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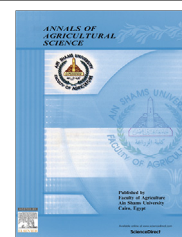

Improved antibacterial efficacy of bacteriophage-cosmetic formulation for treatment of *Staphylococcus aureus in vitro*

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Abstract Currently phages are used as alternative antibiotics for treating pathogenic bacteria causing skin disease. However, the efficacy of pure preparations of phage is greatly reduced due to its short longevity on surface of skin. supplemented cosmetic phages [0.5% phage conc./cosmetic] significantly increased phage longevity on skin surface. The phages were isolated by the single plaque assay from the infected skin showing edema and erythema symptoms. The isolated phages had plaques with 3–5 mm diameters and a distinct translucent spreading halo. The morphological phage particles were cubic nucleocapsid with 65–75 nm across with short contractile tails. The supplemented cosmetic phages reduced the bacterial growth to 95.45%, compared with free phages and non-supplemented cosmetic 86.1% and 77% respectively. The phage containing cosmetic was applied for disease treatment and increased the phage longevity from 24 to 100 h and preserved initial phage population. This work indicated the enhanced antibacterial efficacy of fortifying specific bacteriophage in cosmetics to be a promising formulation for efficient treatment of skin diseases.

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Introduction

In recent years, antibiotic resistance has become one of the biggest threats to public health. Conventional antibiotics aim to kill or inhibit the growth of pathogenic bacteria leading to a strong selective advantage for pathogens to develop resistance in many cases (Jo et al., 2016). Therefore, new approaches to develop bioactive preparations as novel antimicrobial agents have been proposed that entail targeting virulence of the pathogens without inhibiting their growth therapy reducing or slowing the selection for resistance (Medellin-Pena et al., 2007). Attachment of microorganisms to skin contact surface can impact antibiotics industry economically and through associated health risks. Pathogenic bacteria have been shown to attach to a wide variety of skin contact and non-contact surfaces (Barak et al., 2005). So finding natural novel biofilm inhibitor products is of much interest (Bazargani and Rohloff, 2016). A biofilm is a functional consortium of microbial cells that adhere to a wet surface and become immobilized in a protective polysaccharide matrix that can entrap nutrients and other microbes, allowing for subsequent microbial growth.

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Attached microorganisms are generally more resistant to sanitation chemicals that are their detached counterparts. This resistant is due to protection from organic materials and the extracellular polymeric substance (EPS) layer, which prevents chemicals from entering the biofilm or causes inactivation of the sanitizer (Lister and Horswill, 2014; McCarthy et al., 2015).

If weather conditions are favorable for disease development, there are no adequate control measures to manage the disease (Kucharek, 1994). Several alternative control methods have been investigated in recent years; another approach for biological control is the use of bacterial viruses to control bacterial diseases. Phages have long been proposed as human disease control agents and have been used in several human bacterial pathogens (Zaccordelli et al., 1992; Balogh et al., 2003).

However, the phages applications were in effective for controlling pathogenic bacteria and phage endurance was significantly reduced (Jones and Pernezny, 2003; Obradovic et al., 2002). Viruses are very fragile and cannot reside long on skin surface because they are quickly eliminated by harmful environmental factors such as temperature moisture and sunlight UV (Mc Guire et al., 2001). Therefore, the need arose to develop for mutations and/or change the application strategy, such as time of application in order to protect phage particles from harmful environmental factors. The accordingly enhanced residual activity of the phages could lead to increase efficacy of phage treatments and to a more convenient application schedule (Balogh et al., 2003; Jones et al., 2007). The objective of this study was to isolate *Staphylococcus aureus* from infected human skin and to investigate *in vitro* efficacy of phage supplemented cosmetic formulation for treatment of *S. aureus* causing skin disease.

Material and methods

Sampling collection

Different samples of clinical symptoms, edema and erythema (can also give rise to focal accumulation of pus or fluid) were collected from forty patients at Benha hospital and were tested for isolation *S. aureus* strains. Swabs carried with nutrient media passing up and down twice on the infected areas. The samples were incubated for 48 h at 37 °C. Swabs were prepared in triplicate for each sample.

Isolation and identification of bacteria

The samples were transferred from the swab and inoculated on the surface of nutrient agar Petri dishes by streaking method and incubated at 37 °C for 48 h for bacteria isolation. The single colony was streaked on Blood agar media and incubated at 37 °C. The pure cultures of unique colony types were obtained and saved for further analysis. The isolated bacteria were identified and classified on the basis of their morphological and biochemical properties following Bergey's Manual of Determinative Bacteriology as well as biochemical analysis (Holt et al., 1994). In addition, Vitek analysis method using VITEK® MS from bioMerieux, France, was applied after biochemical tests as confirmatory test for bacteria, aerobic and facultative bacteria identification.

Isolation and identification of bacteriophages

The swabs rich with nutrient medium and passing up and down twice on *focal accumulation of pus or fluid* were used to inoculate nutrient broth containing flasks. The flasks were shaken on a rotary shaker for 72 h at room temperature on 3000 rpm for 20 min. The flasks were inoculated with *S. aureus* isolates at log phase culture approximately 4×10^6 CFU/ml in nutrient glucose broth (2.0 g/L yeast and 2.5 g/L glucose) to achieve a multiplicity of infection varying between 0.02 and 2.0. The flask cultures were incubated and shaken continuously overnight at 37 °C in shaking incubator. Bacterial cell and debris were removed by centrifugation at 6000 rpm for 15 min. The obtained phages suspension was propagated by plaque assay method to obtain at least 10^8 PFU/ml. The phage mixtures consisted of four phage isolates and had an approximate final titer of 1×10^{10} PFU/ml. The phage mixtures were stored in 2 ml Eppendorf tube at 4 °C in complete darkness.

Phages morphology

Transmission electron microscope (TEM) was used to detected phages specific to *S. aureus* using negative staining method with 1% aqueous urinal acetate. The grids were air-dried and were examined by TEM (JEOL – JEM – 1010 Electron microscope) in The Regional Center for Mycology Al-Azhar University, Egypt, according to Heringa et al. (2010).

Phage infectivity

S. aureus was diluted in sterile distilled water to a density of 10^7 CFU/ml and inoculated on nutrient agar plates. The phage drop (20 µl) of each isolate was over layered on agar. The plates were incubated at 28 °C overnight. Clear confluent lysis, and turbid confluent lysis were recorded as positive result, while extremely faint zones were considered negative result (Heringa et al., 2010).

Formulated cosmetic

Skin cream composed of Steric acid 6%; Propylene glycol 3%; Paraffin oil 7%; Isopropyl myristate 3%; Tocopherol acetate 0.5% and Rosemary 0.2% desolated in water (Capparelli et al., 2007).

Phage treatment

S. aureus culture was harvested from nutrient agar plates 24 h post inoculation and suspended in sterile water and adjusted to A660 = 0.5 by spectrophotometer which approximately is 10^8 CFU/ml. Three Petri dishes of each treatment were inoculated with bacterial suspension using a hand-hold plastic sprayer until completely wet. The suspension phage mixtures were adjusted by spectrophotometer which approximately account for 1×10^{10} PFU/ml and is used for formulated and non-formulated biological treatment. As well as three Petri dishes were without phages as control. Inoculated Petri dishes were incubated in growth incubator at 37 °C for 48 h.

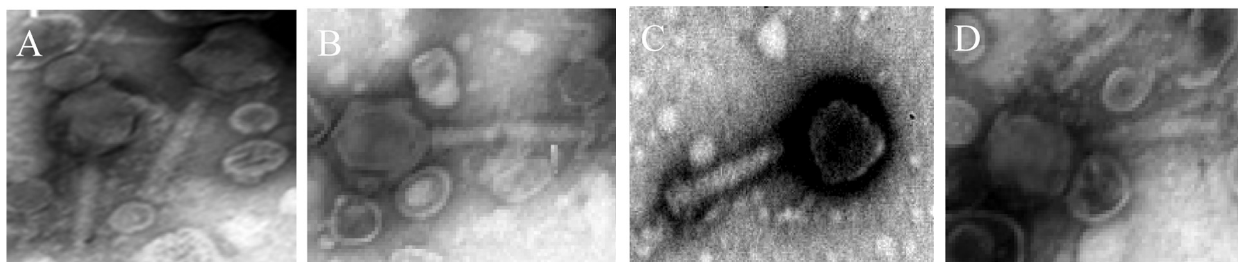

Fig. 1 Photograph of TEM showing four different phages specific to *S. aureus* isolates: (A) IS-1 phage, (A) IS-2 phage, (A) IS-3 phage and (A) IS-4 phage.

Table 1 Plaques morphology of *S. aureus* phages.

Staphylophage isolates	*S. aureus* isolates	Plaques morphology[a]
IS-1 phage	*S. aureus* -1	Turbidity, Large circular irregular with 2–3 mm
IS-2 phage	*S. aureus* -2	Clear, Large circular irregular with 1.5–3 mm
IS-3 phage	*S. aureus* -3	Clear, mediate circular regular with 1.5–2 mm
IS-4 phage	*S. aureus* -4	Clear, small circular regular with 1–2 mm

[a] Growing plaques after 24 h of incubation.

Inhibition of biofilm formation

Tissue culture plate method is used as a quantitative test, is considered the gold standard for antivirulence activity of specific phages detection against *S. aureus* isolate and determined viable cells as follows.

The phage free cosmetic and phage supplemented cosmetics were assayed for their potential to prevent biofilm formation of *S. aureus* isolate. The bacterial cells were added to 200 ml trypticase soy broth medium at the time of inoculation and the cells were allowed to form biofilm. The cosmetic and/or phages were added to *S. aureus* isolate by 50 µl cosmetic; 50 µl specific phage cm/(5×10^5 PFU/mL) and 100 µl of *S. aureus* isolate. Individual wells of flat bottom polystyrene tissue culture plate (96 wells, Sigma Aldrich, USA) were filled with 150 µl of treated *S. aureus*. Negative control wells were filled with 150 µl of broth medium positive bacterial control. The plate was incubated at 37 °C for 24 h. After incubation period content of each well was removed (free floating bacteria) by gently tapping. The wells were washed three times with 2.0 ml of saline phosphate buffer (PH 7.2) and then dried. Biofilm formed by bacteria adherent to the wells was fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess of stain was removed by washing with distilled water and then dried and kept. Optical density (OD) of stained adherent biofilm was obtained by using ELISA microliter plate reader (Sun Rise –TECAN. Inc. ®, USA) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times.

Data analysis

The percentage cell viability was calculated using the Microsoft Excel as follows:

$$\%\ \text{cell viability} = \text{Mean Abs}_{\text{bacteria}} - \text{Mean Abs}_{\text{cosmetic}}/\text{Mean Abs}_{\text{control}}$$

Statistical analysis

The results of each sample were transformed using log transformation [$z = \log 10\ (Y + 1)$] and then subjected to ANOVA.

Results

Identification of bacterial isolates

The four isolates of *S. aureus* from clinical samples were identified based on morphological and biochemical tests. These isolates revealed negative results with oxidase test, mannitol, sorbitol and inositol as sole carbon sources. The isolates were cocci and positive for gram staining. The isolates produced brown diffusible pigment on king B medium. Based on morphological and biochemical tests and confirmation by Vitek analysis method, four isolated bacteria were identified as *S. aureus*.

Table 2 Anti-biofilm activity of different cosmetic concentrations.

	Control	Cosmetic concentration (mg/mL)			
		0.05	0.10	0.15	0.20
Biofilm formation (0.D)	1.10	0.957	0.758	0.455	0.252
Biofilm inhibition (%)	0.0	13	31	59	77

Table 3 Anti-biofilm activity of different concentrations of *S. aureus*-1 phage.

	Control	Phage concentration (PFU/mL)			
		5×10^5	4×10^5	3×10^5	2×10^5
Biofilm formation (0.D)	1.10	0.152	0.255	0.457	0.508
Biofilm inhibition (%)	0.0	86.18	76.82	58.45	53.82

Table 4 Anti-biofilm activity of different cosmetic concentrations mixed with different concentrations of specific *S. aureus*-1 phage.

Cosmetic	Content	Cosmetic concentration			
Phage		0.2	0.15	0.10	0.05
Control	1.10	0.23 79.09%	0450 59.09%	0.750 31.82%	0.95 13.64%
5×10^5	0.15 86.87%	0.05 95.45%	0.12 89.09%	0.157 85.74%	Not Applicable
4×10^5	0.25 77.27%	0.123 88.81%	0.152 86.18%	0.175 84.09%	0.245 77.73%
3×10^5	0.45 59.09%	0.154 86.00%	0.172 84.36%	0.215 80.45%	0.272 75.27%
2×10^5	0.50 54.55%	0.200 81.81%	0.220 80.00%	0.250 77.27%	0.950 59.09%

Identification and propagation of Phage isolates

Phages specific for *S. aureus* isolates were detected in infected skin samples. Crude phages suspension prepared from furuncles and vesicles and assayed by the over layer technique. Single plaque isolate was picked up and put 2 ml of *S. aureus* broth culture (1×10^8 CFU/ml) and macerated then incubated. The phages were assayed quantitatively by plaque assay. The plaques produced by phages showed different degrees of lysis [Clear confluent lysis, turbid confluent with large and small circular with halo and without halo] were mixed for using as biocontrol agent for anti-biofilm activity (4.5×10^{10} PFU/ml). Electron microscope of *S. aureus* phage particles revealed that phages are belonging to Myoviridae family with short-contractile tail. The phage particles have an isometric head with different diameter size 65.2–75.5 nm and the tail with 200.3–245.5 nm in length and 15.4–18.5 nm in width (Fig. 1). The four phage isolates produced different types of plaque with different degree of lysis [Clear confluent lysis], turbid confluent with large and small circular halo with diameter about 1 to 3.0 nm after 24 h of incubation as shown in Table 1. The phage isolates exhibited host specificity when tested with four pathogenic *S. aureus*. Four phage isolates were reacted with four *S. aureus* isolates with different types of lysis except 3 isolates did not develop plaques.

Biotreatment in vitro

The phage infectivity

The phage suspension against *S. aureus* isolates showed the plaque diameter of inhibition ranged from 17 ± 0.78 to 24.8 ± 0.55 (mm) and mean growth inhibition percentage was 100 ± 00% against all tested bacteria.

Longevity of formulated phages

The change in phage populations was determined at room temperature. The changes in phages reflected in phages populations appeared in a host–free environment. On the other hand unformulated phage populations were quickly reduced and practically eliminated within 48 h and/or 36 h after spraying. The cosmetic formulated phages decreased the reduction rate of phage populations at 2 days but not at 7–10 days.

Biofilm formation

S. aureus isolates 1, 2, 3 and 4 were grown, colonized and attached to form biofilm on surface. The obtained results illustrated that, tested *S. aureus* isolates biofilm formation was strong with 1.10 (OD; 570 nm); 0.95 (OD; 570 nm); 0.75 (OD; 570 nm); and 0.35 (OD; 570 nm) respectively. A biofilm is a functional consortium of microbial cells that adhere to a wet surface and become immobilized in a protective polysaccharide matrix that can entrap nutrient and other microbes, allowing for subsequent microbial growth. Attached microorganisms are generally more resistant to sanitation creams that are their detached Counterparts. This resistance is due to protection mediated by secreted organic materials and the EPS layer, which prevents chemical, from entering the biofilm or causes inactivation of the bioactives present in the sanitizer.

Biofilm inhibition

Biofilm formation by cosmetics and specific phage was successfully inhibited. The results in Tables 2 and 3 show the potential activity of cosmetic and specific phage against *Staphylococcus sp*. biofilm formation.

The optical densities of produced biofilm by *Staphylococcus* isolate grown with cosmetic concentrations (0.05, 0.10, 0.15 and 0.20 mg/ml) were 0.957, 0.758, 0.455 and 0.252, respectively, as compared to the control (1.10) of *Staphylococcus* grown in phage free cosmetic.

Table 4 shows that the combination between different concentrations of both phage and cosmetics resulted in reduction of biofilm formation as measured by optical density for *S. aureus*. The inhibition percentage of biofilm ranged from 54.55% to 95.45% by applying mixtures of different concentrations of

phage (2×10^5, 3×10^5, 4×10^5 and 5×10^5 PFU/mL) and different cosmetic concentrations (0.05, 0.10, 0.15 and 0.20 mg/mL). The highest inhibition percentage was found in case of mixing the highest phage titer (5×10^5 PFU/mL) and the highest cosmetic concentration (0.20 mg/mL). These results indicated that the biofilm inhibition increased with increasing specific phage titer and/or cosmetic concentrations.

Discussion

S. aureus globally has become a major clinical problem. In an effort to develop effective control strategies against these genotypically diverse organisms, a mixture with antibacterial activity was formulated and contained lytic bacteriophage specific for *S. aureus* and ready to use cosmetics (Tanaka et al., 1990). In previous studies, antibacterial coatings containing silver, nitric oxide and antibiotics were developed to inhibit biofilm formation on medical devices (Desrousseaux et al., 2013). In addition, small molecules, enzymes and biocides were reviewed to inhibit biofilm formation (Chen et al., 2013).

Lytic bacteriophages specific for *S. aureus* were isolated before (Son et al., 2010). In this work, the isolated phage was a Myoviridae phage and found to have antibacterial and anti-biofilm activity against *S. aureus*. Also, a novel bacteriophage lysin with broad spectrum activity protects against *Streptococcus pyogenes* as well as methicillin-resistant *S. aureus* was described (Gilmer et al., 2013). In addition, two types of phages from Podoviridae and Myoviridae families were reported for *S. aureus* but not from Cystoviridae family (Balogh et al., 2003; Kousik and Ritchie, 1996). Based on International Committee on Taxonomy of Viruses (ICTV) only one bacteriophage (named phage ϕ 6) was reported to act against *S. aureus*.

The results indicated that the efficacy of phages supplementation in cosmetics could provide increased longevity for phages and controlling timing of application, properly. Several protective formulations were identified in earlier studies (Balogh, 2002; Saccardi et al., 1993) and three of these were selected for disease control trials. These formulations increased the longevity of phages' viability 2 days after the application (Iriarte et al., 2007).

In this study a protective formulation consists of specific bacteriophage for *S. aureus*; a global life-threatening pathogen and cosmetic increased the efficacy of phages as alternative treatment and a promising therapeutic agent for disease control. Bacteriophages supplemented Cosmetics gave the best results *in vitro* as compared to preparations of free phages. Formulating a mixture from bioactive cosmetic and bacteriophages with antibacterial and biofilm removal ability could be of interest as alternative for overcoming antibiotic resistance stemmed from misuse of antibiotic drugs.

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