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The Effectiveness and Safety of Phage KA95 Treatment against Staphylococcus aureus in Vitro and Mice

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Abstract
***Background**: A new approach using phages to treat diseases caused by drug resistance Staphylococcus bacteria has been a contentious problem. **Objective:** Therefore, this study sought to understand the safety and effectiveness of phage treatment against Staphylococcus aureus in mice. **Methods:** A phage was isolated from sewage, screened through a double-layer assay, and incubated at 37°C for 24 hours. The isolated phage was confirmed against Staphylococcus aureus using a spot test. Staphylococcus strain was tested against various antibiotics to know the resistance of the bacteria. **Results:** The mice infected with phage showed 100% lytic activity against the host bacteria more than clindamycin post-infection. Treatment with clindamycin and phage showed 75% post-infection at 24 hours and 100% post-infection at 72 hours. The antibiogram test showed that the host Staphylococcus aureus was a drug-resistant bacterium. The in vitro test revealed that the phage KA95 was lytic after culture at 37°C for 24 hours. Also, the toxicity test proved that phage KA95 was safe and effective. **Conclusion:** This showed that the isolated phage KA95 is safe and effective, and its application should be studied and considered a bio-control treatment for Staphylococcus infection.*

***Keywords:** Antibiotics, Antibiogram test, Mice, Phage KA95, and Staphylococcus aureus.*

I. Introduction

Bacteriophage is a virus that infects and lives on bacteria to multiply. A phage is specific to a bacterial strain where they have a narrow host range. However, some phages infect more than one bacterial species as their host shows a broad host range. They contain proteins that enclose with DNA or RNA. Phage multiplies within a host cell by injecting its DNA or RNA into the bacterial cytoplasm [1].

Staphylococcus aureus is an opportunistic bloodstream bacterium that can cause disease in humans. The bacterium causes food poisoning resulting from eating food contaminated with harmful substances. As a result, patients suffered from diarrhea, vomiting, and sometimes death. The bacteria caused to skin, soft tissue, and healthcare-associated infections. The rates of disease in the community are increasing. Nurses in various communities and homes are at the highest risk of acquiring multi-drug resistance Staphylococcus aureus infections.

The rate of infection of Staphylococcus aureus in the world is different from country to country, and 27% infect bone, skin, and soft tissue. Besides, 6.3 to 13.9% of urinary tract diseases are caused by Staphylococcus aureus in Ghana, Senegal, and Nigeria.The bloodstream disease outbreak occurs in Africa, approximately 3.28 cases per 100,000 infected children in South Africa each year. Between 101 and 178 cases per 100,000 populations in Mozambique had the highest rate of infection in children aged less than 0-5 years [2]. In 1994, 1,273 cases in which 63.8% were males infected with Staphylococci bloodstream disease, and two-thirds of the populations were older than 70 years, which is 36.6% in Australia [3]. In 2010 different antibiotics were produced for animal health care and humane treatment. Despite the challenges, the cost of research and production of antibiotics increases while Staphylococcus bacteria continue to resist drugs and create a healthy disease. In 2014, the Staphylococcus strain severely infected humans and animals and resulted in a higher risk of infection to Public Health. As a result, Staphylococcus aureus resistance to antibiotics is more than the production of new drugs. Therefore, a new way of treatment is required to fight against these bacterial strains [4]. This study showed a new approach to combat drug-resistant Staphylococcus aureus using bacteriophage KA95 as an antibacterial agent.

Moreover, the phage KA95 used in this study is not a new way of treatment. In this regard, we isolated bacteriophage from sewage and wastewater to obtain a lytic phage KA95 that would be investigated and used against Staphylococcus aureus. In 2017, 120,000 people got infected with Staphylococcal bloodstream infection, and about 20,000 associated deaths occurred in the United States of America [5].

1.1. **Objective**

This study's principal goal is to investigate further the new approach to fighting multi-drug resistance Staphylococcus aureus using phage KA95 as an antibacterial agent.

- To establish the safety and effectiveness of lytic phage KA95 against Staphylococcus aureus in sewage and wastewater in Liberia
- To find out in vitro the lytic ability of phage KA95 against Staphylococcus aureus in Liberia, West Africa
- To understand the effectiveness and safety of phage KA95 treatment against Staphylococcus strain in vivo and mice.

II. Materials and Methods

2.1. Study Area

However, we conducted this study in the Microbiology Laboratory, Department of Biomedical Engineering, College of Life Science, Huazhong University of Science and Technology, China. The residents in this community have poor opportunities for drinking water, sanitation, and health care services [6]. The lack of these basic needs makes them vulnerable to severe infections caused by multi-drug resistance Staphylococcus bacteria.

2.1.1. Sample Collection

In this study, the researchers collected sewage samples in a sterile dark container from the sewage treatment plant in Wuhan, China. The raw sewage was placed in 50ml tubes and centrifuged at 10,000g for 15 minutes to remove cell debris. After that, 10ml of the solution was added to a 50ml Luria broth medium and mixed with 10mM calcium chloride and 10mM Magnesium chloride solution. Further, 10ml of phage KA95 samples was mixed with 100µl of Staphylococcus culture and incubated at 37^{o}C for 24 hours. The next day the sample was centrifuged at 10,000g for 15 minutes. And the phage lysate was filtered through a 0.22µm paper and kept at 4^{o}C before use.

2.1.2. Staphylococcus aureus Cultured

In this research, we collected a sewage sample from the sewage treatment plant, filtered and centrifuged it at 10,000g for 15 minutes. The bacterial strain collected was mixed with a sterilized 0.85% sodium chloride solution and incubated at 37^{o}C overnight. As a result, host Staphylococcus aureus formed on the culture plate were sub-cultured and stored at 37^{o}C before being used [7].

2.1.3. Isolation of Staphylococcus Phage KA95

However, 40ml of phage sample was mixed with 0.7% agar, incubated at 37^{o}C for 24 hours in a shaking incubator, and centrifuged for 15 minutes at a 10,000g rate per minute. The phage filtrate was filtered through a 0.22µm filter and serially diluted. Subsequently, 250µl of the tenfold diluent, mixed with 300µl of Staphylococcus culture and combine with 0.7% overlay and spread on a 1.5% agar plate, and incubated at 37^{o}C for 24 hours. The plaques formed were picked and purified using the double layer assay. The plaques were incubated at 37^{o}C for 24 hours and then centrifuged at 10,000g for 15 minutes. The plaques were filtered using 0.22µm paper and stored at 4^{o}C before being used in this study.

2.1.4. Staphylococcus Phage KA95 Multiplication and Purification

Phage KA95 sample was infected with the host Staphylococcus cells at the multiplicity of infection of 0.01 and incubated at 37^{o}C for 24 hours. The next day, the bacterial cell burst in the culture media, and 2% of chloroform was added to the phage lysate and incubated at 37^{o}C for 30 minutes with gentle shaking. The phage was centrifuged at 10,000g for 15 minutes, filtered through the 0.22µm paper and purified three times using agar plates. The high titer phage KA95 stocks at 10^{9} PFU/ml were multiplied in Luria broth media containing 10mM Magnesium Chloride and 10mM Calcium Chloride and centrifuged at 10,000g for 15 minutes while the researchers stored the phage KA95 lysate at 4^{o}C before use.

2.1.5. Staphylococcus Phage KA95 Confirmation

The host range of Staphylococcus phage KA95 was determined using the spot testing method and verified by a double layer agar assay. However, the phage lysate 10^{8} PFU/ml was mixed with 100µl Staphylococcus culture in 0.7% top agar and incubated at 37^{o}C for 24 hours. As a result, the phage KA95 infected and killed only Staphylococcus cells, while Klebsiella pneumonia and Escherichia coli were resistant to the KA95 phage [8].

2.1.6. The antimicrobial testing

The antimicrobial testing used in previous studies allows us to study the efficacy of the antibiotics against Staphylococcus aureus, including Cephalosporin, Penicillin, Vancomycin, Cefuroxime, Gentamycin, and Erythromycin. [9].

2.1.7. In Vivo /Animal Study

In this work, the researchers fed six mice aged 6 to 8 weeks with antibiotics and water from the Biomedical Research Institute in China.

2.1.8. Safety and Effectiveness of Phage Treatment

2.1.9. Preparation of Staphylococcus culture and phage KA95 sample for infection and treatment

The number of phage samples and Staphylococcus culture were centrifuged at 2,000g for 5 minutes, washed three times, and dispensed in a sterilized concentration of sodium chloride. Five (5ml) Staphylococcus culture, a 1ml phage KA95 sample mixed with 20ml Luria broth media, and incubated at 37^{o}C for 4 hours. Subsequently, the culture was centrifuged at 10,000g for 15 minutes and filtered through 0.22µm filters. The phage obtained was stored at 4^{o}C before use [10].

III. Results

3.1. Isolation of Staphylococcus Phage KA95

In this work, the researchers isolated phage KA95 from a rich sewage sample using the plaque assay to verify the presence of the phage in the sewage water. The technique was applied to purify phage KA95. The Staphylococcus aureus culture was then mixed with the phage dilutions, cultured on Luria broth agar plates, and left for 20 minutes to be dried. We spotted 10µl of Staphylococcus culture on the agar plates. The plates were left for another 20 minutes to be air dried, followed by incubation at 37^{o}C for 24 hours. When checked, clear zones of lysis formed on the host Staphylococcus cells, and the plaques were picked and serially diluted in sulfate-magnesium (SM) buffer and confirmed by the double-layer agar method.

The isolated phage KA95 was lytic, showed a narrow host range, and killed only Staphylococcus aureus, while Klebsiella pneumonia, Pseudomonas aeruginous, and Escherichia coli strains, were not burst by the phage KA95 [11].

3.1.1. Bacteriophage KA95 Isolated and Screened against Staphylococcus aureus in Vitro

In this research work, we isolated a lytic phage KA95 against Staphylococcus aureus to determine the virulent activity of the phage using a spot test. As a result, lytic phage KA95 formed lysis on culture plates.

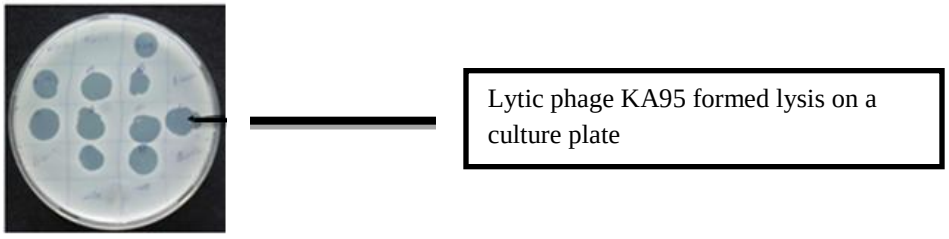

Figure1. Spot test showing clear plaques

The lytic bacteriophage KA95 isolated killed Staphylococcus aureus in vitro when used in the Luria broth at 37^{o}C for 24 hours.

3.1.2. The Antimicrobial Testing

In this study, the authors used an antimicrobial test to compare the susceptibility of phage KA95 and antibiotics against Staphylococcus aureus. Following this, antibiotics used include; Penicillin, Erythromycin, Vancomycin, and Gentamycin. As a result, Staphylococcus aureus was sensitive to Cephalosporin. Further, some of the drugs indicated smaller zones of infections which revealed a resistant range and therefore are considered non-effective against Staphylococcus aureus. Subsequently, drugs sensitive to the hostStaphylococcus aureus showed zones of lysis on the culture plates [12].

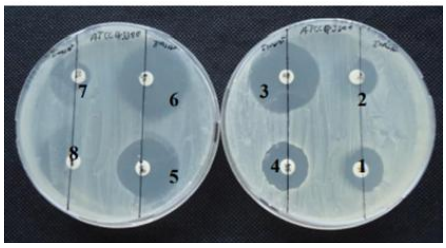

Figure 2: An antibacterial test showing the sensitivity of Staphylococcus aureus to antibiotics used in this study: Cephalosporin 30µg (resistant), Penicillin 1µg (resistant), Vancomycin 30µg (resistant), Cefuroxime 30µg (sensitive), Gentamycin 10µg (resistant), and Erythromycin 15µg (resistant).

3.1.3. In Vivo Study

3.1.4. Safety of Phage Therapy

This study revealed that the isolated phage KA95 was not virulent, infecting the treated mice, and none of the mice died. As a result, all the mice used in this study show no sign of rumpled fur or weaknesses. When infected with Staphylococcus culture with the non-infected and phage-infected mice groups, no change occurred in the body structure of the treated mice at 24 hours and 72 hours post-infection [13].

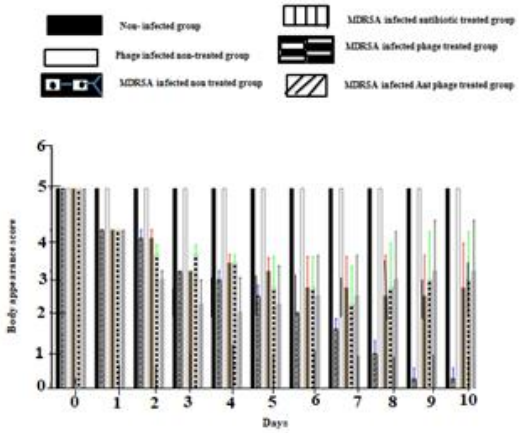

Figure 2.A graph showing the body structure of mice treated at 24 hours post-infection

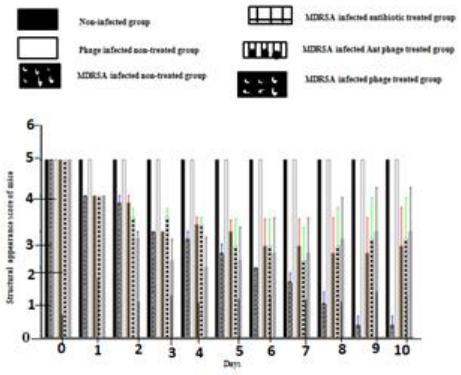

Figure3. Schematic graph showing body structure of mice treated at 72 hours post-infection

However, the highest survival rates of Staphylococcus aureus-infected mice at 24 hours post-infection compared to those treated at 72 hours post-infection to know how many mice infected. In this study, we compared the survival rates of the non-infected and phage-infected groups to the number of mice safe after treatment at 24 hours and 72 hours post-infection. From the starting point of infection at 24 hours and 72 hours post-infection, 100% of the survival rates were recorded. Immediately after treatment at 24 hours post-infection, 90% of the treated mice

survived in the treatment groups, while at 72 hours post-infection, only 10% of the mice survived [14].

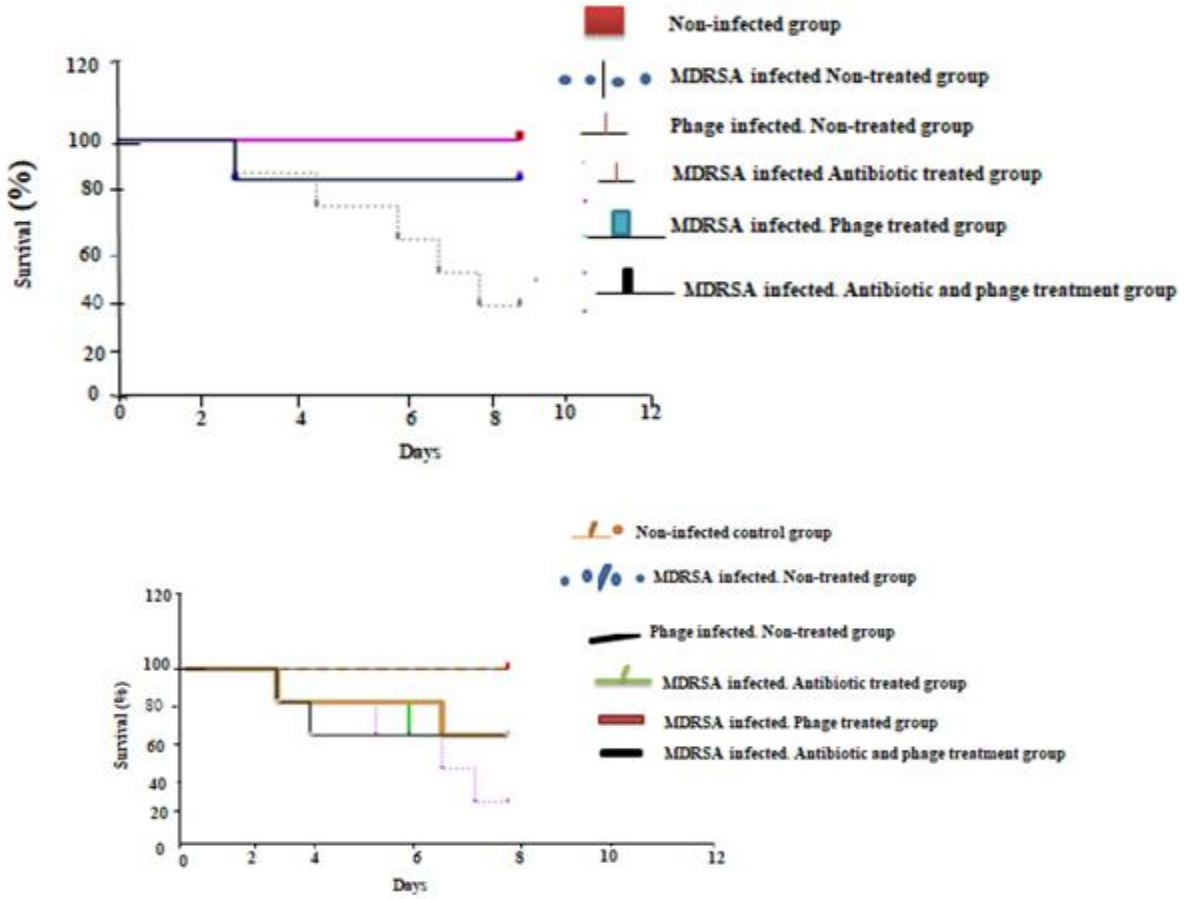

Figure4. Schematic graph showing mice groups that survived after infection at 24 hours post-infection (A) and 72 hours post-infection (B).

3.1.5. The Effectiveness of Phage Treatment

The multi-drug resistance Staphylococcus culture treated with a non-infected group at 24 hours and 72 hours post-infection showed the presence of Staphylococcus strain in the blood of the mice on day 10. Following infection, the phage control mice group treated at 24 hours and 72 hours post-infection showed no Staphylococcus bacteria in their blood from day 4 to day 10 [15].

72 hours post-infection treatment graph

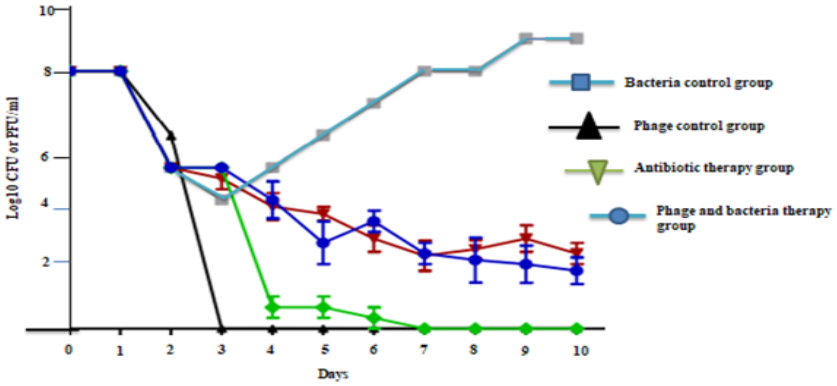

Figure 5.A schematic diagram showing blood samples on each day of the study. By day 10, all Staphylococcus bacteria had cleared from the phage-infected group compared to all other treatment groups.

In comparing both treatments, drug-resistant Staphylococcus aureus, when infected with phage, had no bacteria in the blood of the mice from day 9 to 10. Then a group of mice treatedwith clindamycin and phageKA95 10^8PFU/ml showed no bacteria in their blood. Therefore, when comparing the blood of non-infected and phage-infected control groups, Staphylococcus bacteria were not present in the blood of the treated mice [16]. On the other hand, the mice group treated with clindamycin and phage at 24 hours and 72 hours post-infection had no Staphylococcus bacteria in their blood.

IV. Discussion

This study has acknowledged that Staphylococcus aureus is resistant to penicillin, erythromycin, gentamycin, and vancomycin [17]. Consequently, the bacteria showed resistance to antibiotics. The epidemiology of Staphylococcus aureus has posed a Public Health problem in the world, especially in Africa and Asia, where the infectious rates increased from 39.8% to 81%. Furthermore, we infected a dose of phage 10^8PFU/ml with a group of mice and showed that all of the mice were safe in the results of the antibacterial testing [18].

In the comparative analysis of this study, previous work showed that mice infected with a dose of phage from day 7, at 24 hours and day 9, at 72 hours post-infection, did not show Staphylococcus strain in the blood samples of the treated mice. This finding proves that a dose of phage at 10^8PFU/ml is considered the best treatment against Staphylococcus aureus than a dose of clindamycin 8mg. As a result, mice treated with phage KA95 appeared healthy in their body appearance, and 100% of the mice survived. These results indicated that lytic phage KA95 did not produce any side effects nor discredit the hypothesis, which states that phage treatment is safe and effective against Staphylococcus infection. Further, those phages are specific to infect specific multi-drug resistance Staphylococcus strains. These findings agreed with past studies in which bacteriophages were considered the most effective treatment against Staphylococcus disease in infected mice than antibiotics [19].

Studying the effectiveness of phage KA95 against Staphylococcus aureus in mice, a single dose of clindamycin 8mg was used. And 10^8PFU/ml phage sample mixed with clindamycin 8mg infected with the mice to ensure the efficacy of the treatment against the treated mice. As a result, these treatments were not enough to cure the treated mice's bacterial infection. Consequently, the combined treatment plus clindamycin 8mg failed because of the resistance of the Staphylococcus strain against the drug [20]. The researchers evaluated the effectiveness of phage KA95 against Staphylococcus disease using the mice's body structure and the presence of bacteria in the blood of these mice. The overall findings showed that the infected mice were free from the infection in treatment this will allow the phage to become the best option to cure Staphylococcus disease patients.

V. Conclusions

- To conclude, the Staphylococcus strain is multi-drug resistance bacteria that live in sewage treatment plants.
- A dose of 10^8PFU/ml phage KA95 is most effective than a dose of clindamycin 8mg against Staphylococcus infection in mice.
- Staphylococcus phage KA95 is an antimicrobial agent, safe, and showed no side effects in treated mice.

VI. Problem Statement

The misuse of drugs has caused an increased risk of Staphylococcus infections against human health and food security. As a result, these antibiotics are ineffective against bacterial diseases. These have posed economic loss, especially in sub-Saharan Africa, where drug resistance to Staphylococcus infection increases due to poor public health policy and overuse of antibiotics in hospitals and clinics. Staphylococcus strains have caused cross infection in food production, water sources, and animal breeding. Because of these conditions, it is hard to control Staphylococcus diseases in water sources and food production sites. If these challenges are not solved, Staphylococcus strains will cause more diseases and death in humans and animals in communities. On that account, we called upon world scientists and drug companies to develop and produce new drugs for alternative treatment using phages as a bio-control agent against drug-resistant Staphylococcus bacteria.

VII. Recommendations

- To research phage treatment, there is a need to characterize and understand the role of lytic phages against Staphylococcus infections.
- To carry out a campaign on phage treatment, policymakers, and health practitioners should use phages as an antibacterial agent against multi-drug resistance Staphylococcus aureus diseases.

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