

Evaluation of bacteriophage therapy against *Staphylococcus aureus* infections using a silkworm larval infection model

Introduction: *Staphylococcus aureus* (*S. aureus*), which is a Gram-positive coccus, it can cause suppurative disease, food poisoning, and opportunistic infection in humans and animals. Some *S. aureus* strains can become resistant to methicillin, and they can attenuate the therapeutic effect of the conventional chemotherapy. Methicillin-resistant *S. aureus* (MRSA) is now spread in not only hospital but also community, and so it causes serious infections and possibly lethality in some cases. Thus, MRSA infection is needed to be paid with much of attention.

In such the critical situation led by MRSA, the newly therapeutic measures, which are potentially different from the conventional chemotherapy, are clinically desired to be introduced. A possible solution to this is bacteriophage (phage) therapy. Phage is a bacterial virus which can infect particular host bacteria and can lyse them at the end of the infection. Phage therapy is therapeutic application of phage to treat bacterial infections using phage host specificity and bacteriolytic property. In phage therapy, each therapeutic phage is a key tool for successful treatments. Thus, safety and antibacterial effectiveness of each therapeutic phage candidate should be carefully examined.

Animal models used in the safety and antibacterial effectiveness examination in phage therapy development have been generally higher mammals such as mice, rats, and rabbits. However, the usage of the higher mammals raises many issues related to ethics, equipment, spaces, and facilities and costs. To circumvent these issues, invertebrates such as nematodes and insects have recently been proposed as alternative to mammalian models of infection. The silkworm larval model has been used to study bacterial pathogenicity, and drug pharmacokinetics, pharmacodynamics and toxicology. Silkworm larvae are readily commercially available and more accessible because of silk production. Hence, silkworm larvae can become reliable and effective animal model in phage therapy developments. To our knowledge, the silkworm larval model has not been considered for phage therapy developments.

Aims: In this study, (1) we newly isolated *S. aureus* phages from sewage, and selected those with broad host ranges and strong bacteriolytic activity. (2) The therapeutic applicability of the phages was then investigated using the silkworm larval model, in comparison with the septic mouse model.

Material and Methods:

(1) Selection of appropriate therapeutic phage candidates.

Isolation of phage: *S. aureus* phages were isolated from sewage in Kochi City, and were cultured with *S. aureus*.

Analysis of phage: Phages were cultured with *S. aureus* strain SA27 in 300 mL of liquid medium, and were purified by CsCl density-gradient ultracentrifugation or iodixanol density-gradient ultracentrifugation. The purified phage was observed under transmission

electron microscopy, the genome size was estimated by pulsed-field electrophoresis, and the virion proteins were separated by SDS-PAGE and sequenced by N-terminal amino acid sequencer.

(2) Examination of safety and therapeutic effects using silkworm larval infection model.

Preparation of S. aureus-infected silkworm larval model: Silkworm larvae (the second day of the fifth instar) were subcutaneously inoculated on the dorsal part with 0.05 mL of *S. aureus* strains SA27 and SA14 at 27 °C.

Phage therapy experiments in the silkworm infection model: Lethal doses of *S. aureus* strains SA27 and SA14 were inoculated into the silkworm larvae. Each purified phage was diluted in HIMC to MOIs of 1, 10^{-1} , 10^{-2} , or 10^{-4} relative to the lethal dose. The phage suspension (0.05 mL) or an equal volume of the control (HIMC) was injected into the hemolymph of each infected silkworm larva through the opposite side of the dorsal surface at 10 min, 6 h, 12 h, or 24 h after the infection.

Phage therapy experiment in the mouse infection model: Fourth week-old mice were intraperitoneally inoculated with the lethal bacterial dose 2.0×10^9 cells / 0.2 mL. The purified phages diluted with HIMC to MOI 100, 10, 1, 0.1, and 0.01 relative to the inoculated bacteria quantity were intraperitoneally inoculated. Mouse survival rates were calculated during at least 1 week.

Results and discussion:

(1) Twenty-nine *S. aureus* phages were isolated from sewage, and two of them, S25-3 and S13', were selected based on their broad host ranges and bacteriolytic activity. Both of them showed host range of 83.1% (74/89) with strong lytic activity on *S. aureus* including the MRSA strains.

(2) The lethal doses of the *S. aureus* strains SA27 and SA14 in silkworm larvae were determined to be 3.8×10^7 and 1.6×10^7 bacteria in 0.05 mL, respectively. Next, the effects of the delayed administration of phage were evaluated. The phage-treated groups at an MOI of 1 exhibited significantly higher survival rates than the HIMC-treated control groups on day 2 ($P < 0.05$). The bacterial and phage concentrations were measured in the silkworm larval hemolymph: either phage S25-3 or S13' (at MOI = 0.01) was administered 10 min after pre-inoculation with the *S. aureus* strain SA27. The bacterial concentration was time-dependently lower in the phage-treated groups than in the control group 24 h after the infection with statistical significance. On the contrary, the phage concentrations increased time-dependently in the groups treated with phages S25-3 and S13' with statistical significance. These phage concentration increases imply that phage replication and bacterial destruction by phage occurred in silkworm larval infection models, suggesting the distinct feature of phage therapy.

Moreover, in septic mouse model infected with strain SA27, the groups treated with phages S25-3 and S13' showed 100% and 50% survivals at MOI = 1 on day 2, respectively. The therapeutic effects in the mouse infection model were similar to those (83.1%) in the

silkworm larval infection model.

Conclusion: The results suggest that phages S25-3 and S13' are eligible as therapeutic candidates, and the silkworm larval model is valid for the evaluation of phage therapy as well as mouse models.