

Novel Bacteriophage Cocktail, Endolysin-Hydroxyethyl Cellulose Gel, and Mixture Gel Therapy for Acne Vulgaris Caused by Multidrug Resistant *Propionibacterium (Cutibacterium) acnes*

Haneen Emad Hussain, Ahmed Sahib Abdulmir¹, Iqbal Ghalib Farhood², Ahmed R Abu Raghif³, Mohammed Razzaq Ali¹, Muhannad Abdullah Al-azzawy⁴

Department of Microbiology, Al-Forat General Hospital, Al-Karkh Health Director, Iraqi Ministry of Health, ¹Departments of Microbiology, ²Medicine, ³Pharmacology, College of Medicine, Al-Nahrain University, Baghdad, ⁴Department of Medical Microbiology, College of Medical Technology, Al-Kitab University, Kirkuk, Iraq

Abstract

Background: *Propionibacterium (Cutibacterium) acnes (P. acnes)* is a bacterium closely associated with the pathophysiology of acne vulgaris. **Objectives:** First to prepare a hydroxyethyl cellulose (HEC) gel containing *Propionibacterium*-specific cocktail of lytic bacteriophages along with *Propionibacterium*-specific phage endolysin, and second to test the efficacy of the prepared gel using *in vitro* antibacterial validation tests and *in vivo* clinical trials targeting facial multidrug resistant (MDR) *P. acnes* by applying the gel topically on moderate-severe acne lesions. **Materials and Methods:** Thirteen isolates of MDR *P. acnes* bacteria were obtained from acne lesions of human skin by using comedone extractor. Two isolates of bacteriophages with specific lytic activity to MDR *P. acnes* were isolated by conventional microbiological methods and bacteriophage cocktail was accordingly prepared; moreover, a purified endolysin extracted from one of the isolated bacteriophages was used with the phage cocktail. Gel-based formulation from bacteriophage cocktail and endolysin-HEC was prepared. *In vitro* validation tests, namely spot lysis, top layer plaque, and antibacterial activity assays were performed. *In vivo* topical application of the prepared gel for 30 minutes three times daily for 1 week on moderate-severe facial acne lesions of volunteers aged above 25 years old caused by MDR *P. acnes*. **Results:** Gel-based formulations showed 100% *in vitro* spectrum of lysis against MDR *P. acnes* isolates. No allergic reaction was found from the topical therapy of the prepared gel; moreover, a remarkable clinical improvement was observed in terms of decreased inflammatory signs, size, and number of comedones, along with complete decolonization of MDR *P. acnes* in gel-treated acne lesions when compared to the untreated acne lesions (control). **Conclusion:** Bacteriophage cocktail, endolysin-HEC gel, and mixture gel therapy may offer a real chance for patients with chronic acne lesions caused by MDR *P. acnes* and may protect teenagers and young adults from antibiotic overuse, anxiety, and depression.

Keywords: Bacteriophage, cocktail, endolysin, HEC-gel, MDR, *P. acnes*

INTRODUCTION

Propionibacterium “*Cutibacterium*” *acnes* is a Gram positive, anaerobic, commensal, and opportunistic bacterium that plays a major role in the inflammatory process of acne vulgaris.^[1] The lipoglycogan-based cell wall envelope and the extracellular secreted “lipase, endoglycoceramidase, hyaluronate lyase, neuramidase, and (CAMP₃) factors” help in the bacterial adherence and colonization to the sebaceous follicle which aids in the progress acne process.^[2] *Propionibacterium acnes* isolated from patients with acne lesions proved highly resistance rates to macrolides antibiotics as “erythromycin, azithromycin, and clindamycin.”^[3] Scientists have raised the alarm that an alternative strategy

to topical antibiotics should be available.^[4] Currently the role of phage in the treatment of acne lesions has attracted the concern of researchers and clinician.^[5] Bacteriophage therapy has been used in human for several types of infections with interesting results.^[6] *Propionibacterium acnes*

Address for correspondence: Dr. Muhannad Abdullah Al-azzawy,
Department of Medical Microbiology,
College of Medical Technology, Al-Kitab University, Kirkuk, Iraq.
E-mail: nnn1952005@gmail.com

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phages have high specificity to their strains and did not damage the others within the family of *Propionibacterium* bacteria.^[7] Even though the bacteria developed resistance to phages they remain sensitive to endolysin, because endolysin enzymes were highly conserved at the level of amino acid in various *P. acnes* phage strains,^[8] therefore bacteriophage endolysin therapy could be used as antibacterial agent with good results.^[9] Even though the bacteria developed resistance to phages they remain endolysins sensitive. This suggests developing a novel topical treatment of acne that target bacterial cell wall via the genetic engineering of enzymes.^[10] The aim of this study was to prepare bacteriophage cocktail, endolysin-hydroxyethyl cellulose (HEC) gel, and mixture gel from the two, and then to test its efficacy via *in vitro* antibacterial validation tests and clinical trials targeting facial multidrug resistant (MDR) *P. acnes* when applied topically to moderate-severe acne lesions.

MATERIALS AND METHODS

Samples of bacteria were collected from Al-Yarmouk Teaching Hospital, Baghdad, Iraq. A total of 13 *P. acnes* isolates were collected from patients at age more than 20 years old whom attended the dermatology consultant unit of hospital and complained from skin lesions, which diagnosed clinically by dermatologist as a lesions of acne vulgaris. All isolates were obtained directly from the pus formed in the bottom part of the inflamed follicle of acne lesions by aseptic techniques using a comedone extractor and a sterile disposable cotton swabs, then inserted into a test tube containing 5 mL of thioglycolate broth. After 2 days of an aerobic incubation at 37°C, the isolates were inoculated onto blood agar and trypticase yeast extract media with 0.025% Tween 80, Glycerol 1%, Bromocresol purple 0.002% and 2 mg/L furazolidone antibiotic. Plates incubated under an aerobic condition with an aerobic jar at 37°C for 3 days.^[11] *Propionibacterium acnes* were identified based on conventional microbiological methods such as Gram staining and biochemical tests.^[12] Antibiotic susceptibility test was designed according to the Kirby-Bauer disk diffusion method, antibiotic disk applied on inoculated agar plates and incubated anaerobically at 37°C for 48 h. Accordingly, the size of inhibition zone determines whether isolated bacteria were resistant, intermediate, or sensitive. The antibiotic disks used were metronidazole, gentamicin, clindamycin, erythromycin, azithromycin, tetracycline, and levofloxacin.^[13] Isolates of bacteria were stored at freezing -20°C by using medium containing 1.5 mL of glycerol to 8.5 mL Brain Heart Infusion broth.

Bacteriophages sampling, processing, and preparing as cocktail

Primary bacteriophages isolated from collected disposable cotton skin swabs of lipid areas of human skin as forehead, two sides of nostrils, comedones and deeply sebaceous gland of hair follicles, then mixed with salt magnesium

buffer and stored at 4°C until used. Propagation of primary bacteriophages was carried out according to the methodology conducted in Hyman^[14] with some modifications.

- Stocks of bacteria were prepared by incubating *P. acnes* anaerobically for 48 h at 37°C in nutrient broth, and then 100 mL of each bacterial isolates were mixed together in a sterile 5 mL test tube.
- Swabs preserved into sterile tubes containing 5 mL of salt magnesium “SM” buffer filtered by using cellulose filter paper to discard any solid materials were added to the mixture.
- Nutrient broth (2–3 mL) and SM buffer (2 mL) were added to the mixture as well, the mixture was incubated anaerobically for 48 h at 37°C.
- The mixture was filtrated using micropore syringe filter (0.20–0.22 Mm) and transferred to new Eppendorf tubes then equal volume of SM buffer was added.
- Spot lysis assay was used for screening the virulent phages on a nutrient agar^[15] and determined the coverage rate of the specific lytic phages via the following formula:

$$\text{Coverage rate} = \frac{\text{Number of bacteria lysed by phages}}{\text{Total number of bacteria}} \times 100\%.$$
^[16]

Endolysin extraction and purification

Up to (100 mL) of broth containing *P. acnes* bacteria was incubated anaerobically at 37°C for 48 h, and then the bacterium (1×10^{12}) was put in 1000 mL of broth for 3 h. A total of 300 mL of bacteriophage at titer (1×10^{13}) PFU/mL was added for 20 min (MOI 1:10) and the total volume was divided in 50 mL tubes, put directly in ice, centrifuged the tubes at 10^4 rpm for 15 min and the sediment was taken and put in (6 mL) of (0.05 M) phosphate buffer with deoxyribonuclease “5 mg,” incubated for 60 min at 37°C. Ethylene diamine tetra acetic acid (0.005 M) was added and centrifuged at 10^4 rpm for 1 h and the supernatant was taken. Disodium tetrathionate “0.3 M” was added and mixed for 1 h at 4°C. In the next day, centrifuged at 10^4 rpm for 1 h. Then the mixture was resuspended in 5 mL of (0.05 M) phosphate buffer (pH 6.1). Dialysis against (200 mL) of the phosphate buffer saline (PBS) with 2× concentration. At 4°C overnight was done. Purification was done by adding to column chromatography sephadex G.100 in (0.1 M) phosphate buffer. They were collected in (10 mL) plane tubes at 10 min intervals. Absorbance of each fraction was measured at “280 nm.”^[17] From each fraction 10 mL was dropped by automatic pipette to bacterial lawns of the specific bacteria to see which plane tube contain the endolysin. Concentration of extracted endolysin was calculated accordingly to the method of Bradford.^[18] Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were evaluated by using broth dilution method.^[19]

Gel-based formulation preparation

1% from hydroxyethyl cellulose (HEC) powder dissolved in 10ml phosphate buffer saline (PBS) with addition 10% glycerol as a humectant substance to prepare gel-base, then mixing with (10% v/v) of (5mg/ml) from purified extracted endolysin as an active ingredient to prepare endolysin-HEC gel.^[20] 10% bacteriophage cocktail 1mL at titer (1×10^{10}) was gradually mixed with 10mL of endolysin-HEC gel and gently homogenized to prepare the mixture gel. Fusidic acid cream 2% and Bactroban “Mupirocin” cream 2% commercially available as topical antibiotics were used for comparison with the formulated gels (endolysin Sa1-HEC, endolysin Sa3-HEC).

In vitro lytic activity of bacteriophage cocktail and the formulation gels

The efficacy of endolysin-HEC gel and mixture gel of bacteriophage cocktail with endolysin-HEC gel was determined via a plate lytic assay.^[21] The culture of *P. acnes* (1×10^7 CFU) was spread onto nutrient agar. The antibacterial activity of each gel formulation was detected by adding 10mL of gel onto nutrient agar plates, followed by incubation anaerobically for 48h at 37°C. The plates were examined and a clear zone indicated the efficacy of endolysin-HEC gel and mixture gel to lysis bacteria.

The effectiveness of bacteriophage cocktail within the mixture gel was examined by taking 100mL of mixture gel into 900mL of SM buffer to perform tenfold serial dilution. The titers of effective bacteriophage cocktail (PFU/mL) were examined after incubation at 37°C, and then 10mL of these dilutions was placed onto a lawn plate with 1×10^7 CFU/mL of *P. acnes* bacteria, followed by incubation of a clear lytic zone indicated the activity of bacteriophages against bacteria.

In vivo topical antibacterial activity of bacteriophage cocktail, endolysin-HEC gel and mixture gel therapy on MDR *P. acnes* facial acne vulgaris

The protocol of interventional study and the subjects consent forms were approved by IRB No. 204/3/2 at College of Medicine/Al-Nahrain University. Subjects with the following criteria were chosen for the study: firstly skin swab from facial acne lesions of each subject at age more than 25 years old with previous treatment for at least three years with multiple regimes (topical and systemic) with no response and did not take any topical or systemic acne vulgaris treatment at least 6 months ago. Swabs 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, endolysin-HEC gel, and mixture gel on intact skin to check for immediate allergic reactions. Then applied therapy topically on 2cm² patch of facial acne lesions for 30min, three times daily and for 1 week, whereas another patch also containing MDR *P. acnes* were left as a control

without any treatment. After 1 week of topical applied patches examined and the efficiency of therapy was evaluated according to the clinical improvement response of the treated patch by determining the decreased in size, number of acne lesions and decrease of inflammatory symptoms of facial skin with acne vulgaris caused by MDR *P. acnes* as compared with control patch. Also the efficiency of therapy was determined via decolonization in the growth of MDR bacteria after cultured swabs of the treated patch and compared with untreated (control) patch.

RESULTS

An aerobic *P. acnes* isolates were collected directly from the pus formed in the bottom part of the inflamed follicle of acne lesions from patients at age more than 20 years old with acne lesions by a septic techniques using extractor comedon and a sterile disposable cotton swabs which inserted in the tubes contain 5ml of thioglycolate broth and incubated at 37°C under anaerobic condition with gas jar for 2 days, then cultured on blood agar and trypticase yeast extract glucose media.

All *P. acnes* generated pinprick sized to 0.5 mm in diameter colonies, which appeared glistening, circular, and opaque, with different colors, including white, yellow, or gray with weak or no hemolysis on an aerobic blood agar as shown in Figure 1A.

Fermentation reactions of *P. acnes* were studied on trypticase yeast extract glucose media with Tween 80 and Bromocresol purple as indicator for the bacterium produced propionic acid, succinic acid and lactic acid as the end products of glucose fermentation, which lead to turn the color of medium from purple to yellow as shown in Figure 1B.

The microscopic examination of *P. acnes* isolates showed Gram positive, pleomorphic cells that appeared in different forms, such as bacillus or spherical with different arrangement: single, in pairs, clustered, short chains in “V” or “Y” configuration as shown in Figure 1C. Diagnosis of the isolated bacteria relied also on conducting catalase, indole, and nitrate reduction tests for positivity reaction.

Antibiotic susceptibility testing was performed on Muller Hinton agar by Kirby Baur disk diffusion method against six different antibiotics and was interpreted according to CLSI (Clinical and Laboratory Standard Institute) 2022 criteria. It revealed that all of the tested isolates were resistant to three and more antibiotics classes. Isolates that were resistant to three or more antibiotics classes were designated as MDR bacteria as shown in Figure 2.

Accordingly, all of 13 *P. acnes* isolates were MDR bacteria. The highest resistance was observed to erythromycin (100%), metronidazol (77%), clindamycin (54%), and gentamicin (38%). Resistance to Tetracycline was found in



Figure 1: *P. acnes* identification. (A) *P. acnes* colonies on blood agar, under anaerobic condition at 37°C were done between (48 and 72) h. (B) *P. acnes* ferment glucose and turn the medium from purple to yellow. (C) *P. acnes* were Gram positive, with different forms and arrangements



Figure 2: Antibiotic susceptibility test of *Propionibacterium acne* isolates on Muller-Hinton agar plates at 37°C for 48 h of incubation

(31%), and there was no resistance to Levofloxacin (0%) as showed in Table 1.

In this study, two lytic specific bacteriophages active against *P. acnes* were isolated and purified, and both of them formed visible plaques in the early stage when tested on bacterial lawn of specific MDR *P. acnes*. They were isolated from lipid area of human skin as forehead, sides of nostrils, comedones, and sebaceous gland of hair follicles via sterile comedones extractor by showing lysis on bacterial lawns of MDR *P. acnes*. Characteristics of these bacteriophages were determined by the margin cut, diameter, clarity/turbidity, and the shape of their plaques. The size ranged between (2.7 and 3.9mm) with a mean of (3.3mm), the plaques morphology of the two bacteriophages were circular, margin cut clear, and the clarity ½ semi clear and ½ clear plaque as shown in Table 2.

When mixing the two bacteriophages of *P. acnes* at titer (2×10^8) together an extensive overlapping was observed and yielding 100% coverage. Two isolated bacteriophages of *P. acnes* at titer (1×10^8) with high lytic activity formed

bacteriophages cocktail with highly effectiveness which produced obvious clear lytic zone on target bacteria and demonstrated high titer (1.7×10^{10} PFU/mL) as shown in Figure 3A, B.

Endolysin was successfully extracted from one of the *P. acnes* bacteriophages by using sephadex G100 column chromatography as shown in Figure 4. The results were (57 µg/mL), (1 µg/mL), (4 µg/mL) for concentration, MIC, and MBC respectively.

The results showed that endolysin-HEC gel has potential lytic activity against bacterial isolates which covered approximately 2cm of the bacterial lawn when compared with lytic activity of commercial antibiotics (Fusidic acid 2%, Bactroban “Mupirocin” cream 2%) through applying 10mL from gel and commercial antibiotics on bacterial lawn as shown in Figure 5.

The visual appearance of gel formulations was examined every week; the result showed that the gel formulations were able to maintain their white color, consistency, and characteristic odor for 30 days, the average pH (7.1). Assessment of the formulation gels showed that mixture gels retained full lytic capacity and bacteriophage cocktail with high effectiveness at titer (1×10^9 PFU/mL) after 30 days when stored at 4°C, whereas endolysin-HEC gel retained full lytic capacity by 21 days after formulation and stored at 4°C. The formulation mixture gels of bacteriophage cocktail with endolysin-HEC showed reduced lytic capacity and decreased in the effectiveness bacteriophage cocktail to the titer (1×10^4 PFU/mL) by 21 days and complete loss (100%) of lytic capacity at 25°C after 30 days, while the formulation gels of endolysin-HEC retained the lytic capacity at 25°C by 14 days and completely loss the lytic activity after 21 days.

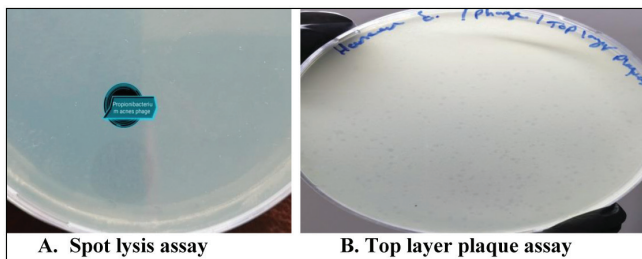
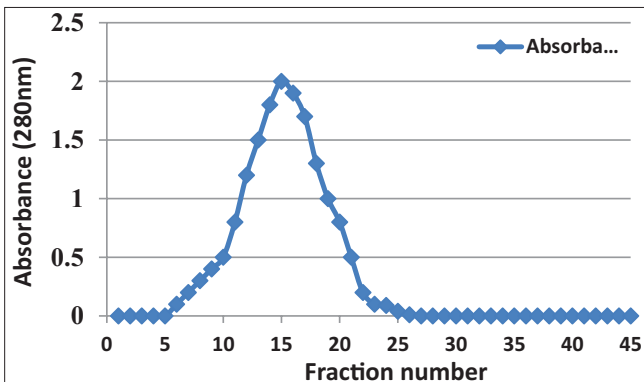
The results showed success of therapy when comparing the treated patch of acne lesions on facial skin of subjects whom applied the therapy topically with patch of acne lesions without any treatment, yet the response was determined by clinical improvement in the signs of lesions and by decolonizing in bacterial growth and without any allergic reactions against therapy as shown in Figure 6.

Table 1: Antibiotic susceptibility percentage of *P. acnes* isolates

Antimicrobial class	Antimicrobial agents	<i>P. acnes</i> isolates	Mean of percentage of resistance
Macrolides	Erythromycin	13	100%
Nitroimidazole	Metronidazole	10	77%
Lincosamides	Clindamycin	7	54%
Aminoglycosides	Gentamicin	5	38%
Tetracyclines	Tetracycline	4	31%
Fluoroquinolones	Levofloxacin	0	0

Table 2: Characteristics of bacteriophages plaques (Bp-Pa1 and Bp-Pa 2)

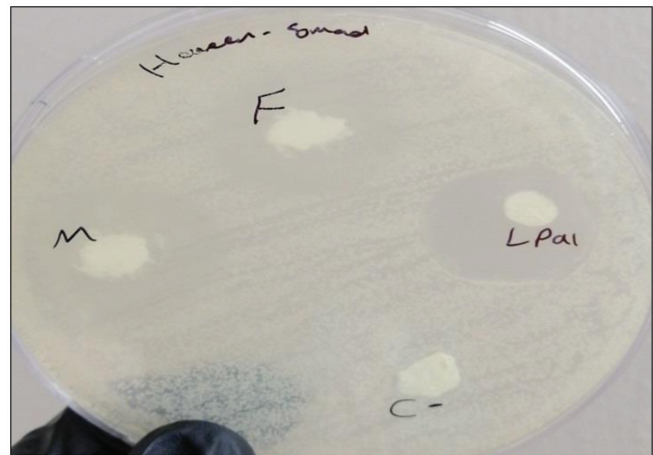
Bacteriophage isolated	Size (mm)	Clarity	Shape	Plaque edge
Bp-Pa1	3.9 mm	Clear	Circular	Regular
Bp-Pa2	2.7 mm	Semi-clear	Circular	Regular

**Figure 3:** (A) Spot lysis assay of *P. acnes* bacteriophage. (B) Top layer plaque assay of *P. acnes* bacteriophage**Figure 4:** Gel filtration chromatography on Sephadex G 100 column (25 × 3 cm) equilibrated with 0.05 M sodium phosphate buffer pH 7 with 30 mL/h flow rate to extracted endolysin

DISCUSSION

After 2000, increasing numbers of the scientific reports have been published on experimental treatment trials that have used bacteriophages to treat infections caused by resistant bacteria when the classical therapies have been unsuccessful.^[22-25]

The results of the current study showed different antibiotic resistant profiles by different bacterial isolates of *P. acnes*. All of the bacterial isolates were resistant to three or more of antibiotics, which considered as multidrug resistant isolate in this study. This reflected that widespread use of antibiotic and the misuse of

**Figure 5:** *In vitro* lytic capacity of endolysin-HEC gel-based formulation and commercial antibiotics against MDR *P. acnes* bacterial isolate. Endolysin-HEC (L Pa1) gel, Mupirocin (M) cream, Fusidic acid (F) cream, and PBS-HEC gel as a negative control (C -ve) against MDR. *Propionibacterium acnes* isolate

inappropriate antibiotic have led to rise in the emergence of multidrug resistant bacterial infections. Increasing the prevalence of resistant bacterial strains increased serious concerns by the World Health Organization. This is a serious medical problem, and this study showed that Trypticase yeast extract-glucose-Tween 80-Bromocresol purple- media with furazolidone antibiotic was effective as a selective and differential media for isolation anaerobic *P. (Cutibacterium) acnes* bacteria in this study. As a selective media by its ability to inhibit the growth of *Staphylococcus aureus* bacteria due to the activity of furazolidone antibiotic, and by adding Tween 80 which provides a suitable surfactant for enhancement anaerobic growth of these bacteria. Acts as a differential media because it contain bromocresol purple as an indicator for differentiating the acidity of *P. acnes* bacteria that turned the purple color of media to yellowish color due to production of propionic acid, succinic acid, and lactic acid as the end products of glucose fermentation.



Figure 6: Selected subjects treated with bacteriophage cocktail and endolysin-HEC gel

Bacteriophages can be called the elixir of life for their natural role in controlling microbial population and their highlight effective behaviors in treating topical and systemic bacterial infections.

This study showed the best source to isolate specific lytic bacteriophages for *P. acnes* was the lipid areas of the human skin “mainly the foreheads, nostrils, and comedones.” This result agreed on with the previous study which showed the correlation *P. acnes* bacteriophages with the distribution of their host *P. acnes* bacteria in the pilosebaceous unit and lipid rich areas of human skin, since they depend on their hosts for replication.^[26] The two isolated bacteriophages in the present study with unique profile 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 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 included in the therapeutic bacteriophage.^[27]

Bacteriophage extract endolysin therapy is a possible alternative to antibacterial therapy; it has proved to be medically superior to antibacterial agents in treatment bacterial infections.^[28] Our findings support this notion, as the bacteriophage extracted endolysin used in this study were successfully produced in their soluble forms, purified with high yield purity, and with high efficiency against MDR *P. acnes* bacteria. Extracted endolysins have many

advantageous which belong to their bactericidal effect, rapid activity, and their safety for human cell. For all these reasons, endolysin appears to be attractive candidate as alternative antibacterial agent for treating antibiotic MDR infections.^[29] Our results agreed on with this notion, as the minimum inhibitory concentration MIC (1 µg/mL) and MBC (4 µg/mL) of extracted endolysin was used in this study reflect that bacteriophage extracted endolysin with bactericidal effect and had rapid action on MDR bacteria of skin acne lesions. Thus endolysin enzymes will be of direct benefit in environments where antibacterial resistant Gram-positive pathogens are a serious problem, such as hospitals, nursing homes, and day care centers.^[30]

The bacteriophage cocktail characterized so far possesses the desired formulation parameters: they remain stable at 4°C storage.^[31] The results agreed with this notion, bacteriophage cocktails remain with the same stability and activity at 4°C for more than 3 months, and within mixture gel formulations “bacteriophage cocktail with endolysin-HEC gel” bacteriophages remain stable at 4°C for more than 30 days with the same effectiveness and potential lytic activity.

While endolysin-HEC gels formulation protected the potential lytic activity of extracted endolysin at 4°C for more than 21 days and this is in agreement with a previous study showed that lysin ointment formulation preserved the potential lytic activity of enzyme for more than 15 days at 4°C.^[32] On the other hand, Bactroban “Mupirocin” commercial antibiotic cream inhibits the bacterial protein synthesis by interfering with charging of tRNA-11e.

However, there are many alternative metabolic pathways in bacteria. Thus, it is not surprising that mupirocin-resistant strains develop very easily. This situation does not apply to endolysin. The bactericidal activity of endolysin is independent of active host metabolism. Additionally, as far as we detected, resistance was not identified for any reported endolysins.^[33]

The results obtained in these *in vivo* topical application trials is encouraging, because our finding and those of others support the improvement pharmaceutical characteristics of formulation gels “endolysin-HEC gel, and mixture gel” to use as commercial therapy against MDR bacteria in a near future, and support the development of modified procedures in a more efficient way to improve the use of bacteriophage cocktail and extracted endolysin therapy, for example, the recombinant production of phage lysin in *Escherichia coli* which displayed abroad bacteriolytic spectra within the MRSA strain.^[34-36]

CONCLUSION

Bacteriophage cocktail, endolysin-HEC, and mixture gel therapy were safe during this interventional study with no allergic reaction and did not show any side effect on all human subjects whom topically applied it, and showed a brilliant *in vitro* validation tests and *in vivo* topical applications results by clinically improvement response which associated with decolonized in viable MDR *P. acnes* bacterial growth of facial acne lesions as compared with control “untreated” lesions.

Ethical Approval

Not Applicable.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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