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P-27/HP Endolysin as Antibacterial Agent for Antibiotic Resistant *Staphylococcus aureus* of Human Infections

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Abstract *Staphylococcus aureus* causes a wide range of suppurative infections in humans and animals. Due to its high virulence, ability to adopt various environmental conditions, and acquired multiple drug resistance, treatment of such infections has become difficult. Therefore, there is an immense need to develop alternate drug modalities to control this pathogen. In past few years, phage-encoded endolysin therapy has emerged as a new hope not only due to its ability to specifically kill the target bacteria irrespective of their antibiotic sensitivity but also because of minimum or no side effects, a problem associated with antibiotic therapy. In this article, we report purification of a broad spectrum anti-staphylococcal endolysin (P-27/HP endolysin) encoded by phage P-27/HP isolated from sewage water. On SDS-PAGE endolysin resolved in three polypeptides of molecular weights 33.5, 48.6, and 62.2 kDa. Endolysin exhibited maximum in vitro lytic activity at temperature between 35 and 40°C and pH 7.0. In vivo experiments revealed considerable (99.9%) elimination of *S. aureus* 27/HP from spleens of endolysin-treated mice and had saved them from death due to bacteremia caused by *S. aureus* 27/HP challenge infection. Thus, P-27/HP endolysin offers suitable substitute of antibiotics to control *S. aureus* infections.

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Introduction

Staphylococcus aureus is commensally occurring opportunistic pathogen of human and other animals. In humans, it causes several nosocomial and community acquired infections leading to relatively milder skin diseases to severe conditions such as pneumonia, meningitis, endocarditis, toxic shock syndrome, and septicemia [3, 12]. Its enormous ability to adopt various environments and resistance to most antibiotics including methicillin and vancomycin has ensued it high virulence attributes [4, 13] and as a consequence has created serious problem to physicians and attracted attention of the researchers to explore appropriate alternative therapeutic regimens with the potential of eliminating such infections irrespective of their antibiotic sensitivity [20, 21].

Bacteriophage therapy has emerged as potential substitute of antibiotics against pathogenic bacteria. Phage virions have been extensively employed to control *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Klebsiella*, and other bacteria in human and animals [6, 7, 10, 17]. Although phages lytic activity against bacteria is natural and non-toxic, but, resistance development in host bacterium, presence of bacterial toxins in phage preparations, development of phage neutralizing antibodies, complicated pharmacokinetics, and transducing nature are the key issues associated with their therapeutic applications [8, 19]. To overcome these limitations of whole virion therapy experimental approaches aiming to utilize endolysins (lysins) as antibacterial agent have been evaluated in last few years [1, 5]. Lysins are dsDNA bacteriophage-encoded

enzymes produced at the end of lytic cycle to degrade the cell wall of infected bacteria, thereby allowing progeny virions to be released [11].

This study was conducted with the objective of purification, characterization, and application of P-27/HP endolysin to control human infection-associated multidrug resistant *S. aureus* 27/HP in vitro and in vivo in experimental mice.

Materials and Methods

Bacterial Strains

Multidrug resistant *S. aureus* 27/HP and other staphylococcal strains used in this study were isolated from human clinical samples (acne, blood, cerebrospinal fluid, ear, eye, nasal, throat, surgical sepsis, and wounds) collected from hospitals in India and have been reported earlier [7]. Standard bacterial strains, *S. aureus* ATCC 6538, *Micrococcus luteus* ATCC 9341, and *Bacillus subtilis* ATCC 6633, were procured from Himedia.

Lytic Bacteriophage

Broad lytic spectrum phage P-27/HP used to produce P-27/HP endolysin was isolated from sewage water against indicator host strain *S. aureus* 27/HP [7].

Endolysin Preparation and Purification

To produce endolysin, early log phase *S. aureus* 27/HP broth culture growing at 37°C in NZCYM medium was inoculated with phage P-27/HP at 0.01 multiplicity of infection (MOI) as per Gupta and Prasad [7] and incubated until the complete lysis occurred. Cell lysate was concentrated to obtain crude endolysin following the method of Sonstein et al. [22] with appropriate modifications. In brief, after removing the cell debris by centrifugation (25,000×*g* for 15 min at 4°C), supernatant was concentrated by repetitive ammonium sulfate precipitation followed by dialysis against sodium phosphate buffer saline (0.07 M) for 48 h. Crude endolysin was purified by gel filtration chromatography using Sephadex G–200 (Amersham Biosciences) matrix. After draining off the void volume from the column, 44 elute fractions of 1 ml volume each were collected and analyzed for the endolysin with the help of spectrophotometer at 280 nm. Endolysin-enriched elute fractions constituting a sharp peak were pooled to obtain purified endolysin. Protein content of crude and purified endolysin was determined by Lowry's method. Endolysin was stored at 4°C for further application.

Purified P-27/HP endolysin was analyzed for its constituting proteins through 12.5% (w/v) SDS-PAGE gel electrophoresis (Bio-Rad mini gel apparatus) conducted in Tris–Glycine buffer at constant voltage (150 V) for 1 h. The gel was stained with Coomassie Brilliant Blue R-250. Broad range (10–200 kDa) protein molecular weight marker (Fermentas) was run alongside to determine the molecular weight of constituting polypeptides of the purified endolysin. Gel image was captured and analyzed with Quantity-one software (Bio-Rad).

Endolysin in Vitro Lytic Activity

Endolysin dose and treatment time required to inhibit *S. aureus* 27/HP growth below a certain threshold was optimized in vitro. Likewise, temperature and pH conditions for endolysin activity were also standardized. In brief, exponentially growing ($\sim 10^9$ cfu/ml) *S. aureus* 27/HP broth cultures in NZCYM medium at 37°C were centrifuged to pellet the cells which were re-suspended in equal volume of fresh NZCYM medium and distributed in 5 ml volumes in glass culture tubes. For dose optimization, cultures were added with endolysin so as to keep the final endolysin concentration as 2.5, 5.0, and 10.0 µg/ml and incubated at 37°C for a period of 4 h. Treatment time was standardized by incubating endolysin-treated (10 µg/ml, final concentration) cultures at 37°C for 0, 1, and 2 h. Optimum temperature and pH conditions were determined by incubating endolysin added (10 µg/ml, final concentration) cultures at a range of temperatures (4, 10, 15, 20, 25, 30, 35, 40, 45, and 50°C) and pH (4, 5, 6, 7, and 8), respectively. Endolysin unaided cultures served the control in each of the four cases. Experiments were conducted in triplicate. Declination in bacterial viable counts in terms of colony forming unit (cfu) were determined by spreading 100 µl appropriately diluted aliquot from each culture onto the Brain Heart Infusion (BHI) agar plates. Plates were incubated overnight at 37°C and scored for bacterial colonies.

Endolysin Lytic Range

In vitro lytic range of P-27/HP endolysin was tested against 40 staphylococcal isolates [28 *S. aureus*, 4 coagulase positive staphylococci (CPS), and 8 coagulase negative staphylococci (CNS)] reported earlier [7], *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, *M. luteus* ATCC 9341, and *E. coli*. In brief, exponentially growing bacterial cultures were added with endolysin at concentration 10 µg/ml and incubated at 37°C for 2–6 h. Endolysin unaided cultures served the control. Endolysin activity was assayed by clearance in turbidity of bacterial culture.

Safety Test

Safety test of P-27/HP endolysin was conducted in mice model. Healthy adult mice of either sex injected with 250 μg of endolysin via intra-muscular, subcutaneous, intra-venous, and intra-peritoneal routes were monitored for 20 days.

Endolysin in Vivo Lytic Efficacy in Mice

In vivo lytic efficacy of P-27/HP endolysin against *S. aureus* 27/HP was assessed in experimental mice. Healthy adult mice of either sex weighing not less than 18–20 g were divided in 3 groups (G1–G3) of 5 mice each. Single replicates of G1 (negative control) and G2 (positive control) and triplicates (Subgroup 1, 2, and 3) of G3 (endolysin treated) were used. Each mouse except from G1 was infected with 0.2 ml (5×10^8 cfu/mouse) exponentially grown ($\sim 10^9$ cfu/ml) *S. aureus* 27/HP culture (following the lethal dose recommended by the Capparelli et al. [2] and Matsuzaki et al. [15]) through intra-peritoneal route. G1 mice were injected with normal saline. 24 h after challenge infection, mice of group G3 were subcutaneously post inoculated with 0.2 ml (250 μg/mouse, following Jado et al. [9]) purified P-27/HP endolysin. Mice from G1 and G2 were left untreated. Each mouse that survived was sacrificed at 4 day after the challenge infection. Spleens were dissected aseptically, homogenized in 0.85% normal saline solution, and analyzed for the presence of *S. aureus* 27/HP on the BP (Baird-Parker) agar plates.

Results

Endolysin

The phage–bacteria system used for endolysin preparation gave a high yield of crude P-27/HP endolysin (1.8 mg/ml). Gel filtration chromatography resulted with the elution of endolysin in three successive fractions with optical density (OD) 0.459, 0.523, and 0.412 (Fig. 1a) that were pooled to obtain final purified endolysin. Total protein concentration of purified endolysin was 1.25 mg/ml. SDS-PAGE analysis of P-27/HP endolysin envisaged three distinct polypeptides of molecular weights 33.5, 48.6, and 62.2 kDa. The polypeptide corresponding to 48.6 kDa appeared as most intense band (Fig. 1b).

In Vitro Lytic Activity

Dose optimization assay revealed that P-27/HP endolysin at concentration 10 μg/ml was adequate to drop *S. aureus* 27/HP population by 99.99%, i.e., more than 4.0 log units

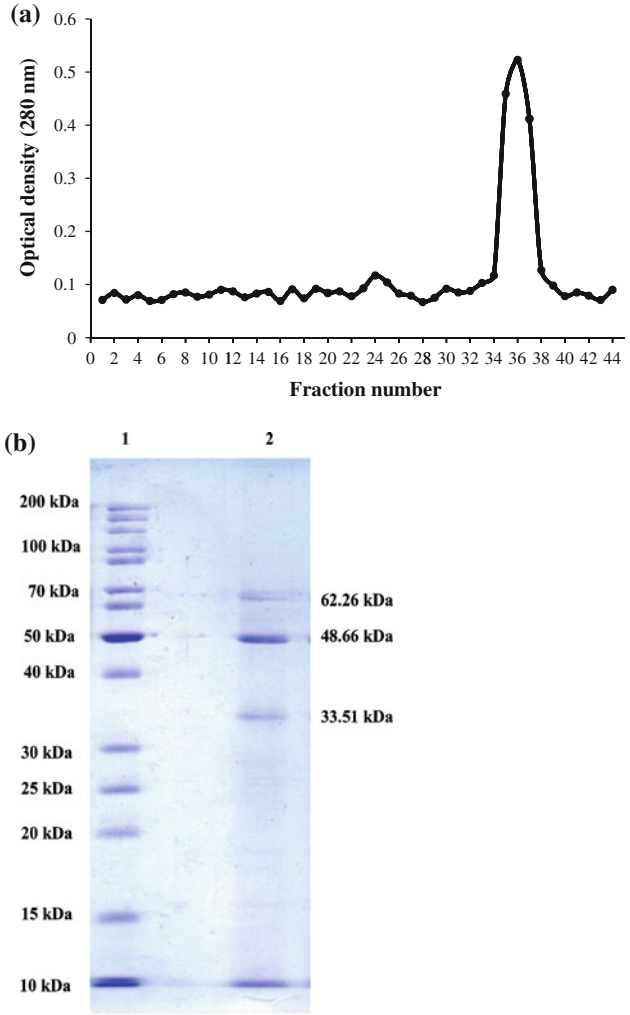

Fig. 1 Endolysin purification. **a** Elution profile of P-27/HP endolysin obtained by Sephadex G-200 gel filtration chromatography. Endolysin eluted in three successive elute fractions namely 35, 36, and 37 which made a sharp peak on the chromatogram and **b** SDS-PAGE of P-27/HP endolysin. *Lane 1*: Broad range (10–200 kDa) protein molecular weight (MW) marker. *Lane 2*: P-27/HP endolysin. Endolysin resolved in three distinct polypeptide bands of MW 33.5, 48.6, and 62.2 kDa. Polypeptide band of 48.6 kDa MW appeared as the major component of endolysin

($P < 0.00001$) from 9.32 ± 0.07 to 4.59 ± 0.05 mean $\log_{10}$ cfu in 4 h. Endolysin worked poorly at lower concentrations. At 2.5 and 5.0 μg/ml concentration, endolysin could reduce the bacterial count (mean $\log_{10}$ cfu) only by <1.0 ($P < 0.0001$) and ~1.0 log unit ($P < 0.00001$), respectively (Fig. 2a). Similarly, in case of time optimization assay, it was observed that 2 h incubation with endolysin (10 μg/ml) conferred substantial declination of ~4.0 log units ($P < 0.00001$) equivalent to 99.99% of *S. aureus* 27/HP viable count which was reached to 4.82 ± 0.43 ($t = 2$ h) from 9.18 ± 0.03 ($t = 0$ h) mean $\log_{10}$ cfu. An incubation period less than 2 h was suboptimal for endolysin lytic activity (Fig. 2b). Hereafter, for all in vitro experiments,

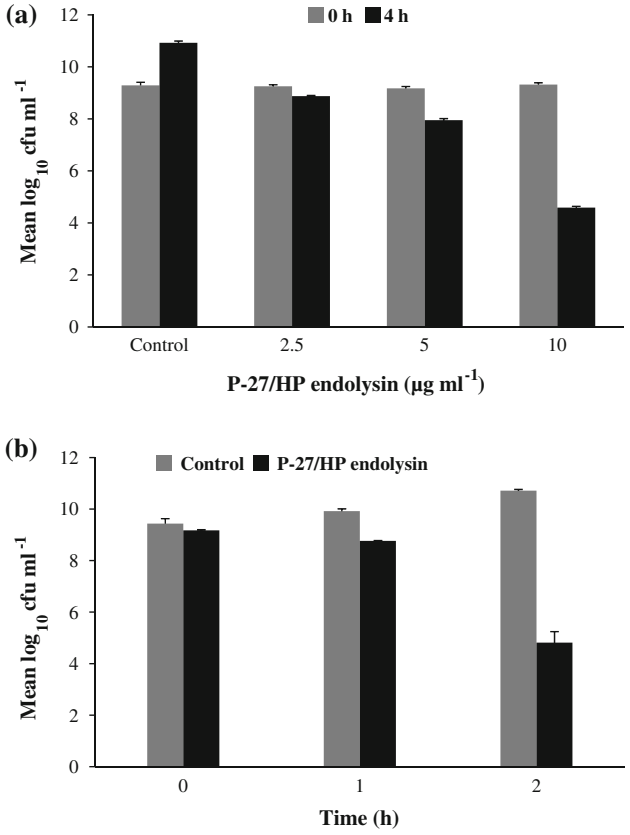

Fig. 2 Endolysin in vitro lytic activity against *S. aureus* 27/HP. **a** Dose optimization. Endolysin at the dose (10 µg/ml) could decline bacterial growth (cfu) substantially by more than 4.0 log units. **b** Time optimization. Endolysin needs minimum 2 h to reduce bacterial population (cfu) considerably (~4.0 log units)

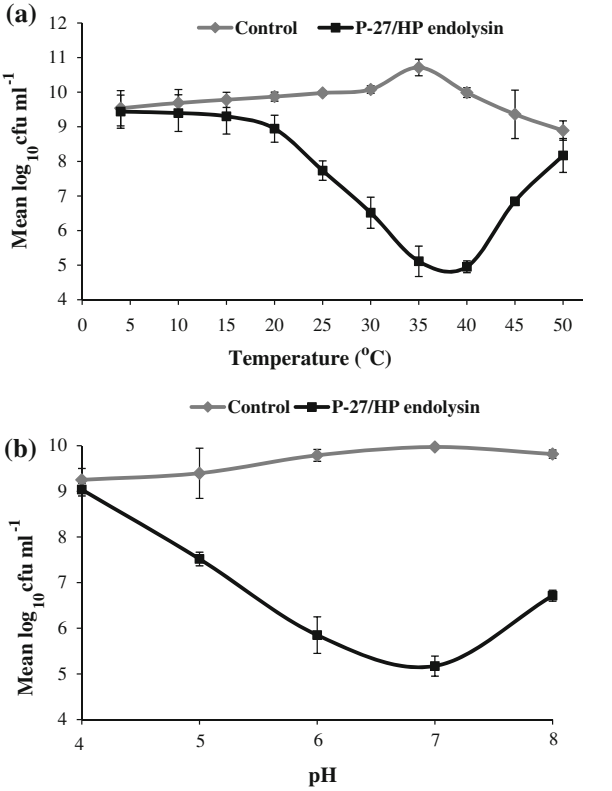

Fig. 3 Endolysin in vitro lytic activity against *S. aureus* 27/HP. **a** Temperature optimization. Endolysin works most efficiently at temperature between 35 and 40°C. **b** pH optimization. From the graph it is evident that optimum pH for endolysin activity is 7.0

endolysin dose and incubation period were kept as 10 µg/ml and 2 h, respectively. Endolysin worked most efficiently at temperature 35 and 40°C. A declination of about 4.0 log units ($P = 0.0001$ and 0.00001 for 35 and 40°C respectively) in *S. aureus* 27/HP count was noted at both these temperatures. A gradually decreasing lytic activity was seen at temperature below 35°C and above 40°C (Fig. 3a). Experiments done to determine optimum pH for endolysin activity envisage that endolysin was most efficient against *S. aureus* 27/HP at neutral pH and mitigated its growth by ~4.0 log units ($P < 0.00001$) in 2 h. Poor lytic activity was noted at pH 5, 6, and 8 which got seized completely at pH 4 (Fig. 3b).

Lytic Range

In vitro lytic spectrum analysis revealed that 17 of 28 tested *S. aureus* isolates were sensitive to P-27/HP endolysin. Endolysin was also effective against standard *S. aureus* ATCC 6538 strain, one CPS and one CNS strain. Conversely, heterologous bacterial strains such as *B. subtilis* ATCC 6633, *M. luteus* ATCC 9341, and *E. coli* were resistant to endolysin (Table 1).

Safety

Endolysin was safe to mice at the dose 250 µg. No adverse physiological effects or mortality in injected mice was recorded during 20 days of observation.

In Vivo Lytic Efficacy

Negative control mice (G1) that received saline only were healthy and exhibited normal performance over a 4 day follow-up period (data not shown). On the contrary, of the positive control mice (G2) that received *S. aureus* 27/HP (5×10^8 cfu/mouse) only, one mouse died on 1 day and two mice died on 3 day after the challenge infection. Remaining two mice were exhibiting chronic symptoms of bacteremia. The mean $\log_{10}$ cfu count enumerated in spleens of these two mice on BP Agar was noted to be 4.44 ± 0.2. Conversely, none of the mice died from G3 which were infected with *S. aureus* 27/HP (5×10^8 cfu/mouse) and injected with P-27/HP endolysin (250 µg/mouse). They showed noticeable clinical improvements at 24 h onwards of the endolysin treatment. Mean $\log_{10}$ cfu in spleens of these mice (Subgroups 1, 2, and 3) were recorded as 1.69 ± 0.23, 1.61 ± 0.14, and 1.52 ± 0.37

Table 1 In vitro lytic range of P-27/HP endolysin

Isolate or strain	Endolysin activity[a]
S. aureus 1/HP	–
S. aureus 2/HP	+
CPS[b] 3/HP	–
CNS[c] 4/HP	–
CNS 5/HP	–
S. aureus 6/HP	+
S. aureus 7/HP	+
S. aureus 8/HP	+
CNS 9/HP	–
CPS 10/HP	–
S. aureus 11/HP	+
S. aureus 12/HP	–
CPS 13/HP	+
S. aureus 14/HP	–
S. aureus 15/HP	+
S. aureus 16/HP	–
S. aureus 17/HP	–
CNS 18/HP	–
CNS 19/HP	–
CPS 20/HP	–
S. aureus 21/HP	+
S. aureus 22/HP	–
CNS 23/HP	+
S. aureus 24/HP	+
S. aureus 25/HP	+
Micrococcus sp. 26/HP	–
S. aureus 27/HP	+
CNS 28/HP	–
Micrococcus sp. 29/HP	–
S. aureus 30/HP	+
CNS 31/HP	–
S. aureus 32/HP	+
S. aureus 33/HP	–
S. aureus 34/HP	–
S. aureus 35/HP	–
S. aureus 36/HP	+
S. aureus 37/HP	+
S. aureus 38/HP	+
S. aureus 39/HP	+
S. aureus 40/HP	–
S. aureus 41/HP	–
S. aureus 42/HP	+
S. aureus ATCC[d] 6538	+
M. luteus ATCC 9341	–
B. subtilis ATCC 6633	–
E. coli	–

[a] Endolysin activity was determined by bacterial turbidity clearance assay. "+" clearance seen, "−" no clearance

[b] Coagulase positive Staphylococci

[c] Coagulase negative Staphylococci

[d] American type culture collection

which account for about ~7.0 log units (99.9%) declination in *S. aureus* 27/HP count (Fig. 4).

Statistical Analysis

Statistical analysis was done on Microsoft Office Excel. The mean $\log_{10}$ cfu values have been given as group mean ± standard deviation. *t*-test was performed to calculate *P*-values.

Discussion

High yield of P-27/HP endolysin obtained from the lysate of phage P-27/HP infected *S. aureus* 27/HP cells suggests that phages isolated from sewage water could be employed efficiently to isolate endolysins against human clinical staphylococcal isolates. Occurrence of three polypeptides of varying molecular weights (33.5, 48.6, and 62.2 kDa) assessed through SDS-PAGE explicates this endolysin to be the heterogeneous mixture of proteins. However, being appeared as most intense band it can be speculated that the polypeptide with molecular weight 48.6 kDa constitutes the functional endolysin whereas other two polypeptides are contaminants, though possibility of 33.5 and 62.2 kDa polypeptides for being active endolysin can not be ruled out. Endolysins of various molecular weights have been reported earlier. For instance, SDS-PAGE revealed the molecular weights of *Streptococcus pneumoniae* phage lytic enzymes Cpl-1 and Pal as 39.1 and 34.6 kDa, respectively [9]. Nelson et al. [16] have purified a phage lysin against group A streptococci and determined its molecular weight as 50 kDa by SDS-PAGE. On reducing

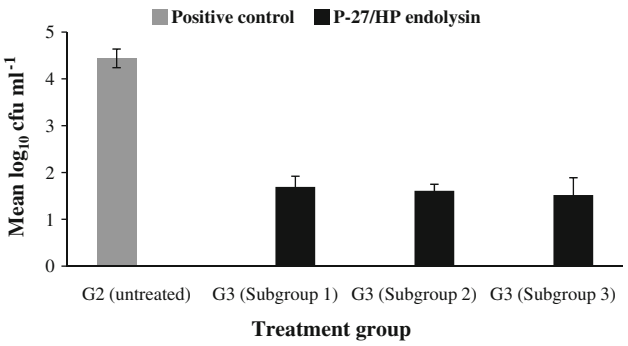

Fig. 4 In vivo lytic efficacy of P-27/HP endolysin in experimental mice. Each mice was infected with *S. aureus* 27/HP (5×10^8 cfu) except negative control (not shown in the graph). G2 (untreated, $n = 5$): mice without endolysin treatment. G3: Three independent subgroups (1, 2, and 3) of 5 mice each were treated with endolysin 1 day after the challenge infection. Mice were sacrificed 4 day after infection, and spleens were analyzed for *S. aureus* 27/HP. Graph shows the considerable reduction in bacterial population in endolysin-treated mice as against untreated controls

gel electrophoresis, P68 phage-induced lytic protein active against *S. aureus* showed a single polypeptide band of ~70 kDa [23].

Our findings of in vitro assay elucidate that the treatment with 10 μg/ml P-27/HP endolysin for a period of 2 h is sufficient to reduce *S. aureus* 27/HP growth significantly below threshold (>99%). Manoharadas et al. [14] reported that P16-17 endolysin at concentration 10 μg/ml was adequate for dropping the survival of exponentially growing *S. aureus* strain 68 by 95% in 1 h. The maximum in vitro lytic efficacy of P-27/HP endolysin observed at temperature nearing 37°C and neutral pH infers its adequate functionality in human body. Although relatively poor but having shown the lytic activity at a range of temperatures (25–45°C) and pH (5–8) endolysin would be able to stand diverse physico-chemical environments and would retain its functional integrity and, therefore, could be employed as therapeutic agent. Similar to P-27/HP endolysin, anti-staphylococcal LysH5 endolysin also works optimally at pH 7 and temperature 37°C [18].

Foregoing results obtained from in vitro lytic spectrum analysis suggest that P-27/HP endolysin possesses a wide lytic range specific to staphylococcal strains. Staphylococcal strains sensitive to P-27/HP endolysin were isolated from a range of human clinical samples such as wound, acne, surgical sepsis, and infections of eye, ear, nose, throat, cerebrospinal fluid, blood etc. which implies that this endolysin can be used as a generic antibacterial agent against staphylococcal infections of diverse body parts. The broader lytic spectrum exhibited by P-27/HP endolysin correspond with the findings of Obeso et al. [18] who isolated a wide lytic spectrum endolysin, LysH5, from bovine *S. aureus* infection which was capable of killing several pathogenic bovine and human *S. aureus* strains. LysH5 also found to be efficacious against various *Staphylococcus epidermidis* strains from human infections. However, it did not kill bacteria from heterologous genus such as *Bacillus*, *Streptococcus*, *Clostridium*, *Listeria*, and *Enterococcus.*

Mice administered with endolysin (250 μg/mouse) did not exhibit adverse physiological effects suggesting that endolysin preparation is safe to use as therapeutic agent. Studies by other groups also advocate the safety of endolysins in experimental mice [1].

Results of in vivo lytic efficacy assessed in experimental mice connote that P-27/HP endolysin conferred drastic elimination (99.99%) of targeted bacteria *S. aureus* 27/HP from spleens of infected mice. Occurrence of high splenic mean $\log_{10}$ cfu value in positive control mice also supports the above concept. These findings suggest that a therapy employing this endolysin may be the candidate to control *S. aureus* infections in human. In last few years, several studies have shown the successful application of endolysins purified from wild-phage strains as well as recombinant ones to protect bacteremia and death in mice experimentally infected with antibiotic resistant *S. aureus* and other pathogenic bacteria [5, 17, 24].

In conclusion, this study provides a model for large scale production of endolysin from phage–bacteria system and gives strong evidence for the safe and efficacious application of endolysin against antibiotic resistant staphylococcal strains of human clinical origin. However, further characterization of the endolysin is necessary before using it for human trials.

Conflict of interest Authors declare that they have no conflict of interest.

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