Extraction, purification and therapeutic use of bacteriophage endolysin against multi-drug resistant *Staphylococcus aureus*: *In vivo* and *in vitro* study

Mohammed R. Ali,[a] Ahmed S.Abdulamir,[a] and Shurooq R. Kadhim[b]

[a]Department of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq.
[b]College of Pharmacy, Al-Mustansiriya University, Baghdad, Iraq.
Correspondence to Mohammed R. Ali (email: dr_mohamadrazak@colmed-alnahrain.edu.iq).
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Background Resistant infection with multidrug-resistant *Staphylococcus aureus* representing a real problem for health care providers. Bacteriophage lytic enzymes or lysins are highly evolved molecules that have been specifically developed by phages to quickly and efficiently allow their progeny to be released from the host bacterium while destructing that bacterium.
Objectives Isolation of endolysin from *S. aureus* bacteriophages, and administering them systemic *in vivo* lab animal and measure the therapeutic efficacy, as well as evaluation of their biosafety.
Method This study was performed from March 2015 to August 2017, 50 bacteriological samples of *S. aureus* were collected, and examined with their anti-biogram, then bacteriophage cocktails were done for five resistant strains of them. Endolysins were extracted from their corresponding bacteriophages, they were characterized and the enzymatic and antibacterial activities assays were performed on them in addition to *in vivo* trial on animals regarding the therapeutic effect of these extracted endolysins on laboratory mice.
Results This study showed that the extracted endolysin from these bacteriophages was effective in treating laboratory mice from bacteremia with *S. aureus* and saving their lives when injected intraperitoneal.
Conclusion Endolysin can be extracted directly from their bacteriophages and used by injection of mice with bacteremia with the proper dose of the extracted endolysin of the corresponding bacteriophages which was effective in all of them.
Keywords bacteriophage, endolysin, resistant infection, *S. aureus*
Abbreviations EDTA, ethylenediaminetetraacetic acid; LB, Luria–Bertani broth; MDR, multi-drug resistant; PBS, phosphate buffer saline; p*Sa*, phage to *Staphylococcus aureus*

Introduction

Bacterial infections are responsible for significant morbidity and mortality in clinical settings.[1] Many infections that would have been cured easily by antibiotics in the past now are resistant, resulting in sicker patients and longer hospitalizations. The endolysins of the bacteriophages are highly evolved molecules that have been specifically developed by phages to quickly and efficiently allow their progeny to be released from the host bacterium. These enzymes damage the bacterial cell wall's integrity by hydrolyzing the four major bonds in its peptidoglycan component.[2] The endolysins that have been characterized are amidases; usually do not have signal sequences to translocate them through the cytoplasmic membrane and cleave their substrate in the peptidoglycan. Instead, the endolysins' translocation is controlled by a second phage gene products called holins.[3] The bacteriophages during development in the infected bacterium, endolysin accumulates in the cytoplasm in until bacteriophage maturation. During a specific time controlled genetically, holin molecules inserted in the cytoplasmic membrane are activated, resulting in the formation of pores so the preformed endolysin in the cytoplasm can access the peptidoglycan, thereby causing cell lysis and the release of the new bacteriophages.[3] Bacteriophages are able to lyse their targeted bacterial hosts, and this has been known for almost a century, and since the late 1910s bacteriophages have been used to prevent and treat human and animal diseases of bacterial origin. But bacteriophage-encoded enzymes have only recently begun to be used for various applications; e.g.: reducing bacterial contamination in dairy products and the preparation of bacterial vaccines.[4,5]

Materials and Methods

Sampling and Processing of Bacteria

Samples of bacteria were collected in Al-Imamein Al-Kadhymain Medical City Hospital. A total of 50 different *Staphylococcus aureus* isolates were collected from Bacteriology laboratory. The specimens were collected from hospitalized patients and outpatients suffering from severe urinary tract infection, otitis media, skin infection, and septicemia. The specimens were cultured in screw universal tubes containing nutrient broth wrapped by parafilm or by using sterile swabs; both were put in ice bags and were transferred at the same day to the laboratory of Medical Microbiology Department in the College of Medicine, Al-Nahrain University to subculture on nutrient agar or to be stored in refrigerator at 4°C for 24 h.[6]

After storing the samples, under aseptic conditions, for 24 h at 4°C in a refrigerator, specimens were plated on nutrient agar in streaking methods. *Staphylococcus aureus* formed smooth round colonies with a fluorescent golden color in nutrient agar. Single colonies of *S. aureus* were isolated from a growing stock by ABC streaking on nutrient agar plates to isolate single discrete colonies. The media were prepared according to the manufacturing instructions and were sterilized by autoclaving at 121°C for 15 min. The bacteria were stocked in Luria–Bertani broth (LB) containing glycerol (30% v/v), preserved at –20°C.

Diagnosis of the isolated bacteria relied first on conducting catalase test to distinguish from streptococci. Then coagulase test was done and positive results confirm the diagnosis of

S. aureus with Gram staining, which shows Gram-positive cocci in clusters. Hence, the identification of *S. aureus* was confirmed.

Sampling and Processing of Bacteriophages

Different crude samples for phage isolation were obtained from different regions in Baghdad including sewage (30–40 ml), waste water (30–40 ml), feces of sheep (20 g), chicken litter (15–20 g), swab from surgical lounge in Al-Imamein Al-Kadhymain Medical City Hospital during the period from January 2015 to June 2015. The samples were put in clean test tubes wrapped by parafilm, put in ice bag and transported to the laboratory in the same day. Part of the samples was stored in the refrigerator at 4°C until used, other part was worked in the same day.

Primary phages are those phages that were isolated from environmental specimens when were mixed with target bacteria. The procedure of isolating and propagating primary phages was done according to the methodology conducted in a recent patent.[7]

(1) Bacterial stocks were prepared by growing bacteria overnight on nutrient broth. About 100 µl of 10 bacterial isolates were mixed together in a sterile 50 ml test tube. Then, 2–3 ml of crude samples, which were derived from sewage, cattle feces, chicken litter, mastitis discharge swabs that might contain *S. aureus* specific phages were added to the mixture. Then, 2–3 ml, equal volume, of nutrient broth and 2 ml of Lambda buffer were added to the mixture as well. Then, the mixture was incubated overnight at 37°C.
(2) Next day, 5 ml of the crude mixture were dispensed into a sterile 15 ml test tube, centrifuged at 1,000 g for 3 min at room temperature.
(3) One ml of supernatant was transferred to 1.5 ml Eppendorf tube. Then, 1:10 v/v chloroform was added to the supernatant with gentle shaking for 7–10 min at room temperature to lyse the remaining bacteria.
(4) Centrifugation of the Eppendorf tubes at 1,000 g for 3 min; the supernatant was transferred into new Eppendorf tube and equal volume of lambda buffer was added. Thus, the primary phage suspension, if any, was produced.

Virulent phages were screened by phage spotting test on a nutrient-agar. Phage spotting can be used to provide a first approximation of the ability of a phage to lyse certain bacterial isolates. The formation of clear zones suggested the presence of lytic phages.[8]

At first, the target bacteria were refreshed in nutrient broth at 37°C for 24 h. After overnight incubation, 200 µl of the bacterial broth were poured on to the nutrient agar plate to make bacterial lawn. After 2–3 min, the lawn should have been dried. Using a mechanical pipette, 10 µl of primary phage suspension were dropped on to the surface of the bacterial lawn and were allowed to dry before incubating at 37°C for 24 h. On the next day, a lytic and specific phage can be discovered for the target bacteria if zone of lysis was developed at the spot where the primary phage suspension was applied.

Then, specific lytic phages to MDR *S. aureus* were picked up by sterile loop and put into 1 ml of Lambda buffer in 1.5 ml sterile Eppendorf tubes with gentle shaking for 5 min.

About 1:10 v/v chloroform was added to the lysate with gentle shaking for 5–7 min at room temperature. Host cell debris was pelleted by centrifugation at 1,000*g* for 3 min, and the supernatant containing phages was transferred to 1.5 ml sterile Eppendorf tubes and stored at 4°C. The supernatant was called transient phage stock suspension.[9]

Extraction of Endolysin

Up to 100 ml of broth containing bacteria (*S. aureus*) was incubated for 18 h at 37°C. Next day, the bacteria was put in 1 L of broth for 3 h (1×10^{12}). A total of 300 ml of the bacteriophage at titer 1×10^{13} pfu/ml was added for 20 min (1:10 MOI) after dividing the total volume in 50 ml tubes and then were put directly in ice. They were centrifuged at 10,000 rpm for 15 min and the sediment was taken. The sediment was put in 6 ml of 0.05 M phosphate buffer + deoxyribonuclease (5 mg). Then it was incubated for 60 min at 37°C. Ethylenediaminetetraacetic acid (EDTA) (0.005 M) was added and centrifuged at 10,000 rpm for 1 h and the supernatant was taken. Disodium tetrathionate (0.3 M) was added and mixed for 1 h at 4°C. Ammonium sulfate was added to 85% saturation and incubated for 18 h at 4°C. Next day, this was centrifuged at 10,000 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×) conc. And pH = 6.1 at 4°C overnight was done. Then it was added to column chromatography Sephadex G100 in 0.1 M phosphate buffer (NaCl = 8 g/l, KCl = 0.2 g/l, Na_2HPO_4 = 1.4 g/l, KH_2PO_4 = 0.2 g/l) pH 6.1, in 18 × 0.5 cm column. They were collected in 0.5 ml Eppendorf tubes at 10 min intervals. From each Eppendorf tube, 10 µl was dropped by using automatic pipette to bacterial lawns of the specific bacteria to see which Eppendorf tube contain the endolysin, if any.

In vivo Therapeutic Challenge of the Extracted Phage Endolysin

Albino *Rattus norvegicus* males of mean body weight 25 ± 1.5 g per mouse; the age of these mice was 2 months. The therapeutic effect of extracted endolysin was evaluated by using three groups of mice for each bacterium (*S. aureus*) each group was composed of five mice.

The first two groups of mice received intraperitoneal (IP) injections of 400 µl aliquots of bacterial suspension at concentration 10^8 CFU/ml. One of these groups, 0.4 ml of (80 µg/ml for *Staphylococcus* and 20 µg/ml for *Pseudomonas*) of a specific endolysin was injected 3 h after the bacterial challenge to evaluate the ability of administered endolysin to rescue the tested bacteremic mice from the inevitable fate of death by a bacterial infection (220). On the other hand, the third group received only IP injection of 0.4 ml of (80 µg/ml for *Staphylococcus* and 20 µg/ml for *Pseudomonas*) of a specific endolysin.

After disinfecting the area of injection by 70% alcohol, the first group, control group, mice were injected IP with 10^8 CFU/ml of the bacterial isolate alone. Then, every hour, the mice of control, test, and endolysin only groups were monitored for their health and physical activities and timely health score was recorded. According to Biswas,[10] certain health scoring system was used as shown in Table 1.

Results

Bacteriophage Cocktails

In this study, three phages active against *S. aureus* were isolated and purified. All of the isolated phages formed visible plaques in the early stage when tested on bacterial lawn of specific MDRs *S. aureus*. They were isolated directly from the environment by showing lysis on bacterial lawns of MDR *S. aureus* as shown in Fig. 1.

Characteristics of these phages were determined by the diameter, clarity/turbidity, margin cut, and shape of their plaques. The size of plaques ranged between 1.5 and 2.6 mm with a mean of 1.97 mm. The plaques morphology of the three phages ranged between oval 1/3(33.33%) and circular 2/3 (66.66%). Plaques clarity is different among the three phages; it ranged between clear 1/3 (33.33%), and semi-clear 2/3 (66.66%). Margin cut of the four phages were irregular 3/3 (100%) as shown in Table 2.

Endolysin Extraction

Endolysin was successfully extracted from all the three *S. aureus* bacteriophages by using Sephadex G100 column chromatography.

Table 1. **The health scoring system of bacteremic mice**

Health signs	Health level
Normal and unremarkable condition	5
Slight illness (lethargy and ruffled fur)	4
Moderate illness (severe lethargy, ruffled fur, and hunched back)	3
Severe illness (severe lethargy, ruffled fur, hunched back, and exudative accumulation around partially closed eyes)	2
Moribund state	1
Death	0

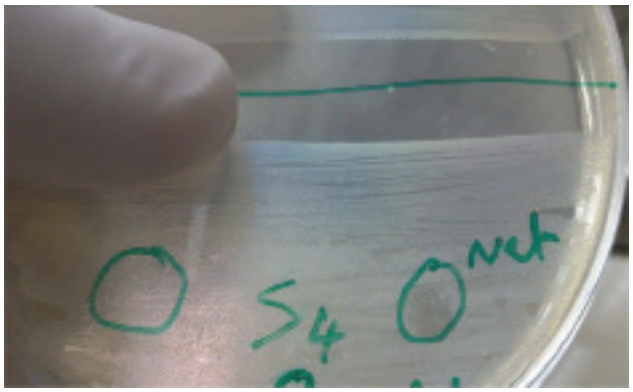

Fig 1. **Phage spot assay of bacteriophage to *Staphylococcus aureus*.**

Table 2. **The plaque characteristics of the isolated phages to MDR *Staphylococcus aureus* bacteria**

Bacteriophage isolates	Plaques size (mm)	Margin cut	Plaques clarity	Plaques shape
p*Sa*1	1.5	Irregular	Clear	Circular
p*Sa*2	2.6	Irregular	Semi-clear	Oval
p*Sa*3	1.8	Irregular	Semi-clear	Circular

p*Sa*, phage to *Staphylococcus aureus*.

After Sephadex G100 chromatography, each bacteriophage lysate gave eight Eppendorf tubes of 0.5 ml eluted fluid, one of them showed positive result on corresponding bacterial lawns as shown in Table 3.

The optical density of bacterial broth for each bacteria was measured initially once at zero time, just before the addition of corresponding endolysin. After the addition of the purified endolysin after 3 days of their extraction with the concentrations (80, 70, and 95 µg/ml), the optical density was measured every 5 min till 1 h. The optical density of the tested bacterial broth was obviously decreasing with time as shown in Tables 4 and 5.

Endolysin Therapy

Fifteen mice were used, ten of them were injected IP with 400 µl aliquots of bacterial suspension of concentration 10^8 CFU/ml, five of these (group two) injected with 0.5 ml of 80 µg/ml of a specific endolysin after 2 h. While the last five mice (group three) were injected with 0.5 ml of 80 µg/ml of a specific endolysin alone.

The result of this experiment showed that the health score of the first group (five mice injected with bacteria alone) started to decrease after 2 h, afterward the health score declined progressively. They died after 8 h. On the other hand the second group

Table 3. **The fraction that endolysin like activity was found for each bacteriophage**

Bacteriophage	Endolysin positive tube
p*Sa*1	4th
p*Sa*2	3rd
p*Sa*3	4th

p*Sa*, phage to *Staphylococcus aureus*.

Table 4. **Changes of optical density of bacterial broth with endolysin addition**

Time (min)	OD of *Sa*1*	OD of *Sa*2	OD of *Sa*3
0	1.34	1.34	1.34
5	1.3	1.32	1.29
10	1.25	1.27	1.24
15	1.21	1.24	1.16
20	1.12	1.19	1.09
25	1.05	1.14	1.02
30	0.96	1.07	0.94
35	0.85	0.98	0.85
40	0.76	0.92	0.74
45	0.64	0.83	0.63
50	0.56	0.74	0.5
55	0.52	0.65	0.46
60	0.49	0.56	0.43
Δ OD/min	0.014	0.013	0.015

*Net OD of *Sa*1–3 after deducting OD of the broth only (0.46). OD, optical density; *Sa*, *Staphylococcus aureus*.

Table 5. **Pace of OD change in every 5 min**

Time (min)	Δ OD of *Sa*1	Δ OD of *Sa*2	Δ OD of *Sa*3
5	0.04	0.02	0.05
10	0.05	0.05	0.05
15	0.04	0.03	0.08
20	0.09	0.05	0.07
25	0.07	0.05	0.07
30	0.09	0.07	0.08
35	0.11	0.09	0.09
40	0.09	0.06	0.11
45	0.12	0.09	0.11
50	0.08	0.09	0.13
55	0.04	0.09	0.04
60	0.03	0.09	0.03

OD, optical density; *Sa*, *Staphylococcus aureus*.

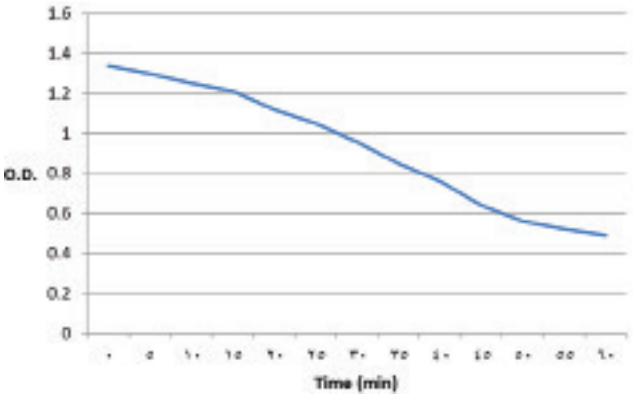

Fig 2. **The decline of OD values for bacterial growth of *Sa*1 due to lysis by (80 µg/ml) endolysin.**

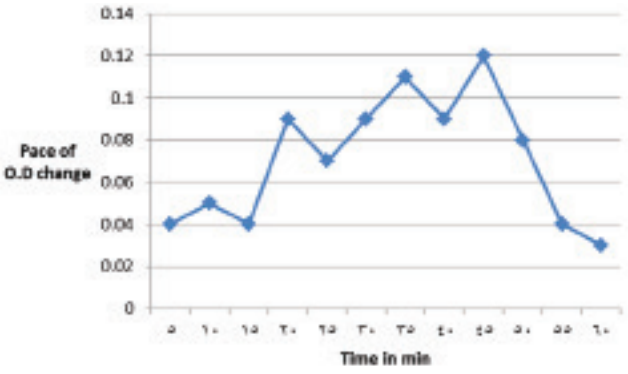

Fig 3. **Rate of OD change in every 5 min for bacterial growth of *Sa*1 due to lysis by (80 µg/ml) endolysin.**

(five mice injected with bacteria and endolysin) lived for more than 4 weeks with full physical activity and the third group (five mice injected with endolysin alone) also lived for more than 4 weeks with full physical activity as shown in Tables 6 and 7.

Discussion

Endolysins have been proven as efficient antibacterial agents against major Gram-positive pathogens such as *S. aureus*,[11] *Bacillus anthracis*,[12] *Streptococcus agalactiae*,[13] and *Streptococcus pneumonia*,[14] but their potential to combat Gram-negative infections remains undemonstrated.[15]

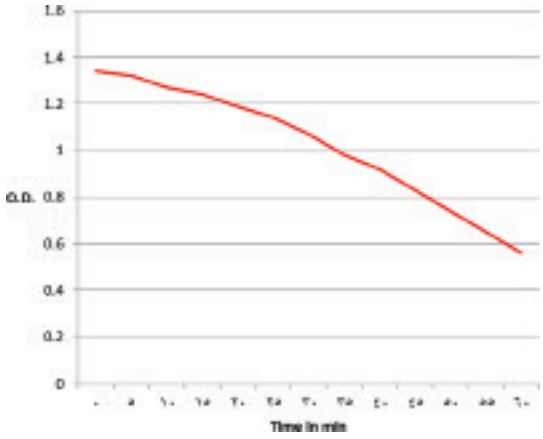

Fig 4. **The decline of OD values for bacterial growth of *Sa*2 due to lysis by (70 µg/ml) endolysin.**

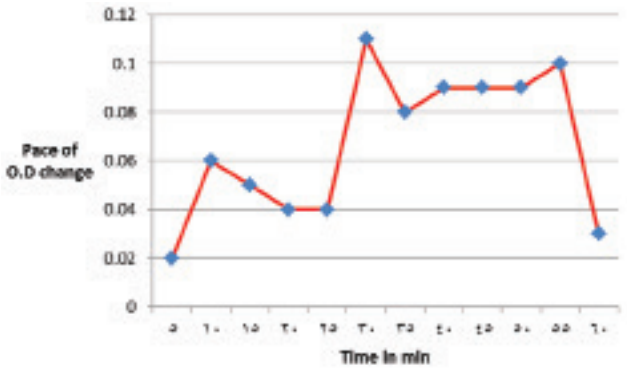

Fig 5. **Rate of OD change in every 5 min for bacterial growth of *Sa*2 due to lysis by (70 µg/ml) endolysin.**

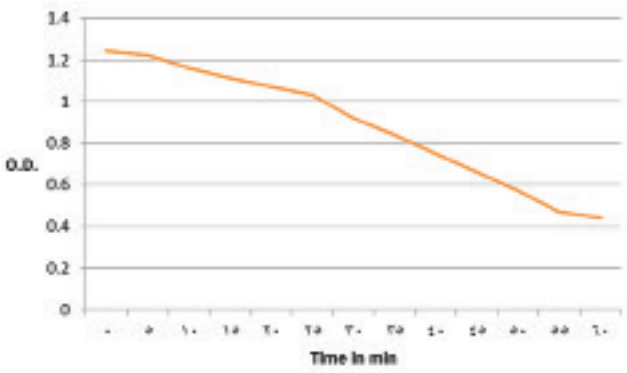

Fig 6. **The decline of OD values for bacterial growth of *Sa*3 due to lysis by (95 µg/ml) endolysin.**

Phage lysin therapy is a possible alternative to antibiotics for the treatment of bacterial infections. Indeed, it has proven to be medically superior to antibiotic therapy.[16,17] Our results support this notion, as phage lysin was shown to be highly efficacious against infections caused by inoculation with antibiotic-resistant *S. aureus*.

In this study, three phages for different *S. aureus* were isolated and characterized. The phages demonstrated high lytic activity underscoring its great potential to treat *S. aureus* infections.

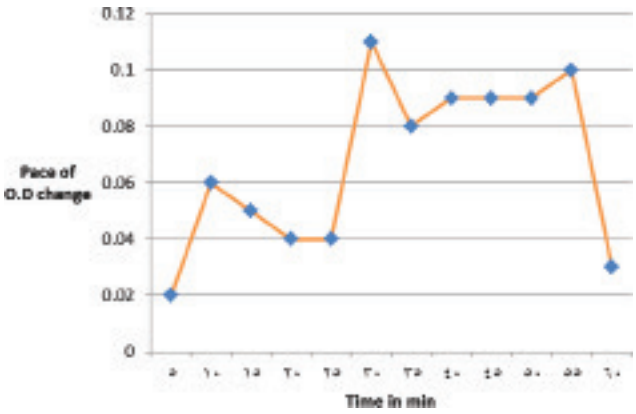

Fig 7. **Rate of OD change in every 5 min for bacterial growth of *Sa*3 due to lysis by (95 µg/ml) endolysin.**

Table 6. **Health score of mice injected with bacteria alone or with endolysin**

Hours	Health scores bacteremic group 1	Median of health scores of bacteremic group 1	Health scores of test group 2 (bacteria + endolysin)	Median of health scores of test group 2 (bacteria + endolysin)	*P*-value (Mann-Whitney test)
0	5,5,5,5,5	5	5,5,5,5,5	5	0.920
2	3,3,4,3,2	3	3,4,3,3,4	3	0.465
4	3,2,3,3,1	3	5,5,4,4,5	5	0.012
6	1,1,1,1,0	1	5,5,5,5,5	5	0.012
8	0,0,0,0,0	0	5,5,5,5,5	5	0.012

Table 7. **Health score of mice injected with endolysin alone or nothing**

Hours	Health scores endolysin only group 3	Median of health scores of endolysin only group 3	Health scores of healthy group 4	Median of health scores of healthy group 4	*P*-value (Mann-Whitney test)
0	5,5,5,5,5	5	5,5,5,5,5	5	0.920
2	5,5,5,5,5	5	5,5,5,4,5	5	0.674
4	5,5,5,5,5	5	5,5,4,4,5	5	0.347
6	5,5,5,5,5	5	5,5,5,5,5	5	0.920
8	5,5,5,5,5	5	5,5,5,5,5	5	0.920

The experiments presented here revealed that a single intraperitoneal injection of the corresponding lysin rescued mice from death due to *S. aureus* even when bacteremia was already well-established. We observed that effective protection was achieved in mice when a single dose of 0.5 ml of 80 µg/ml of a specific endolysin was administered 2 h after inoculation of *S. aureus*.

The results obtained in this experiment is encouraging, since our findings and those of others[18] support the development in a near future of modified procedures to improve the use of lysins in a more efficient way. For example, intravenous administration of the enzymes may confer a superior protection to animals when used later after the challenge. Most interestingly, a single intraperitoneal injection of endolysin was sufficient for a complete cure of mice. It also provides a rapid and specific lytic activity, making these proteins very promising candidates in current antimicrobial therapies.

We have shown here that bacteriophage lysins from *S. aureus* can be used to reduce the bacterial burden of these bacteria efficiently which are multidrug resistant, both *in vitro* and *in vivo*. Our study emphasize the potential therapeutic role of phage lysins for treatment of Gram-positive bacterial infections. Animal studies have generally supported the utility and safety of endolysin phage therapy against bacterial pathogens, like *S. aureus*.[19,20] In this study, endolysin phage therapy of septicemic laboratory mice, through IP injection with MDR *S. aureus*, showed that administration of endolysin resolved the infection dramatically.

It was found that the treatment with endolysin provided significant protection against mortality in mice infected with inocula of 10^8 CFU of highly virulent *S. aureus*. The current results demonstrated the *in vitro* efficacy of endolysin phage therapy and a proof for the concept of successful *in vivo* endolysin phage therapy.

The route of endolysin phage administration was particularly important to the efficacy of the treatment. Intraperitoneal route provides significant protection similar to that of intravenous route.[21] It was stated that endolysin for *S. aureus* phages administered by the IP route are distributed, in high titers, more rapidly and delivered for a more sustained period of time to all of the tissues.[21]

In these experiments, the control group of mice, which were bacterially infected without endolysin phage therapy, showed that all the mice died within just 8 h. Actually using a severe model of sepsis that leads to 100% lethality of mice in less than 24 h is a difficult challenge for every imaginable antibacterial agent. Two hours after bacterial injection, health score of mice began to decline progressively. A single injection of endolysin, rescued 100% of MDR *S. aureus* septicemic mice. Thus, this study showed that endolysin administered in a single dose 2 h post bacterial infection resulted in 100% survival of treated mice when compared to the control group. ■

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