ORIGINAL ARTICLE

UTILITY OF A POLYVALENT PHAGE COCKTAIL: AN INTEGRATED STRATEGY FOR ENHANCED FOOD SAFETY AND MEDICINAL INTERVENTION AGAINST SALMONELLA TYPHI AND SALMONELLA ENTERITIDIS

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ABSTRACT

Considering the spreading antibiotic resistance among pathogenic bacteria, developing novel strategies for combating such microorganisms is essential. *Salmonella enterica* is harmful to humans, but it may also infect poultry, and when it contaminates food, it may become a foodborne pathogen. Several serovars of the genus *Salmonella* have recently been found to be resistant to different antibiotics. Consequently, the isolation of *Salmonella* lytic bacteriophages has gained significance in the search for bactericidal activity. However, extensive study on safety and effectiveness is needed before this procedure can be established and implemented into regular practice. In this work, we report the findings from investigations using two *Salmonella* bacteriophages, STWB21 and SEWB23, that have been previously and recently identified. In addition, phage SEWB23 showed a broad host range, wide pH tolerance, and prolonged thermal stability. In our observations, when each phage was employed against *S.* Typhi and *S.* Enteritidis, both the individual and combined (as a cocktail) phages significantly decreased the number of bacterial cells and the density of the cell culture. These phages additionally showed a successful reduction in bacterial biofilm. The effectiveness of this in-vitro phage therapy was compared with the activity of established antibiotics. Using a novel phage, SEWB23, and a previously reported phage, STWB21 (single and in a cocktail), we demonstrated that phage treatment against strains of *S.* Typhi and *S.* Enteritidis exhibited significant efficacy and acceptable safety profiles. These findings provide promise for a phage trial that might lead to the practical implementation of this approach in chicken production and the food sector.

Keywords:
Salmonella infection, phage, cocktail, foods, biofilms.

INTRODUCTION

Salmonella is a Gram-negative, rod-shaped genus in the Enterobacteriaceae family, causing foodborne illness salmonellosis. Preventing and treating this infection is challenging due to its wide transmission mode and the prevalence of multi-drug resistance[1]. It also develops biofilms for long-term survival in hostile environments, prone to antibiotic resistance[2,3]. Although new medications and combination therapies have been produced with unsatisfactory results, this has become a public health problem. According to studies on their anti-biofilm attributes, bacteriophages have unique characteristics that make them useful for treating biofilms.

Bacteriophages are viruses that evolve and co-survive with their host bacteria, killing them without harming other microflora. They are widespread and present in all-natural habitats. Based on their rapid antibacterial effect, high specificity, and self-replicating abilities, they are an appropriate substitute biocontrol agent for bacterial infections.

Additionally, bacteriophage cocktails have been proven effective in removing *Salmonella* from food and cleaning food preparation surfaces. They significantly reduced *Salmonella* levels on chicken parts when used alone or combined with traditional chemical sanitizers, especially when applied to chicken breast fillets[4].

Our previous studies successfully identified and characterized the *Salmonella* phage STWB21, fulfilling the initial criteria for a possible antibacterial agent that may be utilized for both the prevention and treatment of salmonellosis caused by *S.*

This study aimed to determine the effectiveness of a phage cocktail as a zoonotic *Salmonella* control strategy in raw chicken breast meat as well as the efficacy of antibiotic treatment, phage cocktail, and single phage against *S.* typhi and *S.* enteritidis strain biofilms.

MATERIALS AND METHODS

A water sample was taken from the Ganga River in the West Bengal region of India, located near Kolkata to isolate phages. Phage was isolated using a previously described method[7]. A spot test was conducted on *S.* enteritidis confirming the presence of the phage. Purified phage plaque was obtained by picking up the single plaque using the plaque assay method. The morphological characterization was examined using a Transmission Electron Microscope. Following that, the host range assay and stability test (thermal, pH) of phage SEWB23 were performed by the soft-agar overlay method[8].

The newly isolated phage and a previously isolated *Salmonella* phage STWB21 were employed for the phage cocktail[5]. The phage cocktail utilized in this study consisted of a 1:1 combination of both phages, with a final concentration of 3.7 $\times 10^{10}$ PFU/ml, and was kept at 4°C.

To assess the decrease of *Salmonella* in chicken meat using the individual phage and phage cocktail, four distinct Petri dishes containing small pieces of chicken breast were aseptically cut according to the previously published method with some modifications[9]. These chicken pieces were also given an *S.* Typhi inoculation at 10^8 CFU/ml, followed by a 1-hour incubation at 37 °C. This was followed by adding each phage and a phage cocktail (10^{12} PFU/ml) and for the controls, the same volume of Tris-$MgCl_2$ buffer was used instead of the phage suspension. Immediately after, the chicken pieces were weighed and homogenized with the use of 1.0 mm diameter silica beads. A serial dilution method on HEA agar was used to calculate the colony-forming unit (CFU)/gm tissues of *Salmonella typhi*.

By employing a few modifications from the previously published protocol by Cerca et al., 2006, a 24-hour biofilm of *S.* typhi and *S.* enteritidis was generated in 96-well plates to assess the anti-biofilm efficiency of the phage and phage cocktail[10]. After that, it was treated with phage, phage cocktail, and antibiotic cephalosporin. After that, the plates were incubated for four hours at 37°C. Following the incubation period, 1 ml of 0.1% w/v crystal violet solution was added to the wells, and the plates were then washed three times with 1X PBS. The optical densities of the biofilm were measured at 595 nm of absorbance using a microplate reader.

RESULTS & DISCUSSION

A previously reported typhoidal *Salmonella* phage STWB21 and a newly isolated nontyphoidal *Salmonella* phage SEWB23 were used for this investigation. The SEWB23 phage exhibited distinct and clear plaques (Figure 1A). The morphology of the purified phage SEWB23 was analyzed using transmission electron microscopy (TEM), and the results showed that it had an icosahedral head with an estimated diameter of 64± 4 nm (n = 20) and a long, flexible, non-contractile tail measuring about 121± 6 nm in length (n = 20). According to the ICTV guidelines for phage structure, the phage SEWB23 may belong to the Caudovirales subfamily *Siphoviridae* (Figure 1B).

Spot tests revealed that phage SEWB23 had a broader host range. To confirm its host range, an EOP assay was done for phage SEWB23 (Table 1). A wide range of lytic activity was demonstrated by phage SEWB23. All typhoidal and non-typhoidal *Salmonella* strains, *Sh. flexneri* and *E. coli* were all infected with a low to high efficiency (0.02 to 1.0). The majority of non-typhoidal strains were infected by this phage with high efficiency (0.3 to 1), however, the EOP values were only moderate (0.1 to 0.2) for some typhoidal strains (Table 1).

The thermal stability of the phage SEWB23 was examined; it was shown to be stable for one hour at 40°C but to rapidly decline and lose its lytic activity at 50°C (Figure 1C). Conversely, throughout a wide pH range (5–10), phage SEWB23 exhibited remarkable stability in both acidic and alkaline conditions, with its highest activity occurring at pH 7.0 (Figure 1D).

In chicken meat, the *Salmonella* count is 10 log CFU/gm for the untreated positive control. The treatment of phage STWB21, phage SEWB23, and their cocktail on the chicken breasts reduced the bacterial load of approximately 4 log CFU, 3 log CFU, and 5 log CFU respectively. In comparison to the untreated control, the decrease of biofilm caused by phage STWB21, SEWB23, and phage cocktail infection was statistically significant ($p < 0.001$).

In a 96-well microplate at 37°C, the efficacy of the phage cocktail, consisting of a 1:1 mixture of the phages STWB21 and SEWB23, was assessed in comparison to that of each phage and antibiotic. By using crystal violet staining in 96-well plates, the effect of the phages STWB21, SEWB23, their cocktail, and the antibiotic cephalosporin in the degradation of biofilm was determined. Both the bacterial strains *S.* typhi and *S.* enteritidis were susceptible to the antibiotic cephalosporin. In comparison to the control, biofilm biomass degradation was highly significant ($p < 0.0001$) for the investigated phage titers of single-phage lysates, the cocktail, and the antibiotic against S. typhi and S. enteritidis. On the 96 well plates, treatment with the phage STWB21, SEWB23, phage cocktail, and antibiotic cephalosporin decreased the *S.* typhi bacterial population by approximately 38%, 37%, 44%, and 41%, respectively. However, for the S. enteritidis bacterial strains, it was altered by approximately 43%, 62%, 68%, and 66% respectively.

CONCLUSIONS

This study suggested that the phage STWB21, phage SEWB23, and their combination may be useful in reducing *Salmonella spp.* bacteria viability in chicken breast meat. Therefore, the phage cocktail may be employed in the future by many poultry sectors and has the potential to be a bactericidal agent for the biocontrol of *Salmonella spp.* in raw chicken breast flesh. The findings also showed that the phage cocktail reduced the *Salmonella* Enteritidis bacterial biofilms more efficiently than *Salmonella* typhi bacterial biofilms on a 96-well microplate. Hence, this phage cocktail is an ideal substitute for antibiotics to control *Salmonella* and can reduce the biofilms that are resistant to conventional approaches.

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Table 1: Host range analysis of phage SEWB23 on different strains.

Strains	Strain name	Infectivity	E. O. P
S. enteritidis	(520833) *	(+) ve	1
S. typhi	(KOL 551)	(+) ve	0.262
S. paratyphi	(KOL 534)	(+) ve	0.140
S. typhimurium	(PH-94)	(+) ve	0.370
ETEC	(IDH07942)	(+) ve	0.025
Sh. flexneri 2a	(2457T)	(+) ve	0.170
Sh. flexneri 3a	(UB811)	(+) ve	0.162
Sh. flexneri 6	(UB812)	(-) ve	-
Sh. boydii	(NK02379)	(-) ve	-
V. cholerae O1	(MAK757)	(-) ve	-

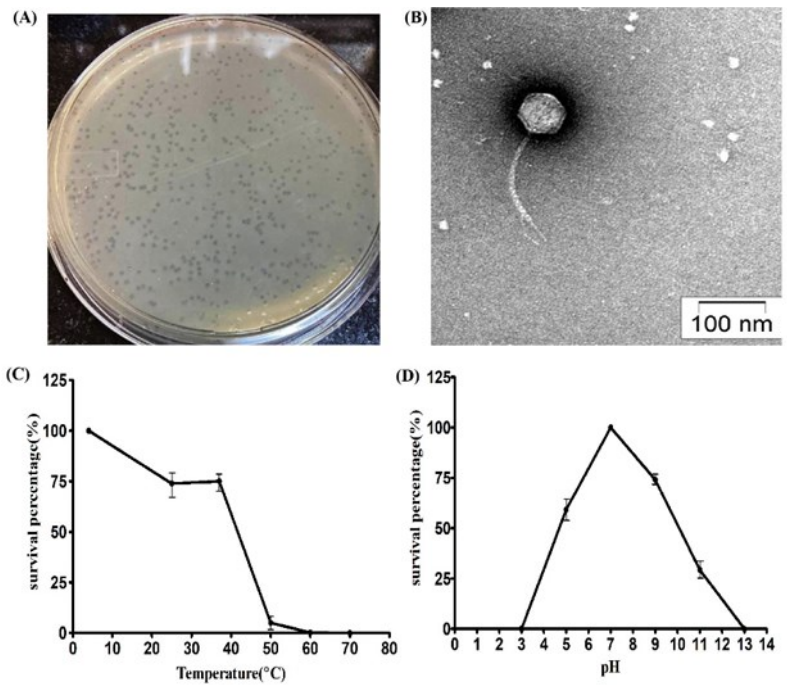

Fig:1 Morphological and physicochemical characteristics: (A) Plaque morphology of SEWB23 bacteriophage on nutrient agar plate. (B) Morphology of SEWB23 bacteriophage using TEM. (C) Thermal stability, (D) pH stability. Given values are the mean of three determinations.

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