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Clinical Investigation of The Bacteriophage Cocktail Efficacy on Acne Vulgaris Caused by Multi-Drug Resistance *Staphylococcus aureus*

Haneen E. Hussain[1] *PhD*, **Ahmed S. Abdulamir**[2] *PhD*, **Iqbal G. Farhood**[3] *FICMS, D&V,* **Ahmed R. Abu Raghif**[4] *PhD*, **Mohammed R. Ali**[2] *PhD*

[1]Dept. of Microbiology, Al-Forat General Hospital, Baghdad, Iraq, [2]Dept. of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [3]Dept. of Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [4]Dept. of Pharmacology, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background Acne vulgaris is a chronic skin disease caused by many factors includes: hormonal disturbance, increase sebum production and inflammatory skin due to multi-drug resistant (MDR) bacteria *Staphylococcus aureus* (*S. aureus*). So, there is an urgent need for bacteriophages (Elixir of life) as an alternative strategy to reduce the overuse of antibiotics and combat MDR bacterial pathogens.

Objective To develop a bacteriophage cocktail specific for MDR *S. aureus* bacteria and investigates its efficacy via *in vitro* validation tests and in vivo topical applied on human skin acne lesions caused by MDR bacterial infections.

Methods Twenty-three bacteriological isolates of *S. aureus* were collected from patients at 20-40 years old with acne lesions that admitted to Al-Yarmouk Teaching Hospital, Baghdad, Iraq. Then, identified and susceptibility tests performed via VITEK2 system. Bacteriophages were isolated from sewage water, then bacteriophages cocktail prepared and determined their lytic activity via in vitro validation tests and investigate their clinical efficacy on the skin of human with acne lesions caused by MDR *S. aureus* bacteria.

Results The bacteriophage cocktail therapy with high antibacterial lytic activity via *in vitro* validation tests proved excellent effectiveness in treating patients from MDR *S. aureus* acne vulgaris via clinical improvement in size, number, inflammatory signs of acne lesions, and decolonization of MDR *S. aureus* bacteria in treated lesions as compared with untreated, control lesions.

Conclusion Bacteriophage cocktail specific for *S. aureus* bacteria can be prepared in laboratory with simple measures, and safe without any side effect on human patients whom topically applied it.

Keywords Bacteriophage, cocktail, MDR, *S. aureus* bacteria, clinical investigate

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List of abbreviations: AST = Antimicrobial susceptibility test, CFU = Colony forming unit, MDR = Multi-drug resistance, MIC = Minimum inhibitory concentration, MRSA = Methicillin resistant Staphylococcus aureus, OD = Optical density, PFU = Plaque forming unit, Staphylococcus aureus = S. aureus

Introduction

Acne is considered as one of the most common skin diseases that affect adolescents and young adults in the world [(1)]. The manifestations of acne can be severing, painful and may cause scarring and

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disfiguration, in some patients, may reduce affect mental health and self-esteem [2].

Acne vulgaris is a disease of the pilosebaceous unit, which is a complex mini-organ of the body that shows considerable microbiological, morphological, and metabolic diversity depending on the affected site. The sebaceous gland usually actively responds to fluctuations in environmental, hormonal and immunological changes [3]. The development of acne is a multifactorial process including both exogenous and endogenous factors including excessive sebum secretion, colonization of skin bacteria, follicular hyper keratinization, and inflammation [4].

The skin acts as a reservoir for commensal normal flora (microbiota), which provides a first-line defense of physical barriers to avoiding the invasion of foreign pathogens [5]. The modulating in the skin ecosystem due to human growth and development process in terms of psychosocial and biological aspects can contribute to causing a high frequency of infectious diseases. Although microbiota protects the human host, they have also been implicated in the pathogenesis of several skin diseases [6].

Studies implicated *Staphylococcus aureus* (*S. aureus*) and *Propionibacterium* (*Cutibacterium acnes*) bacteria in the progression of inflammatory acne vulgaris, the life cycle of pathogenic bacteria is mediated by virulence genes encoding virulence factors which includes toxins, adhesions, invasions, or other factors, present in the pathogenic microorganisms. These products are involved in the pathological damage to the host by promoting interaction between the organism and host, then degrading and damaging the host tissues [7]. For instance, hyaluronate lyse enzyme, Christie-Atkins-Munch-Petersen factor (CAMP), neuraminidases, lipases [8], fibronectin binding protein (FnBp)-A, FnBP-B, adhesins, lipases, proteases, and hyaluronidases are the virulence factors for these bacteria [9].

The main problem with antibiotics therapy of acne is the common tendency to overuse and prolonged use of them. The inflammatory forms of acne may result in permanent disfigurement because they leave scars thus appropriate and correct use of antibiotics in the therapy of acne will help to preserve their utility in the face of increasing antibiotic resistance, and therefore greater awareness of this is required by the doctors. So, it is necessary to treat acne with effective alternatives to antibiotics to reduce the possibility of resistance to this type of therapy and obtain a highly specific agent to kill bacteria effectively [10].

Phage therapy is an innovative concept of a directed action that occurs normally many billion times every second in every ecosystem; from our digestive tract to the large oceans, where numerous numbers of bacteria are killed by bacteriophages every day [11]. Throughout the last years a significant increase in the quantity of discovered *Staphylococcal* bacteriophages has been reported [12]. The other gain of *Staphylococcal* bacteriophages is their scale of specificity. Application of bacteriophage cocktail with approximately two or three bacteriophages can give brilliant coverage, in opposition to Gram-negative bacteria, where a great number of bacteriophages (even an extra 10) are required. This decreased number of bacteriophages required for good coverage produces anti-staphylococcal bacteriophage cocktails much higher commercially interesting [13]. Bacteriophages were effective against local and systemic infections of *S. aureus* bacteria. Mainly, the bacteriophage also lysed the Methicillin resistant *S. aureus* (MRSA) strain. It was proved that bacteriophage therapy can greatly decrease the inflammation that occurred due to the pathogenicity of *S. aureus* [14].

This study aimed to isolate multi-drug resistance (MDR) bacteria (*S. aureus*) from human acne lesions. Additionally, to isolate and prepare specific lytic bacteriophages cocktail for MDR *S. aureus* bacteria and to determine their activity via in vitro validation

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tests. Also, to evaluate the efficacy of bacteriophages cocktail therapy via in vivo topical application on patients' skin acne lesions caused by MDR *S. aureus* bacteria.

Methods

Setting and participants

Patients

Interventional study was approved by Institutional Review Board at College of Medicine, Al- Nahrain University (IRB no. 204/3/2, date January 18th, 2022). Twenty-three S. *aureus* isolates were obtained from 70 (14 female and 9 male) patients at age (20-40) years old whom attended the Dermatology Consultant Unit of Al-Yarmouk Teaching Hospital, Baghdad, Iraq. These patients were complained from skin lesions on face, lesions diagnosed clinically by dermatologist as acne lesions. The therapy was applied topically on acne lesions of eighteen patients from thirty patients ranging from age 25-35 years with moderate -severe MDR *S. aureus* facial acne lesions after getting their agreements and the ethical approval from the ethical committee.

Inclusion criteria

All volunteers with moderate-severe facial acne vulgaris previously treated for at least three years with multiples regimes (topical and systemic) with no response, caused by bacterial MDR *S. aureus* bacteria, age 25-35 years old and did not take any topical or systemic acne vulgaris treatment at least six months ago.

Exclusion criteria

Include patients with aged under 25 years old, patients who having facial acne vulgaris lesions but with negative bacteriological cultures for MDR *S. aureus* bacteria or had treatment against acne vulgaris within six months ago, and patients with lesions of facial acne but combined with others disease for example eczematous skin, sunburn skin.

Bacterial isolation

The hemolytic activity of *S. aureus* isolates was tested using blood agar plates containing 5% blood. An isolated colony from a nutrient agar plate was inoculated on blood agar and incubated at 37°C for 24 hours under aerobic and anaerobic conditions. The hemolytic zones were characterized as α (partial hemolysis), ß (complete hemolysis), and γ (no hemolysis) depending on the extent of each colony. Diagnosis of the isolated bacteria relied first on conducting a catalase test to distinguish it from *Streptococci*. Then coagulase test was done, positive result confirms the diagnosis of *S. aureus* with Gram staining which shows Gram-positive cocci in clusters and mannitol fermentation on mannitol salt agar (MSA). Hence, the identification of *S. aureus* was confirmed [15]. The minimum inhibitory concentration (MIC) of antibacterial agents against *S. aureus* was evaluated using VITEK2 system (BioMerieux, France) all isolates were screened and recognized via the VITEK-2 system according to the manufacturer's instructions. This is a phenotypic type of diagnosis that depends on biochemical reactions to detect the isolates.

Bacteriophage isolating

Primary bacteriophages were isolated from environmental specimen sewage water from Al-Bayaa Central Station Baghdad City, when mixed with target bacterial isolates. These samples were collected in clean tubes 50 ml enclosed by parafilm, then carried to the lab via ice bag to be processed at the same day. The procedure of isolating and propagating primary bacteriophages was carried out according to the methodology conducted by Hyman in 2019 [16]. Bacterial stocks were prepared by incubation *S. aureus* aerobically for 24 hours in nutrient broth at 37°C. One hundred (100 µl) of each bacterial isolates were mixed together in a sterile 5ml test tube. Then, 2-3 ml of crude samples, which were derived from sewage filtrated by using cellulose filter paper to discard any solid materials were added to the mixture. Then, (2-3 ml) of nutrient broth and (2 ml) of salt magnesium buffer (Tris-Hcl 50 mM, $MgSO_4.7H_2O$ 8 mM, NaCl 100 mM, gelatin 0.01%, pH 7.5) were added to the mixture as

well. Then the mixture was incubated for 24 or 48 hours accordingly to the type of bacteria at 37°C.

After incubation, the mixture was filtrated using a micropore syringe filter (0.20-0.22 µm) and transferred to new Eppendorf tubes and an equal volume of SM buffer was added. Thus, the primary bacteriophage suspension was produced.

Spot lysis assay of bacteriophage

Spot lysis assay was used for screening the virulent bacteriophages on nutrient agar. Spotting of bacteriophages can be used as a first investigation for the ability of phage to lyse certain bacterial isolates. The clear zones formation suggested the presence of virulent lytic bacteriophage. Firstly, the target bacteria were activated in nutrient broth at 37°C aerobically for 24 hours, then 100 µl of the bacterial broth was poured onto nutrient agar plate to make a bacterial lawn by using sterile glass spreader. After 20 minutes the plates should have been dried, 10 µl of primary bacteriophage suspension were spotted by using a mechanical pipette onto the surface of the bacterial lawn and were allowed to dry before incubating at 37°C aerobically for 24 hours. The next day, the zone of lysis at the spot area investigates a lytic and specific primary bacteriophage for the targeted bacteria [17]. Then, specific lytic phages to MDR *S. aureus* were picked up by sterile loop and put into 1 ml of salt magnesium buffer in 1.5 ml sterile Eppendorf tubes with gentle shaking for 5 minutes and stored at 4°C. The supernatant was called transient bacteriophage stock suspension.

The coverage rate of the bacteriophage was measured using the following formula:

Coverage rate = Number of bacteria lysed by bacteriophages/ Total number of bacteria x 100% [18].

Top layer agar plaque assay

Serial dilutions of the primary bacteriophage preparation were mixed with a targeted host bacterium and dispersed evenly onto solid medium. Ten-fold serial dilutions (10^1-10^{10}) were made with salt magnesium buffer for the bacteriophage stock solutions by taking 100 µl from solution of the bacteriophage into 900 µl of salt magnesium buffer. One hundred (100) µl of each dilution were transferred for each bacteriophage stock solution into 10 ml volume sterile container containing 1000 µl of 10^7 colony forming unite (CFU) /ml cultures of targeted bacteria. After 10 minutes of incubation at 37°C, 2.5 ml of top layer agar (semi-solid agar or soft agar of concentration 0.80% cooled at 45°C) was added. Immediately, the resulting mixture was poured over plates of nutrient agar. For each dilution, this step was repeated. The semi-solid agar overlay allowed it to be solidified. Then, the plates were incubated aerobically at 37°C for 24 hours for *S. aureus*. After incubation, plaques were counted depending on the best dilution that yields the best rational number of plaques with clear margins and without merging with each other. The plaques were calculated to determine the titer of bacteriophage in stock. Top layer plaque assay is the best assay used for getting highest titers of bacteriophage suspensions [19].

The titer of bacteriophage was calculated by using the following formula (plaque forming unite (PFU)/ml):

Bacteriophage titer = number of plaques per plate × 100 × dilution factor [20].

Purification assay of bacteriophages

From the previous top layer plaque assay, phages were selected based on their; potential lytic activity, the coverage rate of infectivity on the total number of bacterial strains, the diameter of plaques and clarity of lysis. The aggressive bacteriophages were selected to be purified as follows [21]:

1. The soft layer of the selected plaques was penetrated by a sterile pipette tip to obtain a well isolated plaque.
2. The sterile pipette tip was drowned in 100 µl of salt magnesium buffer and mixed gently.
3. By streaking method, the nutrient agar plate was streaked by a sterile loop from the previous suspension and left to dry for 15-20 minutes.

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4. From bacterial growth, a 4.1 ml was added to 3 ml of soft agar (0.8%) and mixed softly.
5. From the C aspect of streaking, the mixture was poured onto the streaked nutrient agar plate to prevent phage redistribution, overlapping, and receive optimal single phage plaque with maintaining the phage dilution by ABC streaking design.
6. The procedure was repeated till receiving the same morphology of plaques, and then the suspensions of purified bacteriophages were stored at 4°C.

Characterization, optimization, and preparation of the bacteriophage cocktail

To acquire the better virulence criteria of the isolated lytic phages, the best plaques (in term of all parameters) should be selected: shape, diameter (mm), depth, margin cut, and turbidity or clarity of the plaque. Since each 1-2 cm^2 of skin acne lesion with approximately (10^6-10^7 CFU/swab) of bacteria, thus the optimum bacteriophage cocktail therapy was prepared by mixing each bacteriophage at titer $1x10^8$ accordingly to the multiplicity of infection (MOI) (10:100) virus to bacterial cell ratio and the kinetic of bacteriophage as follows:

Serial dilution of bacterial growth performed to detect confluent growth (10^6-10^7) CFU/ml, serial dilution of suspension phage cocktail (10^7-10^{10}) PFU/ml, then determined the ratio of multiplicity of infection MOI which give 100% infective rate of bacteriophages cocktail to their specific bacterial cells.

Measurement of the in *vitro* activity of bacteriophage cocktail

The *in vitro* lytic activity for bacteriophage cocktail was measured by antibacterial activity assay which was done by taking the titer of bacterial broth in mid exponential phase (optical density at wave length = 600 nm) of 0.6 and counted using decimal serial dilution then 10 µl were spotted of each dilution on the nutrient agar and inclined then incubated 24 hours aerobically, after incubation, the dilution plate that showed separated countable colonies was used to calculate the bacterial titer using the following equation:

Bacterial titer (CFU/ml) = No. of colonies per plate × dilution factor/ volume of culture plate

At the same time of measuring the titer of the bacterial broth, 100 µl from the same bacterial broth in mid exponential phase (OD600) of 0.6 was incubated for 24 hours with 100 µl phage cocktail.

The titer of bacterial broth with phage cocktail was calculated using serial dilution and culture on the nutrient agar and incubated for 24 hours aerobically, appropriate dilutions of cell suspensions were plated on nutrient agar after incubation; the dilution plate that showed separated countable colonies was used to calculate the titter using the previous equation. The decrease in the titer of the bacterial broth with the bacteriophage cocktail compared to the same broth without any additions was calculated. The antibacterial activity was quantified as the relative inactivation level in log units.

Clinical investigation of the in vivo topical applied bacteriophage cocktail on the MDR bacterial infections acne vulgaris:

A- Ethical approval

Getting the ethical approval from the ethical committee and with agreements of volunteers at age 25-35 years old with moderate to severe facial acne lesions (more than 20 commedones and more than 15 inflammatory lesions) according to the clinical dermatologist who confirmed that these acne lesions [(22)], the facial acne skin swabs were taken and cultured for the growth of *S. aureus* and propagation, The isolated *S. aureus* were diagnosed and subjected to antibiotic susceptibility test. Bacteriophage cocktail was applied to the bacterial lawn by spotting to see if it is positive in spot lysis assay before and after topical treatment with bacteriophage cocktail to evaluate the effect of the candidate therapy.

B- Pre-therapy application

Firstly, skin swabs from facial acne lesions of each volunteer were cultured to determine the type and the susceptibility of MDR bacterial

infection, then the safety was examined by applying the type of bacteriophage cocktail topical therapy on intact skin to check for immediate allergic reactions. Though, an area of redness, swelling and itching may develop (15-20 minutes) after the test, then applied topically on the acne lesions and checks if there is any side effect such as irritation, erythema, photosensitivity, dryness, which remain for a couple of days, if not tolerated should use emollients.

C- Therapy application

Bacteriophage cocktail candidate therapy was applied topically on one or more patches 2 cm^2 of facial lesions caused by MDR bacterial infection *S. aureus* bacteria either as a purified bacteriophage cocktail aqueous solution via impregnated gauze for 30 minutes, three times daily and for one week. While another patch also contains MDR bacteria *S. aureus* facial acne lesions left as a control without any therapy.

D- Post-therapy application

After one week of topical therapy applied, patch with facial acne lesions treated and patch with acne lesions untreated (controlled lesion) were examined for investigating the topical therapy effectiveness on the symptoms of moderate facial acne lesions caused by MDR bacterial infection. Antibacterial activity effect of therapy concentrating on the decolonized bacterial growth either as a negative bacteriological culture or reduction in the titer (log number) of MDR bacteria of the treated patch through re-culture of acne skin swab bacteria pre and post topical treatment.

The efficiency of the topical therapy was pursued under the supervision of the treating clinical dermatologist and depended on the clinical improvement response of the treated patch either a complete response, partial response, or no response through determining the decreased in size, number of acne lesions, and decreased of inflammatory symptoms of facial skin with acne vulgaris caused by MDR *S. aureus* bacteria as compared with control untreated patch.

Results

Bacterial isolation and identification

Identification of *S. aureus*

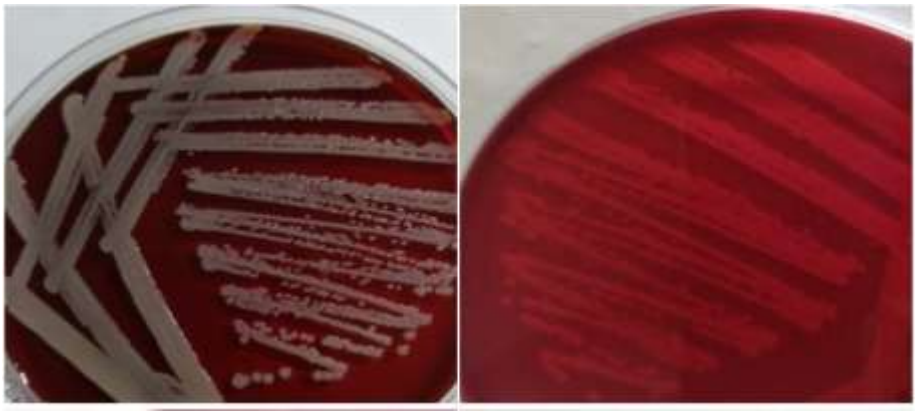
Twenty-three bacterial isolates of *S. aureus* were collected from 70 (14 females from a total of 46 females, and 9 males from a total of 24 males) patients at age more than 20 years old with MDR bacterial acne lesions, then culture on blood agar and mannitol salt agar MSA for the preliminary identification. Identification of *S. aureus* isolates was confirmed by VITEK2 system. S. aureus colonies typically appeared a golden yellow color on MSA, and yellow- gray colonies with β-hemolysis on blood agar as shown in figure (1; A,B,C).

Twenty-one from all *S. aureus* isolates showed yellow-grayish colonies with clear zone β-hemolysis at 37°C under aerobic or anaerobic conditions, but two isolates of *S. aureus* showed yellow- grayish colonies with incomplete hemolytic phenotype (SIHP) at 37°C under anaerobic condition as shown in figure (2).

Diagnosis of the isolated bacteria relied also on conducting catalase test to distinguish it from Streptococci, then coagulase test was done and positive result was confirmed by VITEK2 system.

A. Mannitol Salt agar **B. Blood agar/ colonies** **C. Blood agar/ hemolysis**

Figure 1. *S. aureus* colonies morphology on different media. A: *S. aureus* ferment mannitol and turns the medium yellow. B: *S. aureus* yellow grayish colonies on blood agar. C: *S. aureus* surrounded by clear zone of beta-hemolysis



A. Blood agar/ *S. aureus* colonies with complete hemolysis

B. Blood agar/ *S. aureus* colonies with incomplete hemolysis

Figure 2. Different hemolysis phenotypes of S. aureus on blood agar under anaerobic condition. A: *S. aureus* colonies on blood agar surrounded by complete hemolysis zone. B: *S. aureus* colonies on blood agar surrounded by incomplete hemolysis zone (SIHP)

Antibiotic susceptibility test for *S. aureus*

VITEK2 antibiotics susceptibility system was used to cover MDR *S. aureus* definition based on the Clinical and Laboratory Standards Institute (CLSI) 2022 criteria, isolates that were resistant to three or more antibiotics classes were designated as MDR bacteria.

According to the antibiotic susceptibility test results, all *S. aureus* isolates were identified as sensitive against Vancomycin, table (1) shows the resistance profiles of all *S. aureus* isolates.

A high antibiotic resistance percentage was observed for Oxacillin (100%), Ciprofloxacin (95%), Piperacillin/Tazobactam (91.3%), Gentamicin (69.6%). While moderate resistance to Clindamycin (56.5%), Rifampicin 52.2%, and Erythromycin (49.1%).

In contrast, minimum resistance rates were determined against Tetracycline (26%), Chloramphenicol (21.7%), and Trimethoprim/Sulfamethoxazole (21.7%).

Table 1. Antibiotic susceptibility percentage of *S. aureus*

Antimicrobial class	Antimicrobial agent	*S. aureus* isolates	Mean percentage of resistance
Anti-Staphylococcal- β-lactams/Cephamycin	Oxacillin/Cefoxitin Screen	23	100
Fluoroquinolones	Ciprofloxacin	22	95.7
Penicillin	Piperacillin/Tazobactam	21	91.3
Aminoglycosides	Gentamicin	16	69.6
Lincosamide	Clindamycin	13	56.5
Annamycin	Rifampicin	12	52.2
Macrolides	Erythromycin	11	49.1
Tetracyclines	Tetracyclin	6	26
Phenicol	Chloramphenicol	5	21.7
Folate-pathway inhibitors	Trimethoprim/Sulfamethaxazole	5	21.7
Glycopeptides	Vancomycin	0	0

***S. aureus* Bacteriophages Characteristics**

In this study, three lytic specific phages active against *S. aureus* were isolated and purified. All of the isolated phages formed visible plaques in the early stage when tested on the bacterial lawn of specific MDR *S. aureus*. They were isolated directly from sewage water by showing lysis on bacterial lawns of MDR *S. aureus* as shown in figure (3).

Characteristics of these phages were determined by the diameter, clarity/turbidity, margin cut, and shape of their plaques. The size of Staphylococcus aureus phages ranged between (1.7-3.6 mm) with a mean of (2.73 mm). The plaques morphology of the three S. aureus phages ranged between oval 1/3 (33.33%) and circular 2/3 (66.66 %). The clarity ranged between 2/3 clear, 1/3 semi-clear for the plaques of the three S. aureus phages as shown in table (2).

The Titer of the specific bacteriophages to MDR S. aureus bacteria

High titers of specific and lytic bacteriophages isolated and optimized to the studied bacterial isolated were obtained by using top layer plaque assay which was used to further screen, amplify and measure phages as shown in figure (4).

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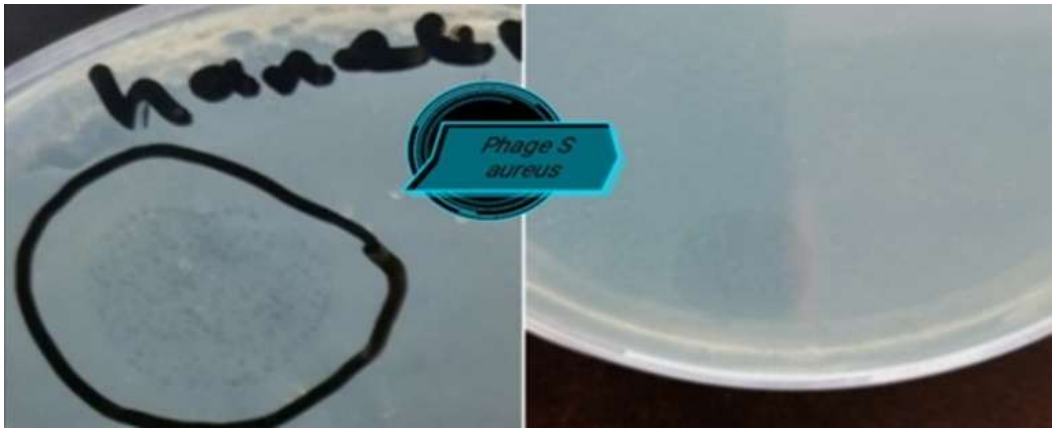

Figure 3. Phage spot lysis assay of bacteriophage to *S. aureus*

Table 2. Characteristics of bacteriophages plaques (Bp- Sa1, Bp- Sa2, Bp- Sa3)

Bacteriophage isolated	Size of plaques	Clarity of plaques	Shape of plaques
Bp- Sa1	1.7 mm	Clear	Oval
Bp- Sa 2	3.6 mm	Semi- clear	Circular
Bp- Sa 3	3.0 mm	Clear	Circular

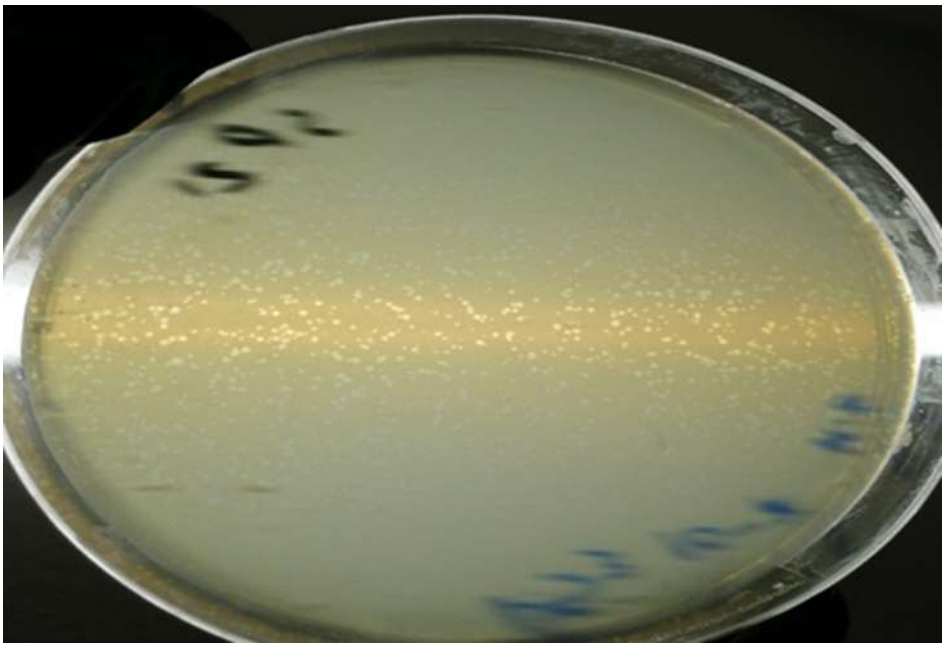

Figure 4. Top layer plaque assay of bacteriophage

Table 3. The titer of specific and lytic phages to MDR S. aureus by using top layer plaque assay

Bacteriophage isolate	Titer (PFU/ ml)	Bacteriophage isolate	Titer (PFU/ ml)
Bp- Sa 1	2.8 x1010	Bp- Sa 1	2.8 x1010
Bp- Sa 2	1.6 x109	Bp- Sa 2	1.6 x109
Bp- Sa 3	2.2 x1011	Bp- Sa 3	2.2 x1011

The coverage rate of bacteriophage

The bacteriophage isolates were highly lytic and produced an obvious clear zone on targeted bacteria. The three different bacteriophages of *S. aureus* were obtained with an approximate coverage rate of infectivity. The bacteriophages Bp-Sa1, and Bp-Sa3 displayed coverage rates as 91.6% of MDR *S. aureus* isolates, and Bp-Sa2 showed 83% coverage rate of 12 MDR *S. aureus* isolates as shown in table (4).

MDR *S. aureus* 8 isolates showed resistance to bacteriophages (BP-Sa1 and BP-Sa2) while MDR *S. aureus* 1 isolate showed resistance to (BP-Sa2), and MDR *S. aureus* 7 isolates showed resistance to (BP-Sa3).

Table 4. Coverage rate of bacteriophages against MDR *S. aureus*

Bacteriophage	Infected MDR bacteria (n=12 isolates *S. aureus*)	Coverage rate (%)	Bacteriophage
Bp-Sa1	11/ 12	91.6%	Bp-Sa1
Bp-Sa2	10/ 12	83.3%	Bp-Sa2
Bp-Sa3	11/ 12	91.6%	Bp-Sa3

***In vitro* measurement of the activity of bacteriophage cocktail on 12 MDR *S. aureus* isolates**

The results showed that the three different bacteriophages for *S. aureus* were obtained with an approximate coverage rate of infectivity, when mixing the three bacteriophages of *S. aureus* at titer ($3x10^8$) together an extensive overlapping was observed and yielding 100% rate of infectivity, all isolated bacteriophages of *S. aureus* with highly lytic activity and formed bacteriophage cocktail with high effectiveness which produced obvious clear lytic zone against all 12 target bacteria isolates and reached to the high titer ($2.3x10^{12}$ PFU/ml) as shown in figure (5 A, B).

The results of antibacterial activity of bacteriophage cocktail against *S. aureus* showed a reduction in the bacterial count of all three isolates of *S. aureus* after 24 hours from being spotted with the bacteriophage cocktail, when treated the bacterial broth with bacteriophage cocktail the reduction was (nine logs, six logs, and seven logs) for *S. aureus* 1, 2, and 3 respectively as shown in table (5).

Data were collected by visual examination of the plates and by manual plaques counting. The titers of the isolated and optimized phages are shown in table (3).

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A: Spot lysis assay **B: Top layer plaque assay**

Figure 5. *In vitro* lytic activity of bacteriophage cocktail of *S. aureus* against 12 MDR *S. aureus* isolates via A. Spot lysis assay. B. Top layer plaques assay

Table 5. The reduction in titer of MDR *S. aureus* bacteria after 24 hours from the addition of bacteriophage cocktail

Bacteria	Bacterial broth titer before any addition (CFU/ ml)	Bacterial broth titer after 24 hours of bacteriophage cocktail addition (CFU/ ml)
Sa1	4.3×10^9	0
Sa2	3.7×10^7	4.1 x 10
Sa3	5.1×10^7	0

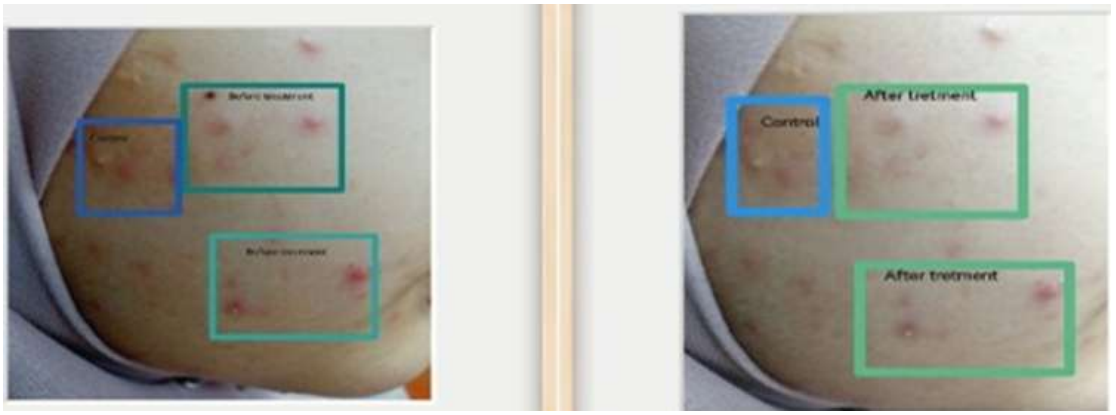
Sa: *Staphylococcus aureus*

Clinical investigation of the *in vivo* topical antibacterial activity of bacteriophages cocktail therapy on MDR bacterial infection facial acne vulgaris

Bacteriophage cocktail therapy was applied topically on one or more patches of 2 cm^2 facial skin acne lesions, with leave patch of facial skin acne lesions as a control (un treated) for volunteers whom age was 25-35 years old with moderate facial acne lesions confirmed the presence of MDR *S. aureus* in all of them with a high rate of resistance against many antibiotics and did not have any treatment for acne lesions at least 6 months ago.

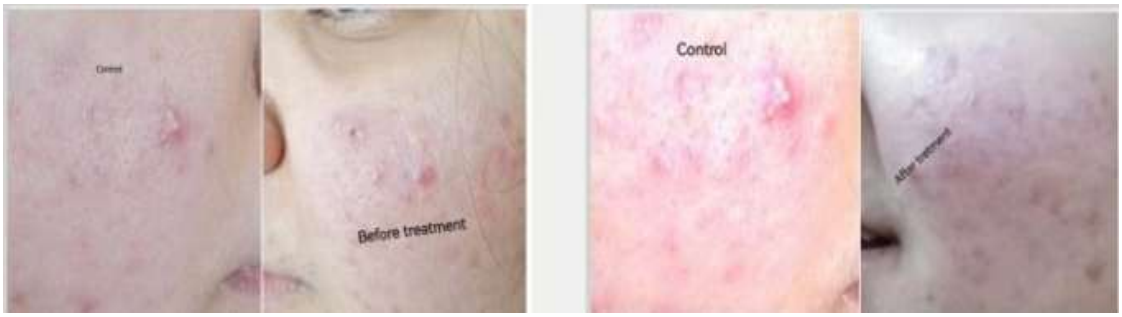
The result of this clinical trial showed that the successful *in vivo* facial topical application of bacteriophage cocktail therapy was achieved in all volunteers when comparing the patches of facial skin acne lesions treated by *in vivo* facial topical application with control acne lesions without any treatment. The response was determined by decolonized bacterial growth and clinical improvement in the signs of facial skin acne lesions. So there was negative bacterial growth in the volunteers with complete response, while there was a reduction in the titer of bacterial growth in the volunteers with partial response, and the results of clinical investigation showed decrease in size, number of acne lesions (white head/ black head, papules/ pustules, and nodulocyst lesions) and decreased in the inflammatory signs in all treated patches of volunteers compared with control patch. No

allergic reactions were observed against bacteriophage cocktail in all treated volunteers as shown in figure (6).



Before **After**

A-Selected volunteer with partial response to bacteriophage cocktail therapy



Before **After**

B-Selected volunteer with complete response to bacteriophage cocktail therapy

Figure 6. Selected volunteers treated with bacteriophage cocktail

Discussion

Acne is a multifactorial inflammatory disease considered as a one cause of hypertrophic scars. Many different treatment options are available that try to target the underlying causes. These include topical, oral (systemic) antibiotics and physical modalities like photodynamic therapy and laser [23].

The results of the current study showed different antibiotic resistant profiles by different bacterial isolates of *S. aureus*. All of the bacterial isolates were resistant to three or more antibiotics, which is considered as MDR isolate. There are many reasons for this problem such as exchange of the genetic material by plasmid or chromosomal change, some individuals being noncompliant with the prescription of the physicians, which leads to the use of antimicrobial agents at doses inadequate to kill all the pathogenic bacteria completely, self-medication with wrong antibiotic or insufficient doses helped in spread and transmission the antibiotic bacterial resistant strains beyond the human ability of management [24].

This study found two isolates of MDR *S. aureus* with SIHP. Hemolysis caused by these SIHP strains is significantly different from the complete hemolytic ring (B–hemolytic phenotype) produced in other *S. aureus* strains. This result is in agreement with a previous study showed that SIHP strains express β–hemolysin highly, but the complete hemolytic ring was never observed due to the effects of

all four hemolysin genes hla, hlb, hlc, and hld, which are expressed in a different way when compared to the control *S. aureus* strain with complete hemolytic phenotype, and these SIHP strains with potential high virulence controlled by tst gene, therefore attention should be paid in controlling and treatment of these MDR strains [25]. Topical and systemic agents are prevented to given as monotherapy because of fast development of bacterial resistance to antibiotics after weeks-months, high rates of resistance *Propionibacterium* acnes (*P. acnes*) and *S. aureus* erythromycin and clindamycin have been reported throughout the world and this correlates with decreased efficacy the antibiotics for acne vulgaris treatment [26]. Tragically, it is approximated by 2050 there will be 10^6 deaths each year globally from bacterial resistance to antibiotics, which is greater than the deaths from COVID-19 in 2020 [27]. The pharmaceutical industry has not produced a new class of antibiotics for more than a decade and the continuing rise of antibiotic resistance is a major health alarm, so urgent actions should be used to reduce the growing problem of antibiotic resistance in medicine like nontraditional agents [28], increasing in the research for inhibiting bacterial virulence by blocking quorum sensing, or targeting systems that confer resistance against antibiotics such as multidrug efflux pumps, and development the novel therapeutic agent like bacteriophage (living drug) and improvement using of its products [29].

Bacteriophages can be called Elixir of life to reflect their natural role in controlling the microbial population and their highlighted effective behaviors in treating topical and systemic bacterial infections. The double agar overlay assay is the common gold standard method for enumerating and differentiating bacteriophages. In this study, 3 bacteriophages for different *S. aureus* were isolated and reached high titers ranged between (10^9 -10^{11} PFU/ml). This result is agreed with previous studies applied on bacteriophages specific for MDR *S. aureus* [30]. In the current study, get lytic bacteriophages with aggressive infective qualities for *S. aureus* bacteria from sewage water; this result is in accordance with Ali et al, 2018, who found that these bacteriophages isolated from sewage water expressed a high degree of lysis with high tolerance to the harsh physical environment [31].

All isolated bacteriophages in the present study with unique profiles of shape, size, clarity, and margin cut of plaques provided evidence that these isolated bacteriophages are unique and no bacteriophages are identical to each other, which is useful in preparing bacteriophage cocktail to cover a wide spectrum of the bacterial pathogen within the same species so can receive more successful bacteriophage therapy. The isolated bacteriophages need to be exhaustively characterized before being included in the therapeutic bacteriophage [32]. The worried concern about applying therapeutic bacteriophage preparations in clinical trials is that transducing bacteriophage-contaminated preparations or lysogenic bacteriophage may cause transferring unwanted genes such as genes that encode bacterial endotoxins into the biosphere [33], bacteriophages used in current study for therapeutic purposes, were lytic (non-transducing), so that they cannot transfer genetic material among bacteria, and not contain undesirable genes that could be released into the environment. An ideal scenario for applying bacteriophages therapeutically would be to custom design, an effective bacteriophage preparation for each patient, based on the lesions, and unique pathogen, that's similar to use for annually updating the influenza vaccine which based on the antigenicity of the influenza strain(s) predominating during that year. Bacteriophage cocktails custom designing was a common practice in Eastern Europe and the former Soviet Union and is still used in Georgia. Results received using custom designed cocktails suggested that they were significantly more effective in managing of bacterial infections compared with non-custom designed preparations [34].

The results of the current study show that using bacteriophage cocktail at MOI 0.01 can be greatly successful in combating bacteria and eradicating MDR bacterial infections. For improving the management of MDR bacterial

infections would be to provide a system for identifying the infecting bacteria and determining its susceptibility to various component bacteriophages. Thus, the most effective bacteriophages could be selected and used to treat the patients whose MDR bacteria were analyzed.

In vivo facial topical application trials presented here revealed that bacteriophage cocktail therapy reduces the inflammation caused by MDR *S. aureus* bacteria in volunteers with moderate-severe facial acne lesions. Some clinical double- blind phase one and two trials have been carried out with variable success, proved that phage therapy with anti-inflammatory properties, which can target the primary cause of mortality in COVID19, and with high specificity (targeted killing) [35-37].

There is an urgent need for novel therapeutic agents that are directed against MDR bacteria. Bacteriophage cocktail therapy is a possible alternative to antibiotics for the treatment of bacterial infections [38].

In conclusions, bacteriophages cocktail specific for *S. aureus* bacteria can be prepared in laboratory with simple measures. The coverage rate of the formed bacteriophages cocktail is high enough to be considered as a practical tool for in vivo topical treating acne vulgaris caused by MDR bacterial infections. Successful results of in vivo topical applied of bacteriophages cocktail showed via mean of clinical improvement which was associated with reduction in viable MDR *S. aureus* bacterial growth of acne lesions compared with control, untreated lesions.

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Author contribution

All authors contributed directly to the creation of this study and approved the final version of this paper that was submitted. Dr. Abdulamir and Dr. Farhood: put the research plan. Dr. Hussain and Dr. Ali: did the in vitro validation tests. The results of clinical investigation of in vivo topical applied of therapy was obtained by Dr. Hussain, Dr. Abu Raghif and Dr. Farhood.

Conflict of interest

The authors declare there is no conflict of interest.

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Correspondence to Dr. Haneen E. Hussain
E-mail: haneen.msc@gmail.com
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