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Isolation and characterization of Salmonella typhi TA98 phage

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Abstract
Background*: The multi-drug resistance Salmonella typhi, like other bacteria, is most often reported as resistant to antibiotics in treating infections. The possible alternative to antibiotics is bacteriophage as anti-infective agents to control Salmonella typhi. The objective of this study was to isolate and partially characterize bacteriophages using Salmonella culture for the host range to determine the effectiveness of phages on Salmonella typhi.*
Methodology*: The bacteriophage specific to Salmonella typhi was isolated from a sewage sample collected from Tonji Hospital through phage enrichment. The presence of bacteriophage by the spot test method was done, following 10-fold dilution of the enrichment filtrate using soft agar 0.7% overlay method.*
Results: *The phage isolated against Salmonella typhi indicated a wide range of heat and pH stability. The phage morphology, measured by transmission electron microscopy, showed a structure comprised of a head with a diameter of 107nm and a contractile tail of 123nm. The phage exhibited good potential that reduced the numbers of Salmonella typhi cells, as shown by a decrease in the optical density at 600nm from 0.40 to 0.12.*
Conclusions: *This study showed that the isolated bacteriophage has much potential in phage therapy and other applications.*

Keywords: *Bacteriophage; Isolation; Salmonella typhi; Sewage; and Typhoid fever*

I. Introduction

Bacteriophage is a virus that infects bacteria. Bacteriophage can only multiply in bacterial cells [1]. It infects Salmonella bacteria by lytic cycle where the phage bursts the host cells. During the lysogenic life cycle, the phage integrates its DNA into the bacterial cytoplasm and divides and allows the prophage to be copied and passed along with the bacteria DNA and does not produce prophage. The bacteriophage reproduction can only stop when there are no host bacteria cells. Bacteriophage is found everywhere in nature and is known to infect and kill available bacterial cells [2]. Hence, the rapid spread of multi-drug resistance genes in Salmonella bacteria increases worry about the efficacy of antibiotics in the future. Bacteriophages have been in various studies like new, safe, and effective biocontrol methods for treating typhoid infections caused by Salmonella typhi. A double layer agar assay was used to purify and measure bacteriophages. The anti-infective ability of the lytic phage was measured by inoculating a 10µl phage sample to Salmonella culture on an agar plate and incubated at 37^{o}C for 24 hours. However, the effectiveness of the phage in different concentrations was studied using plaque assay. On host range activity, the isolated phage was effective against Salmonella typhi.

Salmonella typhi is a gram-negative, aerobic, rod-shaped bacterium characterized by somatic (O) and flagella (H) antigens. The somatic antigen (O) is found on the cell wall of Salmonella typhi, while the flagella antigen (H) is in the flagellated portion of the bacteria. The surface (Vi) antigen is called virulence factor that resists antibiotics and causes severe typhoid disease. Serotyping of Salmonella typhi helps in classifying the various strains that cause disease. Many people die every year from eating infected food. Salmonella typhi is a pathogen that causes typhoid fever in infants with a 10-20% mortality rate. The modes of transmission occurred through the consumption of drinking water or food contaminated with the feces of people who have typhoid fever or people who are chronic carriers of the responsible bacteria.

About 21.7 million cases were identified, from 1990 to 2013 among people and children aged between 5 to 19 years in South-Central and Southeast Asia [3]. In 2004-2005, salmonella typhi infected more than 42,000 people, and 214 deaths occurred in the Democratic Republic of Congo. In 2016 Pakistan experience outbreaks of drug-resistant typhoid bacteria [4]. In addition, 181,000 cases and 161,000 deaths happened in Asia and Africa in 2013. In 2017, 1,098 cases of typhoid fever occurred, and 90.9% of people in Europe were infected from travel to Asia [4]. In the United States, about 400 cases occur each year, and 75% of them are infected while traveling to other countries around the world [5]. In 1992, 7,400 cases of Salmonella typhoid occurred and 84 deaths in Liberia.

1. 1.Objective

The primary objective of this study was to isolate and partially characterize bacteriophage using Salmonella typhi bacterium for the host range to determine the effectiveness of phages against drug-resistant Salmonella typhi.

II. Materials and Methods

2.1. Preparation of Sulfate-Magnesium Buffer

The following compositions were prepared for the buffer solution: 5.8g NaCl, 2g $MgSO_4$. $7H_2O$, 50mL Tris-cl 1M, pH7.4, and 5mL Gelatin, were added to 1000mL of deionized water and mixed. After mixing the solution, it was autoclaved for 15 minutes at 121^{o}C and stored at 4^{o}C until use.

2.1.1.Preparation of Culture Media, Reagents, and Solution

2.1.2. Luria Broth and Agar Media

However, 25g of Luria broth powder was measured and weighed on the electronic balance, and dissolved in a volume of 1000mL of deionized water. The prepared solution was mixed and sealed with aluminum foil and autoclaved for 15 minutes at 121^{o}C.

There was 15g of agar powder measured and weighed on the electronic balance and dissolved in 1000mL of deionized water, and the prepared culture media was mixed and transferred in a 100mL flask and autoclaved at 121^{o}C for 15 minutes. After that, we stored the solution at 4^{o}C.

2.1.3. Preparation of 10mM Calcium Chloride ($CaCl_2$) and Magnesium Chloride $MgCl_2$ solutions

Essentially, 111.02g of Calcium Chloride ($CaCl_2$) was measured and weighed on an electronic balance dissolved in 1000mL deionized water autoclaved for 15 minutes at 121^{o}C and stored at room temperature 20^{o}C.In addition**,** 9.5g of solid Magnesium Chloride was measured and weighed on an electronic balance dissolved in a volume of 1000mL deionized water autoclaved for 15 minutes at 121^{o}C and kept at 20^{o}C until use.

2.1.4.Bacterial Culture and Strains

However, the Salmonella TA98 bacteria was a solid white powder in a glass-like tube purchased by Huazhong University of Science and Technology from the Chinese Center for Culture Collection in China dissolved in a volume of 250mL of deionized water inoculated on the Luria broth agar plate at 37^{o}C for 24 hours. These bacterial strains were placed in a vital tube wrapped with a parafilm tip and stored at 4^{o}C until use. After culturing host Salmonella TA98 bacteria, Escherichia coli, Staphylococcus aureus, and Klebsiella pneumonia were essential for the study.

2.5. Sample Collection and Investigation

During the study, sewage samples were collected from the Tongji and Jimao Hospitals in an open wide sewage tank with lots of enriched sediments to isolate bacteriophage. The sewage samples were placed in 100mL bottles and transported in a vehicle to the laboratory in August and September 2018. The samples were dispensed in 50mL tubes and centrifuged for 10 minutes at 10,000 rateper minute to remove bacterial cells. After that, 10mL of the mixture was added to a 40mL Luria broth medium and mixed with 10mM calcium chloride and 10mM magnesium chloride solutions [6]. After that, 10mL of phage samples mixed with 100µl of Salmonella culture was inoculated and incubated at 37^{o}C overnight. However, we centrifuged the mixture at 10,000 rate per minute for 10minutes. And the phage sample was filtered through a 0.22 µm membrane filter and stored at 4^{o}C. In the research work, Biological Safety Cabinet with the Model: BSc-150011B2-X was purchased from the LABOAO, Zhengzhou, Henan Manufacturer Company in China and used in this study. The culture media autoclaved at 121^{o}C for 15 minutes. After sterilizing the culture media, lab coats, hand gloves, glassware, masks were all sterilized using 70% ethanol to avoid bacterial contamination. According to previous studies, agar plates with 30mL of agar solution with a concentration of 1.5% and Luria broth at 0.7% dispense on base plates [7]. Salmonella typhi was cultured for 4 hours and mixed with enriched phage sample in each overlay before being transferred onto agar plates and incubated at 37^{o}C for 24 hours. After that, plaques noticed were counted and measured.

2.5.1..Isolation of Bacteriophage

The T898 phage was isolated from raw screened sewage using Salmonella typhi as a host bacterium. Importantly, 50mL of the sewage sample with 5mL of Salmonella culture was mixed. The solution was incubated at 37^{o}C for 24 hours with shaking. After incubation, 1% chloroform solution was added to 10mL of the solution with gentle shaking and left for 30 minutes to combine with Salmonella typhi cells. We centrifuged the mixture at 10,000 rate per minute for 10 minutes, and the liquid porting was filtered using a 0.45 µm filter; to detect the presence of phage [8]. Then a ten-fold serial dilution of the phage filtrate was diluted in a sulfate-magnesium (SM) buffer. At the same time, a 100µl diluted phage sample with 200µl of Salmonella culture was inoculated in a 0.7% soft agar tube and placed in the water bath at 50^{o}C before pouring onto the agar plates. The plates were incubated for 24 hours [9]. The plaques obtained were purified using serial dilution with the double-layer agar technique.

2.5.2.Purification of Bacteriophage

To purify the phage isolated. The plaques from the base plate were picked using the sterilized pipette and carried to the 300µl Sulfate-magnesium buffer containing Salmonella culture, incubated at 37^{o}C for 24 hours [10]. After incubation, the mixture was centrifuged at a 10,000 rate per minute for 10 minutes and filtered through a 0.45 µm membrane filter. The overlay method was used to repeat the phage filtrate three times to ensure the purity of the phage lysate. The phage lysate was stored at 4^{o}C and used in this study [11].

2.5.3.Preparation of high titer Phage Stocks

The phage filtrate was serially diluted in 5mL sulfate-magnesium buffer solutions to give the concentration that would provide successful lysis of Salmonella cell in a base plate. Each of the plates diluted was overlaid, and after incubation, the isolated phage was placed into 5mL sulfate-magnesium buffers and left at room temperature for 1 hour [12]. Following the completion, the filtrate collected was transferred into a 20mL sulfate-magnesium buffer solution and centrifuged at 10,000 rateper minute for 10 minutes [13]. The filtrate was filtered using 0.22µm, and 1% chloroform solution was placed before storage at 4^{o}C. And the titer of the phage stock was determined by the double-layer agar technique**.**

2.5.4. Preparation of Phage for Transmission Electron Microscopy

To prepare phage for electron microscopic high titer stocks were made ready in a sulfate-magnesium buffer. The solution was placed into tubes and centrifuged at 10,000 rateper minute for 10 minutes. The clear liquid was collected and transferred into a 50mL tube and centrifuged at 10,000 rateper minute for 10 minutes. The waste collected was discarded, and the liquid portion was placed in 0.5 mL sulfate-magnesium buffer, filtered through 0.22µm, and stored at 4^{o}C [14]. Further, a two-drop of phage stock was added to a 200-mesh grid and left for 2 minutes. At the same time, excess liquid removes using filter paper, and the grid was allowed to be air dry. Then a drop of 2% phosphotungstic acid was added to pH 6.5 for 25 seconds to maintain the phage, excess water was removed using the filter paper and the grid was air-dried and loaded into the electron microscope (Hitachi H-600), the phage was investigated at X10 0000-260 000 magnification [15].

2.5.5. One Step Growth Curve

A ten-forth dilution of the phage sample was put together in a 20mL of sulfate-magnesium (SM) buffer for the experiment. And 100µl of the host culture was mixed with 100µl of a serial dilution of the phage stocks, poured onto a Luria broth agar plate, and allowed to dry for 20 minutes. After the culture plates were dried, phage dilutions were spotted on each plate and incubated for 24 hours. The plaques seen on plates were picked and purified using the double-layer agar method.

Previous studies revealed that bacteriophages isolated against Salmonella typhi produced many phages from each infected site, and the burst size resulted from using the growth curve [16). The host Salmonella culture was mixed with a phage sample of 10^{8}pfu/ml and incubated at 37^{o}C for 5 minutes, and the solution was centrifuged at a 10,000 rate per minute for 10 minutes before use. After that, Salmonella culture and phage sample were mixed at multiplicity of infection (MOI) values in the range of 1, 0.1, and 0.01 optical density (OD 600nm), with an increase, and the host Salmonella strain was observed periodically by measuring the optical density (OD600 nm) until it reaches the stationary phase.

2.5.6. Thermal and pH Stability Tests

After 1 hour of pre-incubation, the phage samples were at different temperatures of 20, 25, 30, 35, 40, 45, and 50^{o}C at pH 7.0. And those that survived were picked by using a pipette and measured by the overlay technique. The stability of the phage was tested by pre-incubating the phage sample at various pH conditions; 2, 4, 6,8,10, and 12 at 25^{o}C for 6 hours. Those phages that remain alive were collected and determined by the overlay plating method and stored at 4^{o}C until use [17].

2.5.7. Bacterial Reduction Assay

The reduction of the growth of Salmonella typhi was described by Jamal et al. in previous studies that Salmonella culture was inoculated into two flasks, each containing 100mL of culture media, and kept in a shaking incubator at 37^{o}C. One of the flasks was treated with a phage sample with the multiplicity of infection of 1, while the second flask was considered a control with no phage sample. The culture flasks were kept at 37^{o}C with constant shaking at 120 rate per minute, and the readings of the optical density (OD), were recorded using a spectrophotometer every 2 hours up to 24 hours during the study [16]

III. Result and Discussion

3.1. Isolation of Bacteriophageagainst Salmonella Typhi

The TA98 phage was isolated from sewage using Salmonella typhi as a host bacterium. The phage isolated formed clear plaques on the host Salmonella culture in a large area on plates at 10^{-9} and 10^{-10} dilutions and continue to increase from 10^{-3} to 10^{-10} on plates. Also, the same result with 10^{-7} dilution on plates [17]. Escherichia coli: show no lysis on plates from 10^{-1} to 10^{-10} dilutions. Staphylococcus strain: from 10^{-1} to 10^{-10} dilutions, no plaques on plates. And Klebsiella strain: did not show any zone of lysis on plates.

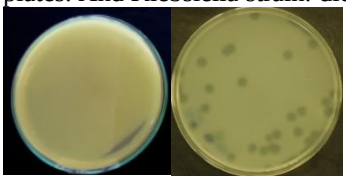

Figure 1.Plaque assay for TA98 phage, A, control,and B, a higher dilution of phage titer showing clear plaques on culture plate with an average diameter of 0.8mm

Table1. The host range of Salmonella TA98 phage

Plus means (+) clear plaques, negative sign (−) represents no plaques

Species Number	Bacterial culture	Clear lysis
1.	Salmonella typhi	+
2.	Escherichia coli	---
3.	Staphylococcus aureus	---
4.	Klebsiella pneumonia	---

3.1.1.Electron Microscopy

The phage isolated has the same structure and is considered to be Salmonella TA98 phage isolated in previous studies [18]. The presence of a tail with a contractile sheath enables the phage to become a member of the family Myoviridae. Furthermore, the phage has a head diameter of 107nm, a tail length of 123nm, and a tail diameter of 20nm.

Figure2.Electron micrograph of Salmonella TA98 phage; measurements are placed in the results.

3.1.2. One Step Growth Curve

Immediately after the phage sample mixed with salmonella culture, there was complete lysis of Salmonella cells at 37^{o}C. After infection, a persistent reduction in the growth of Salmonella typhi again was noticed throughout the experiment. For instance, Salmonella typhi culture was used throughout the experiments and observed that the titer of the phage increased from 1.2 x 10^{7} to 2.9 x 10^{9} pfu/ml at a multiplicity of infection (MOI) 0.1, and 1.0 x 10^{8} to 2.6 x 10^{9} pfu/ml at an MOI of 1. At the same time, the number of Salmonella cells increased to reach a concentration of 8.4 x10^{8}cfu/ml. And the burst size for the phage isolated was 140 PFU/ml and the latent period was 40 minutes. The eclipse phase was 38 minutes. The amount of phage used was 99% within 4 minutes. The latent period and burst size from the past studies are different for the Escherichia coli phage. And the importance of the difference between the latent period and burst size was not explained in detail [19]. However, the host Salmonella typhi was

burst by the isolated bacteriophage and reduced the growth of the bacteria. In about 20 minutes, the growth activities of Salmonella typhi decrease in the optical density (OD) value 1. In 45 minute incubation, the infectious rate of the growth of Salmonella typhi bacteria was lower than the treatment without bacteriophage infection. The bacteriophage was specific to its host bacteria and only infected Salmonella typhi [20].

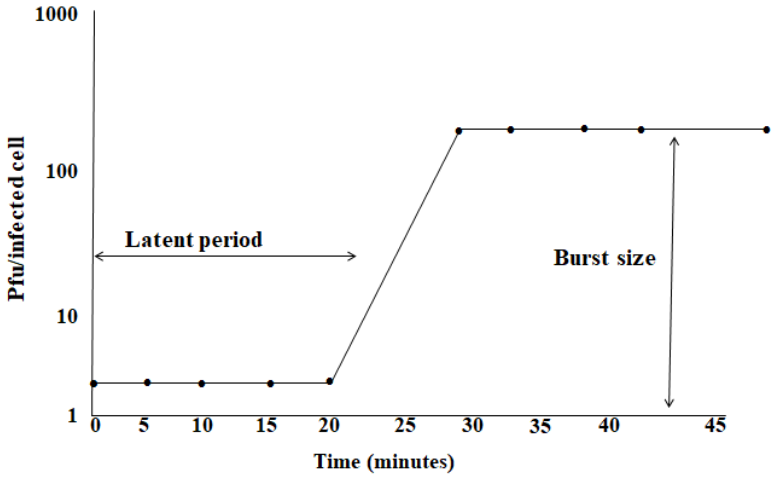

Figure3. This result shows that the host Salmonella typhi cells were burst by the isolated bacteriophage and reduced the growth of Salmonella typhi bacteria in 20 minutes. After infection, the growth parameter of Salmonella cells rapidly decreased in the optical density (OD) value of 1. In 45 minute incubation, the rate of infection of Salmonella typhi was lower than the treatment without bacteriophage infection**.** The bacteriophage was specific to its host bacteria and only burst Salmonella typhi [21].

3.1.3. HostRange

Based on the host range, four bacterial strains were used in this study to establish the specificity of the phage host range. Subsequently, the bacterial strains were tested by spotting methods and verified by agar overlay assays. Interestingly, only Salmonella typhi was burst by the isolated bacteriophage, while Escherichia coli, Staphylococcus aureus, and Klebsiella pneumonia were resistant to the TA98 phage.

3.1.4. Thermal and pH Stability of the phage

Salmonella TA98 phage was stabled 100% after incubation at 37^{o}C for 1 hour at pH 7.0 and showed good stability between 37^{o}C and 50^{o}C. At 70^{o}C, the phage was killed and lost all its lytic activity. As a result, the phage demonstrated a high sense of temperature stability from 25^{o}C to 50^{o}C but lost its infectivity at 60^{o}C. In previous studies, Hazem published the effects of temperatures, pH-values, ultra-violet light, and ethanol on the growth of isolated Salmonella phage, and the stability of the phage was more effective at pH 7.0. After 6 hours of incubation under different pH conditions from pH 5, 9, and 11, no plaques were formed on culture plates. At the pH values of 1, 2, and 3, the phage was nonfunctional, while at high temperatures and low pH; the phage can be destroyed, which may cause impediments to phage therapy.

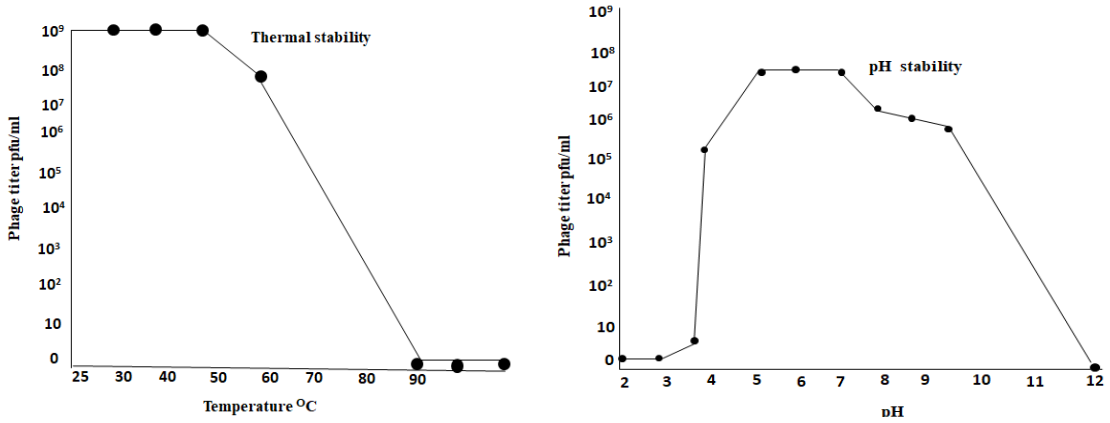

Figure 4. The thermal stability of the phage lytic activity with various temperatures was placed in an oven at 37^{o}C for 1 hour. And the pH of the phage infectivity, at different pH values ranging from pH 2 to pH 12, was incubated at 37^{o}C for 1 hour.

3.1.5. Bacterial Reduction Assay

Bacteriophage sample mixed with Salmonella culture with an MOI value of 1 and a decrease was seen in the bacterial growth for 24 hours during the study. After the infection, an immediate reduction in the optical density (OD) was noticed in the phage-treated Salmonella culture compared to the control culture with no phage, and a rapid decrease in the optical density was seen from 0.40 to 0.12 in the first 4 hours and maintained for about 16 hours. After 16 hours of incubation, the cloudiness of the optical density (OD) value increased because of Salmonella cells in the culture. The infectious concentration of the phage demonstrated the reduction of the bacterial growth throughout 24 hours, as described by previous studies.

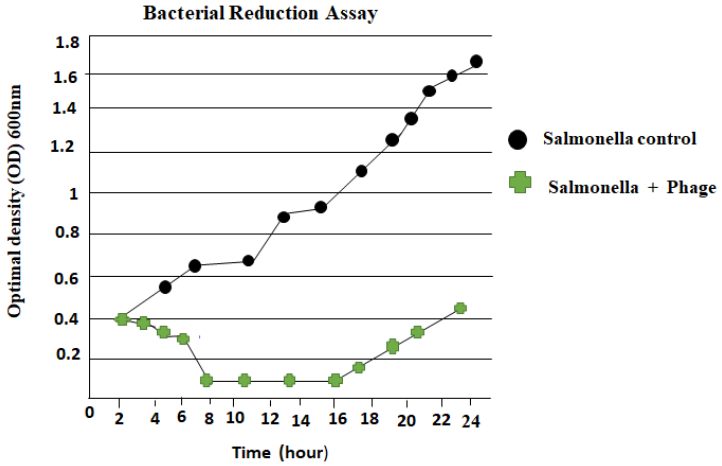

Figure5. Schematic graph of TA98 phage indicating results on salmonella growth culture, compared with a control culture, with no phage.

IV. Conclusion

The results that we got in this research are as follows:

- The plaques found on the agar plate show consistency of the receptor between bacteriophage and Salmonella typhi going through the lytic cycle.
- The bacteriophage isolated from the sewage exhibited a lytic cycle for a specific bacterium but could not infect many strains of bacteria.
- In some bacterial cultures, plaques did not form, which suggests that the receptor did not match or the bacteriophage experience a lysogenic cycle which may end up making the bacteria mutate and even lead to more resistance

V. Problem Statement

The contamination of food and water sources by Salmonella typhi in West Africa, especially in Liberia, created a serious health problem where the mode of transmission increases and Salmonella typhi becomes more resistant to drugs. The relevance of this study was to address the rapid spread of Salmonella typhi infections by introducing phage therapy applications using phages instead of antibiotics to reduce typhoid disease. Bacteriophage was isolated to bio-control the growth of Salmonella typhi in water environments. Where they create serious health problems for humans, to identify the presence of bacteriophage in sewage, and to determine the lytic effect of phage against Salmonella typhi in the application of phages as a future therapy to reduce typhoid fever caused by Salmonella typhi

VI. Future recommendations

In this study, we isolated and partially characterized specific lytic bacteriophages from sewage samples to identify potential phages for phage therapy, which had contributed to the knowledge of science and reduced the growth of Salmonella typhi and purified water. In the future, I would like to recommend that the potential outcome

of lytic phage and its phage-associated depolymerase enzyme production against salmonella bacteria, analyzing the different types of protocols of phage infection against multi-drug resistance bacteria and the entire deoxyribonucleic acid or DNA or ribonucleic acid or RNA pattern of lytic phage should be well studied, to know if the isolated phage could be relevant for the treatment of Salmonella typhoid-related infection.

VII. Acknowledgment

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