Research Article

Efficacy of Bacteriophage-Antibiotic Combinations against *Staphylococcus aureus* Infections: *In vitro* Study

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ABSTRACT

The aim of the study is to evaluate the benefit(s) of bacteriophage activity as antibacterial agent alone and in combination(s) with antibiotic(s) against Multidrug Resistant (MDR) *S. aureus*. Fifty eight samples were collected from patients with multiple types of infections attainted to The Medical City of Al-imamain Al-khadimain (peace being upon them) from February 2014 to April 2014. Combinations of phage with ¼ and ½ MIC of gentamicin, ¼ and ½ MIC of vancomycin and ½ and 1 MIC of tetracycline for isolate 4 and combinations of phage with ½ MIC of gentamicin, ½ MIC of vancomycin and ½ and 1 MIC of tetracycline for isolate showed synergistic effects against *S. aureus* isolates, while combinations of phage with ¼ MIC of gentamicin and ¼ MIC of vancomycin for isolate 7 showed indifference effects. Almost all phage-antibiotic combinations resulted in synergism and only two combinations showed indifference effects. It is recommended to study the synergistic effect of antibiotic-combinations and phage-antibiotic combinations *in vivo* using suitable laboratory animals.

Keywords: *Staphylococcus aureus*, phage, MIC and MOI.

INTRODUCTION

Bacterial infections that prove incurable by antibiotics are a serious clinical problem due to the increasing prevalence of antibiotic- resistant bacteria, mainly resulting from the extensive use of antibiotics[1, 2]. Bacteriophages are bacterial viruses that have genetic material in the form of either RNA or DNA, encapsidated by a protein coat[3]. This capsid is attached to a tail which has fibers that used for attachments to receptors present the on bacterial cell[4]. Phages infect bacteria through lytic life cycle and/or lysogenic life cycle[5]. With the increasing of the incidence of antibiotic resistance and the decreasing introduction of new antibiotics, attention has returned to developing phage as a therapeutic antimicrobial agent that may be used in humans, animals and plants[6]. The acceptance of phage therapy is limited by some factors such as the unknown pharmacodynamics of a replicating agent, as well as the potential for the evolution of resistant bacteria. Therefore combination of phages with antibiotics; Co-administration of phage and antibiotics could increase the phage efficacy by stimulating increased production of phage as in *E. coli* and *Salmonella enterica* or induction of lysogenic phage as in *S. aureus*[7, 8]. Phages have potential advantages over antibiotics including:

1. The specificity of phages for target bacteria that decreases the damage to normal flora of the host. The target bacteria must be identified first or a cocktail of phages should be used. Bacteriophages are self-limiting i.e. they require constantly growing hosts[3].

2. Replication of phages at the site of infection[9].

3. They are safer with no or less adverse effects than antibiotics[9].

4. Phages modify themselves naturally to infect the resistant bacteria, hence reducing the chances of bacterial escape, which scores other advantage of phage therapy over antibiotics[9].

MATERIALS AND METHODS

Fifty eight isolates of *Staphylococcus aureus* were collected from patients with multiple types of infections attainted to Medical City of Al-imamain Al-khadi main (peace being upon them) from February 2014 to April 2014. Collected samples were cultured on manitol salt agar (MSA) for identification in which MSA is a selective agar used for the selective isolation and identification of *S. aureus* by mannitol fermentation then incubated overnight for 18 hours at 37°C. Thirty two of these isolates were *S. aureus*. Identification of *S. aureus* was also done by microscopic examination, biochemical tests like catalase test and coagulase test. VITIK2 compact system is used for identification and sensitivity tests and measurement of minimum inhibitory concentrations in which a sufficient number of colonies of a pure culture are transferred using sterile swab or applicator stick to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted to (0.5-0.63) MacFarland turbidity standard range and measured using a turbidity meter called (DensiChek™)[10]. Phage was obtained from mixture of swage, faeces and others. Two isolates (isolates 4 and 7) were tested for the combination with their specific phages.

Table 1: The summery of the collected *S. aureus* isolates and their sources

Bacteria	No. of isolates	Source
Staphylococcus aureus	17	Blood
	8	Urine
	1	Ear swab
	3	Vaginal swab
	1	Eye swab
	1	Sputum
	1	Abscess swab
Staphylococcus lentus	5	Urine
	3	Swab
	3	Blood
Staphylococcus haemolyticus	2	Urine
	1	Blood
Staphylococcus hominis	2	Blood
Staphylococcus lugdunensis	3	Blood
	3	Swab
	1	Urine
Aerococcusviridans	1	Blood
Alloiococcus otitis	1	Blood
Staphylococcus warneri	1	Swab

Table 2: Antibiotics used to perform the study listed in the following table

Antibiotics	Company
Vancomycin 500mg vial	Julphar (U.A.E.)
Gentamycin 80 mg/2ml vial	Normon (Spain)
Tetracycline capsule 250mg	S.D.I. (Iraq)

The solutions of antibiotics used in this study were prepared as follows:

Vancomycin solution

Stock solution was prepared by dissolving 25 mg of reconstituted powder of vancomycin in 25ml sterilized distilled water and stored in 4°C until used. Then the stock solution was diluted a number of times using sterile diluent normal saline (0.9% NaCl was used) according to equation ($C_1V_1=C_2V_2$) until the target concentration was achieved.

Gentamicin solution

Stock solution was prepared by mixing 1ml of (40mg/ml) gentamicin in 9ml of sterilized distilled water and stored in 4°C until used. Then the stock solution was diluted a number of times using sterile diluent normal saline (0.9% NaClwas used) according to equation ($C_1V_1=C_2V_2$) until the target concentration was achieved.

Tetracycline solution

Stock solution was prepared by dissolving 250 mg of tetracycline capsule in 125 ml sterilized distilled water and stored in 4°C until used. Then the stock solution was diluted a number of times using sterile diluent normal saline (0.9% NaCl was used) according to equation ($C_1V_1=C_2V_2$) until the target concentration was achieved.

The Multiplicity of Infection (MOI) is the average number of phages per bacterium. The MOI is determined by dividing the number of phages added (ml added x PFU/ml) by the number of bacteria added (ml added x cells/ml). The average MOI in the population could be 0.1, 1, 2, 10, etc, depending upon how you set up the experiment. Although the MOI gives an idea about the average number of phages per bacterium, the actual number of phages that infect any given bacterial cell is a statistical function[11].

RESULTS AND DISCUSSION

Among the fifty eight isolates, thirty two isolates were *S. aureus* which obtained from different sources including: Blood (17 isolates), urine (8 isolates), vaginal swab (3 isolates) and only one isolate from each of eye swab, abscess, ear swab and sputum. Upon identification of *S. aureus*, these isolates were gram-positive cocci, mannitol fermenter (grow on mannitol salt agar producing yellow golden colonies with yellow zones around them), catalase and coagulase positive.

Phage-antibiotic combinations

Combinations of phage with each of gentamicin, vancomycin and tetracycline were done for the two selected isolates (4 and 7).

In the current study, the phage showed antibacterial activity and when it was combined with ½ MIC of antibiotics including gentamicin, vancomycin and tetracycline showed synergistic action against the MDR *S. aureus* isolates for all results except for phage-¼ MIC gentamicin and phage-¼ MIC vancomycin combinations for S.A. 7 isolate which showed indifference action.

Kirby[12] found that combined therapy with phage and antibiotic (gentamicin) is a way to control the impact of resistance, to both phage and antibiotics which resulted in more bacterial killing with no detectable phage or antibiotic resistance.

Table 3: The results of VITEK2 GP susceptibility tests and MIC tests of *S. aureus* isolates 4 and 7

S. aureus isolate	Sample Type	*Antibiotics													
		CX	VAN	BP	OC	MOX	GEN	ERY	TOB	LEV	NIT	RIF	TC	SXT	CLM
S.A. 4	Blood	+	≤0.5 S	≥0.5 R	≥4 R	≤0.25 S	≤0.5 S	≤0.25 S	≤ 1 S	≤0.12 S	≤16 S	≤0.5 S	≥16 R	80 R	≤0.25 S
S.A. 7	Blood	+	≤0.5 S	≥0.5 R	≥4 R	≤0.25 S	≤0.5 S	≤0.25 S	≤1 S	≤0.12 S	≤16 S	≤0.5 S	≥16 R	80 R	≤0.25 S

***:** CX= Cefoxitin, VAN= Vancomycin, GEN= Gentamicin, BP= Benzylpenicillin, OC= Oxacillin, NIT= Nitrofurantoin, TOB= Tobramycin, MOX= Moxifloxacillin, LEV= Levofloxacin, ERY= Erythromycin, SXT= Trimethoprim/Sulfamethoxazole, RIF= Rifampicin, TC= Tetracycline and CLM= Clindamycin. R= resistant and S= sensitive.

Table 4: The effects of 1 MOI *S. aureus* phage only

S. aureus isolate	1 MOI *Φ only
S.A. 4	**-ve
S.A. 7	-ve

*: Φ = Phage. **: –ve means no growth.

Table 5: The effect of combinations of 1 MOI *S. aureus* phage with each of ¼ and ½ MIC of gentamicin

S. aureus isolate	1 MOI *Φ-¼ MIC GEN	1 MOI Φ-½ MIC *GEN
S.A. 4	**-ve	-ve
S.A. 7	***+ve	-ve

*: Φ = Phage and GEN=Gentamicin. **: –ve means no growth.***: +ve means growth.

Table 6: The effect of combinations of 1 MOI *S. aureus* phage with each of ¼ and ½ MIC of vancomycin

S. aureus isolate	1 MOI *Φ-¼ MIC VAN	1 MOI Φ-½ MIC *VAN
SA 4	**-ve	-ve
SA 7	***+ve	-ve

*: Φ = Phage and VAN=Vancomycin. **: –ve means no growth. ***: +ve means growth.

Table 7: The effect of combinations of 1 MOI *S. aureus* phagewith ½ and 1 MIC of tetracycline

S. aureus isolate	1 MOI *Φ-½ MIC TC	1 MOI Φ-1 MIC *TC
SA 4	**-ve	-ve
SA 7	-ve	-ve

*: Φ = Phage and TC= Tetracycline. **: –ve means no growth.

1 MIC of tetracycline was also showed synergistic effect when combined with 1 MOI of phage in which the tested isolates were completely killed when phage added producing irreversible effect, while the action of tetracycline alone is usually inhibitory and reversible upon withdrawal of the drug[13].

The synergistic effect between phage-tetracycline was in agreement with study done by Ghareeb[14] in Baghdad in which phage-antibiotic combination therapy increases the sensitivity of *S. aureus* strains to the antibiotic to which the strains were resistant and the sensitive antibiotics also by increasing the diameter of inhibition zones which formed due to the effect of antibiotic disks[15] in which after treatment the bacteria with phage, the results showed increasing of susceptibility toward all used antibiotics and this may be referred to the incorporation of genome of this phage inside the DNA of *S. aureus* and cause inhibition to the genes that responsible for antibiotic resistant so that its became more sensitive to antibiotic[16].

Phage therapy can act as a stand-alone therapy for infections caused by fully resistant strains and able to be used as a co-therapy with antibiotics for infections that are still susceptible to some antibiotics by preventing the development of bacterial mutants against either agent[17].

The phage effect may be attributed to direct bacteriolysis or associated with vaccine-like immune activation by spread bacterial components and to the incorporation of genome of the phage inside the DNA of *S. aureus* and cause inhibition to the genes that responsible for antibiotic resistant. The synergistic action of antibiotic and bacteriophage treatment is likely dependent on the specific combination of antibiotics, bacteriophages and bacterial isolates[18-20].

Bacteria are most likely to form cellular changes and modifications in the molecules that the phage targets such as a cell surface glycoprotein which is a bacterial receptor. In response to this modification, phages will progress in a way that counteracts this change, thus allowing them to continue targeting bacteria and causing cell lysis. As a consequence phage therapy is devoid of problems or disadvantages similar to antibiotic resistance and much of the evidence shows that appropriately administered phage therapy is very effective for treatment and prevention of many types of bacterial infectious diseases, especially those caused by MDR bacteria[21].

CONCLUSION

Most of phage-antibiotic combinations with gentamicin, vancomycin and tetracycline resulted in synergism.

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