%, and 33% to Oxacillin, Tobramycin, Gentamycin, and Levofloxacin, respectively. Unlikely, the susceptibility was found to be with Tigecyclin and Inducible- clindamycin. The susceptibility to Tigecyclin among the isolates from urine samples was similar to a previous study done in our laboratory [11]. The antibiotic resistance genes in *E. faecalis* isolated from urine samples are alarming the researchers, since these genes have the ability to transmit among enterococci [25]. The current study found that only 35% of the isolates were resistant to vancomycin which came with some similarity with a study done by Ghalavand, Z. in 2020, who found that *E.faecalis* isolated from urine sample was sensitive to vancomycin [26]. The goal of the current study was to compare the antibacterial influence of vancomycin and the phage alone or together. Therefore, the susceptibility to vancomycin is required to test its antibacterial effect and compare it with the subjects.

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It is quite clear that the intrinsic and the acquired antibiotic resistance in *E. faecalis* have prone to extensive drug resistance [27]. In addition, mutations of the bacterial cell wall-associated polysaccharide or membrane protein increase the chance of resistance to phage therapy [28]. Therefore, the synergistic effect of phage and antibiotic as a combined treatment shows great promise as an effective therapy in comparison to treatment with phage or antibiotic alone [29]. The strategy behind the combined treatment is due to the variations in the mechanisms of action between phage and antibiotics [30- 32].

The isolation of the specific *E. faecalis* lytic phage was previously done in our laboratory. The effectiveness of the phage cocktail and phage endolysin to diminish bacterial growth was also proved [11]. The effectiveness of endolysin to eliminate *E. faecalis* biofilm was significantly quantified [12]. Hence, further analysis was required to test the effect of the combined treatment with an antibiotic on this uropathogen.

The results estimated that treating *E. faecalis* with a phage cocktail had shown an advantage over a single phage therapy. The infectivity rates were found to be 100%, 76.4%, and 88.2% for BP-EF1, BP-EF2 and BP-EF3, respectively. Though, mixing the three bacteriophages together yields 100% rate of infectivity and can lyse 91.6 % of the tested *E. faecalis*.

The quantitative analysis for the growth reduction level was also significantly reduced when using a phage cocktail than phage BP-EF2 alone or vancomycin alone. In which, four *E.faecalis* were completely defeated by the cocktail therapy.

In contrast, a mixture of vancomcyin and phage cocktail had the power to reduce the growth level to zero in 11 *E.faecalis*. These may be attributed to the mechanism of action of vancomcyin together with the phage cocktail in reducing the growth rate.

Many studies suggest the use of phages as an adjuvant with the antibiotic as an effective therapeutic strategy for treating infectious diseases caused by multidrug-resistant bacteria [33, 34]. Moreover, a synergistic effect was demonstrated when combining bacteriophage lysine with different antibiotics. Bacteriophage lysins or endolysins are hydrolytic enzymes produced by bacteriophages during the final stage of the lytic cycle to allow cleavage through the host's cell and release of the phage's new progeny after multiplication inside the bacteria [35].

In the current study a considerable reduction in EF-4 growth by 3 logs and 5 logs when treated with vancomycin and a single phage, respectively, was detected. However, treatment with phage cocktail or phage cocktail plus vancomycin reduces the growth to a non-detectable level. The same bacteria were treated by different time intervals and the growth was minimized to an undetected level after 30 min treatment with vancomycin or phage cocktail alone. In comparison, 25 min was required to kill the bacteria after lysine treatment and 20 min was enough when lysine was combined with vancomycin.

The restrictions in this study were the need to increase the isolates number to be tested. Further, *in vivo* investigations are required to verify the validity of the antibiotic-phage combination.

It is very clear that the urge to develop a new therapeutic approach to overcome the rapid development of antibiotic resistant strains among *E.faecalis* has become crucial. Thus, many studies are still in need to battle the rapid bacterial transformation.

Conclusion

E. faecalis is known to cause many serious infections and is increasingly producing strains resistant to antibiotics, especially vancomycin. Lytic phages and their endolysins have the potential to affect and lyse certain bacterial cells, making them a potential alternative therapy for antibiotic-resistant strains. An antibiotic resistance profile of 17 *E. faecalis* isolated from urine samples was

established, focusing on susceptibility to vancomycin. Lytic phages were isolated from the local environment. Three lytic phages were identified with infection rates of 100%, 76.4%, and 88.2%, respectively. The synergistic effect of vancomycin with the phage cocktail significantly reduced the growth of 70.6% of the isolates. The combined effects of phage/endolysin and vancomycin have a significant reduction effect on the bacterial growth in a time dependent manner. Interestingly, the combination treatment produced a significant synergy in reducing the growth of *E. faecalis* compared with either treatment alone. Therefore, the synergistic effect could facilitate the future development of the treatment of antibiotic-resistant *E. faecalis*.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: The project was approved by the local ethical committee at University of Baghdad, No. 134 in the 12th of May 2021.

Authors' Contribution Statement

All the authors were contributed in establishing the current study as follows:
Research design: R R. H, Z O H, A A. A and M N. J. Collecting of the samples: Z O H and M N. J
Laboratory works, data analysis: A A. A. Writing and editing of the manuscript: R R. H

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تقديرالتأثير التآزري خارج الجسم الحي ل اندوليسين العاثيات مع الفانكومايسين على العزلات المحلية العراقية ل *Enterococcus faecalis*

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الخلاصة

من المعروف أن المكورات المعوية البرازية تسبب العديد من الالتهابات الخطيرة مع طفرة في تطور سلالات مقاومة للمضادات الحيوية ، وخاصة إلى الفانكومايسين. العاثيات البكتيرية وأنزيمات الإندوليسين الخاصة بها لديها القدرة على التأثير على خلايا بكتيرية معينة وتحليلها مما يجعلها علاجا بديلا ممكنا للسلالات المقاومة للمضادات الحيوية. هدفت الدراسة على تقديرالتأثير التآزري للعاثية / خليط العاثية / الإندوليسين مع فانكومايسين على المكورات المعوية البرازية المعزولة من عينات البول.تم إجراء كشف كامل لنوع المقاومة للمضادات الحيوية ل17 من المكورات المعوية البرازية مع التأكيد على الحساسية للفانكومايسين. تم عزل العاثيات البكتيرية من البيئة المحلية. تم حساب العدوى ومعدل التغطية للعاثية وخليط العاثية. تم عزل إنزيم العاثية البكتيرية بواسطة الكروماتوغرافيا. تم اختبار التأثير التآزري بواسطة اختبار الحد من التعكر لتقييم مستوى الحد من النمو.وجد أن 6/17 من عزلات البراز كانت مقاومة للفانكومايسين ، و 7/17 عزلات كانت متوسطة المقاومة ، و 4/17 كانت حساسة للفانكومايسين. تم تحديد ثلاث عاثيات بكتيرية بمعدل عدوى 100٪ و 76.4٪ و 88.2٪. وأسفر كوكتيل العاثيات عن معدل 100٪ من العدوى و 91.6٪ من معدل التغطية. كان الانخفاض في مستوى النمو للمكورات البرازية بعد العلاج بخليط العاثية / العاثية / الفانكومايسين / خليط من الفانكومايسين وخليط العاثية فعالا. تأثير التآزر من فانكومسيسين جنبا إلى جنب مع خليط العاثية خفضت بشكل كبير نمو 70.6 ٪ من العزلات. تم الكشف عن النشاط المضاد للبكتيريا من فانكومايسين بالإضافة إلى علاج الاندوليسين بطريقة تعتمد على الوقت. أوضح التأثير التآزري للفانكومايسين مع العاثية / الإندولويسين طريقة فعالة لعلاج عزلات المكورات البرازية المقاومة للمضادات الحيوية للتغلب على هذه المشكلة السريرية الناشئة.

الكلمات المفتاحية: العاثيات البكتيرية، الإندوليسين، البكتيريا متعددة المقاومة للأدوية، العلاج بالعاثيات، المكورات المعوية البرازية.