Bulletin of Faculty of Science, Zagazig University (BFSZU)
e-ISSN: 1110-1555
Volume-2024, Issue-1, pp-195-203
https://bfszu.journals.ekb.eg/journal
Research Paper DOI: **10.21608/bfszu.2023.228803.1295**

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Isolation and characterization of a bacteriophage capable of combatting Shiga Toxin-Producing *E. coli* O157:H7

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***ABSTRACT*: The study aimed to identify and analyze a bacteriophage from the Al.Qnayat Wastewater Treatment Plant in EL-Sharika Governorate. A distinctive lytic zone in the bacterial lawn from a plaque assay indicated its presence. Successfully, a novel phage, Vb-Ecm1, was isolated using a phage spot test, demonstrating strong lytic properties against Shiga toxin-producing *E. coli* (STEC) O157 strains LC666912 (stx2) and LC666913 (stx1/stx2). Electron microscopy identified the phage's structural features, hinting at its belonging to the Myoviridae family due to its extended contractile tail. Vb-Ecm1 produced a distinct 1mm plaque with a transparent core. Its host specificity was assessed using spot tests on a variety of bacterial strains from Egyptian labs. Remarkably, Vb-Ecm1 exhibited potent lytic activity against the *E.coli* strain LC589615, sourced from Ainshams University's Botany department. The phage's adsorption rate showed a consistent decrease, bottoming out at 12 minutes, suggesting an adsorption rate of K=1.7x10-10. The phage infection lifecycle was detailed through a one-step growth curve, revealing 10-minute latency and an average burst size of 128 for Vb-Ecm1. Vb-Ecm1 displayed notable heat stability, remaining active up to 50°C. However, it became inactive at 80°C and completely lost its activity at 90°C. Its activity spanned a wide pH spectrum (4 to 11), with optimal survival observed at pH 6 and 7.8. Notably, the phage was more resilient at a pH of 6 compared to a pH of 8.**

KEYWORDS: *Shiga toxin bacteria, Phage therapy, food pathogens, Host range*

Date of Submission: 12-8-2023 Date of acceptance: 21 -08-2023

I. INTRODUCTION

Bacterial infections continue to pose a significant threat to public health globally, with the emergence of antibiotic resistance further complicating the effective management of such infections (Ventola, 2015). Among the bacterial pathogens of significant concern is Shiga Toxin-Producing Escherichia coli (STEC) O157:H7, known for causing severe foodborne illnesses and associated with life-threatening complications such as hemolytic uremic syndrome (Karmali et al., 2010). Conventional antimicrobial treatments are often challenged by the development of bacterial resistance and the risk of exacerbating disease progression by inducing the release of Shiga toxins (Mellmann et al., 2008). Hence, there is an increasing need to explore novel and effective strategies to control and treat such infections. Bacteriophages or phages, viruses that infect and kill bacteria, have emerged as a potential alternative or adjunct to conventional antibiotics (Clokie et al., 2011). Their inherent specificity allows for targeted eradication of pathogenic bacteria without disrupting beneficial microbiota, making them attractive for therapeutic applications (Loc-Carrillo & Abedon, 2011). Moreover, phages are ubiquitous in nature, with the capacity to evolve alongside bacterial resistance, presenting a renewable resource against bacterial pathogens (Pirnay et al., 2011). Several studies have demonstrated the successful isolation and application of bacteriophages against various bacterial species, including STEC O157:H7 (Raya et al., 2006; Niu et al., 2009). However, the effectiveness of phage therapy is often contingent on the detailed understanding of the isolated phage's characteristics, such as host range, burst size, adsorption rate, and resilience to environmental conditions (Abedon et al., 2011). This study aims to isolate and characterize a bacteriophage from the Al.Qnayat Wastewater Treatment Plant capable of combating STEC O157:H7. The isolated phage, named Vb-Ecm1, was analyzed for its morphology, host range, adsorption rate,

growth curve, and stability under varying temperature and pH conditions. The findings from this study contribute to the ongoing efforts in developing phage-based therapeutic approaches against bacterial infections.

II. MATERIALS AND METHODS

Host Strains and Growth Conditions

Two isolates of *E. coli* O157:H7 strains (Stx2 producing strain LC666912 and bi Stx1,2 producing strain LC666913) isolated by collecting cattle faeces samples from various cattle pens in AL-Sharkia Governorate- Egypt and increasing the internal bacterial mass by adding Tryptone Soya Broth media nine time more to 25gm of sample and incubated at 37 °C for 24 h. By using most probable number technique and streaking the swap of positive tubes in Sorbitol MacConky agar media and incubated at optimal conditions, the white colonies picked up and tested for antibiotic susceptibility to detect the high resistant isolates. The high resistant isolates examined as Shiga toxin producing *E. coli O157:H7* by agglutination test using O157and flageller H7 antisera and PCR test usingStx1 and Stx2 primers .**(El-Tahan, *et al*., 2023)**

Isolation of STEC O157:H7 bacteriophage

According to **Adams (1959)** four waste water samples collected from various locations in Elsharkia, Egypt were used as a source of virus. The collected samples were processed by avoiding the large impurities using Whatman filter paper and centrifuged for twenty minutes at 6000 rpm then samples were filtered by 0.45 micro-pore membranes. The enrichment of phage by adding equal volume of filtered sewage and nutrient broth medium (20:20 ml) and 1 ml of different bacteria, and the mixture was cultured and incubated for day at room temperature with shaking. After incubation the culture precipitated by centrifuging for minutes, and 10ml of supernatant was filtered through membranes filter. The filtered sources were tested for phage existence by spotting of the 15µl droplets of supernatant on solidified double layer of agar plates, first layer containing pure solid nutrient agar media and the other layer containing 3ml semisolid media injected with100µl of bacterial cultures. After drying and incubation, the plates were investigated by showing clear lysed area (**Adams, 1959**).

Plaque assay

According to earlier investigations **(Adams, 1959, Viazis *et al*., 2011),** the sources caused lytic zone were used in plaque assay by adding of 100µl of each source to 100µl of each bacterial culture to 3ml semi solid media and poured on plate containing solid agar media ,the plates leaved to solidified and incubated for 19 h.

Purification and Propagation of Bacteriophage

Phage isolates were purified by obtaining homogeneous plaques by choosing single plaques three times in a row using a sterile Pasteur pipette. plaques were selected, placed in 500 µl of broth media containing 100 µL of bacterial host and then After incubation at normal condition,and few drops of chloroform was added and used as phage source and was kept at 4 °C **(Askoura *et al* ., 2021).**

Morphological Characteristics (Electron Microscopy)

The phage morphology was examined through use (TEM) Transmission electron microscopy as described before **(El-Telbany *et al*., 2021).** 4 drops of phage particles with high concentration were deposited on 200 mesh carbon-coated copper grids with formvar films (Sigma-Aldrich, Saint Louis, MO, USA) to allaw adsorbtion of 1 min, then 2% phosphotungstic acid staining with for 30 s (Sigma-Aldrich). The phage was examined in the

Faculty of Agriculture, Mansoura University with a Hitachi H600A electron microscope at the Electron Microscopy Unit. Phage pictures were taken using digital micrograph software, and measuring phage particles.

Adsorption rate of STEC O157:H7 bacteriophage

The **Adams (1959)** approach was used to calculate the rate at which phages adsorb. Phages suspension (10^8 PFU/mL) were added to a bacterial culture of Stx2 producing *E. coli* O157:H7 LC666912 strain at(19×10^7 CFU/mL) at (MOI=1.0) .2 ml of the samples were filtered by micro-filter every 5 min to stop adsorption then cultured with bacteria in double agar plats and incubated .The adsorption rate estimated by counting the current plaques of free phage and using equation: K= (2.3/BT) x log (Po/P) **(Rattanachaikunsopon, 2006) .**

One-step growth curve

In accordance with the methodology outlined by (Pajunen et al., 2000), the growth curve was used to estimate the burst size and latent period of the phage. Equal volume of phage and host *E. coli* O157 bacteria (MOI=1.0) were mixed phage suspension was added to the E. coli O157 bacteria as the phage's host before allowing time for adsorption and incubating at 37°c. After centrifuging serial dilutions were made and incubated at 37 °C. Then, 1ml was taken after every 5 minutes, added to 1 litre of chloroform, and thoroughly mixed before being kept at 4°C overnight. In order to count the phage particles released from the infected bacterial cells.The formula used to calculate the relative burst size was relative burst size = [(Final titer - Initial titer) / Initial titer]. To calculate the latent period and burst size, the relative burst size at various intervals was plotted against time.

Determination of STEC O157:H7 bacteriophage Host Range

Phage host rang was investigated using the spot test technique. We tested the host range of these phages using bacterial lawns of 7 various strain (E.coli, *Pseudomonas aerginosa, Klebisiella pneumoniae Sp, Staphylocccus aureus, Morganella sp*.) obtained from various laboratories in Egypt

Thermal and pH Effect on phage activity

According to **Capra et al. (2004) and Hammeral *et al*. (2014),** the temperature stability of phages was assessed by subjecting the phage to various temperatures (30, 40, 50, 60, 60, 70, 80, and 90 °C for 10 min using a water bath, and then directly cooling under tap water treated phage was diluted and assayed by the plaque assay). The ability of the phage to persist at various degrees of pH was tested by exposing the phage suspension to acid and base pH values .phage suspension was added to different buffer solutions at range (1:9).the law degree of pH because of the acidity which occur at using glycin-HCl buffer, while the high alkalinity as result of using sodium and potassium hydroxide and the normal or middle pH was obtained by using acid and base solutions.the phage was treated to all pH values. (**Jamalludeen *et al*., 2007 and Hammeral *et al*., 2014).**

Statistical Analysis

All experiments were performed in three times. Data were compared using Student's *t*-test. Analytical statistics were undertaken using GraphPad PRISM version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA). A significant level of 0.05 was applied in all cases.

III. RESULTS

Phage Detection in Sewage Sources:

We were able to detect phage activity in a single sewage sample collected from Al.Qnayat Wastewater Treatment Plant in EL-Sharika Governorate. A significant lytic region was visible on the bacterial lawn LC666912 after the plaque assay, suggesting high phage concentration.

Table (1) Isolation of *E.coli O157* bacteriophages from different sewage samples:

Formation of lytic area after spotting of for different sewage sample	Isolates No.	
	LC666912 (stx2)	LC666913 (stx1 /stx2)
Sewage from Zagazig hospital university. (EL-Sharika Governorate).	-	-
Sewage from Ain shams hospital university (Cairo Governorate).	-	-
Ibrahimya treatment plant from (EL-Sharika Governorate).	-	-
Wastewater treatment plant Al.Qnayat (EL-Sharika Governorate)	+++(6×10^5)	+++(2×10^2)

+= Formation of lytic area. - = no formation of lytic area.

Phage Spot Test:

The isolated phage, Vb-Ecm1, demonstrated lytic capabilities against two STEC O157 bacterial host strains, LC666912 (stx2) and LC666913 (stx1/stx2), with concentrations of 2×10^8 and 3×10^6, respectively (Table 2).

Table (2) Phage Spot and Plaque assay

Phage	Bacteria	Pfu/ml
Vb-Ecm1	LC666912 (stx2)	2×10^8
	LC666913(stx1 /stx2)	3×10^6

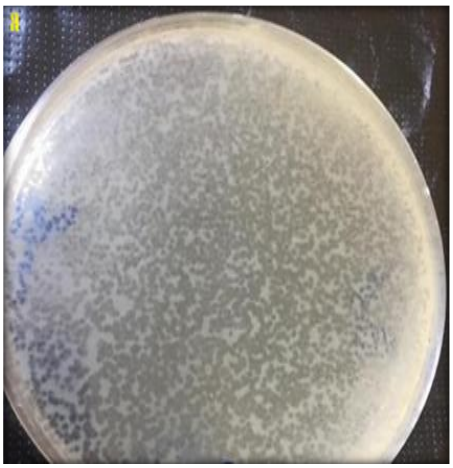
Phage and Plaque Morphology

Plaque morphology size was 1mm with clear center and was picked up for purification and characterization. Electron microscopy of the isolated phages particles Revealed that, the Vb-Ecm1 **bacteriophage** with hexagonal head with 86.9 nm diameter and long contractile tail measured 173 nm which belong to Myoviridae family see Figure 1(A and B).

Host Range:

The host range of Vb-Ecm1 was assessed using spot tests on bacterial strain lawns obtained from various laboratories in Egypt. The bacteriophage Vb-Ecm1 exhibited lytic activity against a specific E.coli strain, LC589615, procured from the Botany department of Ain Shams University. The other bacterial strains were resistant to Vb-Ecm1 (Table3).

(B)



(A)

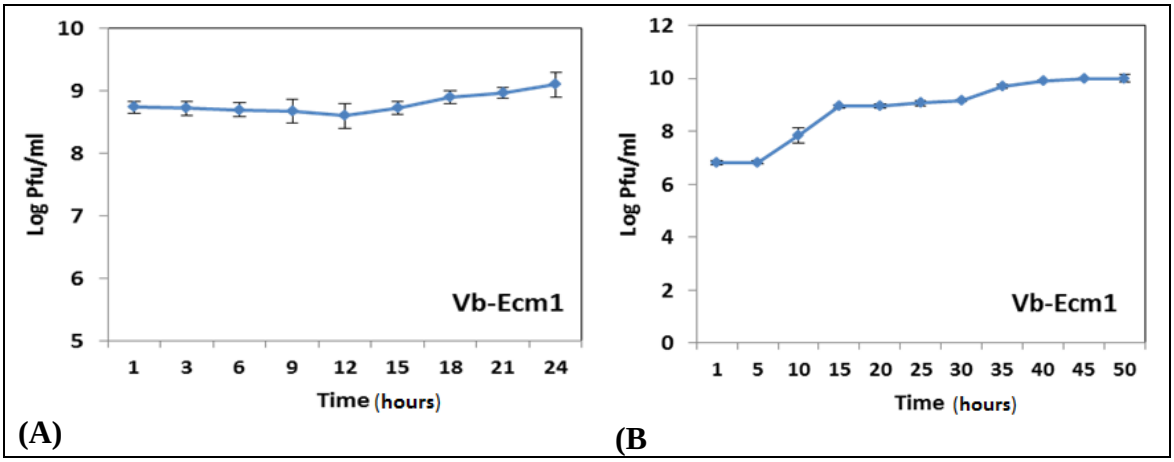
Fig (1) shows the plaque and phage morphology,at (A) picture the plaque appear with clear center. In (B) picture Vb-Ecm1phage appear with hexagonal head have 86.9 nm diameter and long contractile tail measured 173 nm which belong to Myoviridae family

Table (3) Phage Host range

Bacterial sp	Isolates sources	Vb-Ecm1
E.coli	Botany department of Ainshams university(Gene bank accession number :LC589615)	+
E.coli	Microbiology department of Zagazig university Gene bank accession number :LC649234)	-
E. coli	Microbiology department of Zagazig university	-
Pseudomonas aerginosa	Botany department of Ainshams university Gene bank accession number :LC586427)	-
Klebisiella pneumoniae Sp	Botany department of Ainshams university	-
Staphylocccus aureus	Botany department of Ainshams university Gene bank accession number :LC596095)	-
Morganella sp	Microbiology department of Zagazig university	-

+= Formation of lytic area. - = no formation of lytic area

Phage Adsorption Rate and Growth Curve *E.coli* O157:H7 Bacteriophage:
Vb-Ecm1 were adsorbed on host cell and the number of free not adsorbed phages decreased gradually over time, reaching the lowest concentration at which the time of maximum adsorption were recorded at 12 minutes, indicating a phage adsorption rate K = 1.7×10-10 for Vb-Ecm1 showed in (Figure 2A).Our findings in one-step growth experiment revealed that the latent period of 10 minutes for Vb-Ecm1, with an average burst size of 128 PFU per infected *E.coli* O157 cell (Figure 2B).



Figure(2) Vb-Ecm1 phage adsorption and single-growth step curves **(A)** Phage adsorption and the plaque forming units (PFUs) per infected cell in cultures of *E.coli O157* LC666912 (stx2) strain at different adsorption time.**(B)** Single-step growth curve for *E.coli* O157 bacteriophage .Samples were taken at intervals every 5 min. Each data point is a mean of three independent experiments, and the results are shown as means ± standard.

Thermal Stability and Acid-Base Condition Activity:
The phage remained infective up to 50°C, with infectivity drastically decreasing at 60°C and 70°C. The phage titers at 50°C and 60°C were approximately 107 and 105 PFU/ml, respectively. Phage titers further dropped to 104 and 103 PFU/ml at 70°C. Vb-Ecm1 lost its infectivity entirely following treatment at 80°C and exposure to 90°C.showed in (Figure 3A). The results in Figure 3B show that Vb-Ecm1 persistence in a pH range of 4 to 11, with optimal survival at pH levels 6, 7, and 8. It exhibited superior survival in an acidic environment (pH 6) compared to an alkaline one (pH 8) (Figure 3).

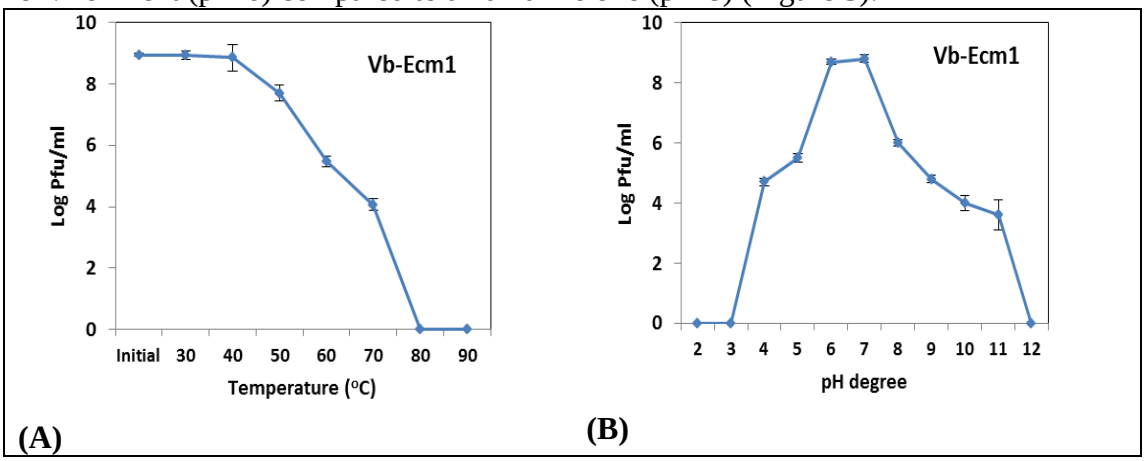


Figure 3. The temperature and pH effect on the stability of *E.coli* O157:H7 Bacteriophage **(A)** The stability of Vb-Ecm1 phage at different temperatures. **(B)** The stability of Vb-Ecm1 phage at different pH values. Number of phages were estimated through plaque assay method using *E.coli* O157 LC666912 (stx2) strain. Each data point is a mean of three independent experiments, and the results are shown as means ± standard error.

IV. Discussion:

The successful isolation and characterization of a unique bacteriophage, Vb-Ecm1, capable of lysing Shiga toxin-producing *E. coli* (STEC) O157 strains LC666912 (stx2) and LC666913 (stx1/stx2), signifies an important advancement in bacteriophage research and its potential

use in controlling harmful bacterial strains (**Sulakvelidze, *et al*., 2001**). The strategies of prevention and improved management must be used to control infections caused by multidrug-resistant Shiga toxin producing *E. coli* O157:H7. Many studies aimed to using of bacteriophages instead of antibiotics to overcome the bacterial infections in poultry and, thus, for food safety and public health **(Pereira *et al*., 2022)**. Phages have been considered promising agents against pathogenic bacteria due to their specificity, abundance, and ability to evolve, bypassing bacterial resistance (Kutter, *et al*., 2010).Bacteriophages are abundant in the environment and can be separated from ecosystems of fresh water, sewage, soil, and ocean. Due to fecal contamination, sewage generally contains a wide variety of coliforms. As a result, enteric pathogens are stored in sewage water, followed by sewage samples from sewage treatment plants and domestic animal drainage (cows, pigs) **(Johnson, 2003**). In this study from four wastewater sample locations, plaque were originally isolated from one samples Al.Qnayat Wastewater treatment plant EL-Sharkia Governorate. The electron microscopic analysis revealed a long contractile tail characteristic of the Myoviridae family, consistent with previous studies highlighting the lytic capabilities of phages within this family (**Kropinski, 2006)**.and also Olsen demonstrated studies of isolated phages infecting *E.coli* have belong to the Myoviridae family (**Olsen *et al* ., 2020**). The clear zone observed within a 1mm plaque confirms the strong lytic activity of the isolated phage. Interestingly, Vb-Ecm1 was found to display unique lytic activity against a specific *E.coli* strain LC589615, showcasing its host specificity, a desirable trait when considering bacteriophages as biocontrol agents (**Gill & Hyman, 2010**). It minimizes the potential disruption to non-target beneficial bacteria, a common issue with broad-spectrum antibiotics. The declining phage adsorption rate until it reached its lowest level at 12 minutes is a crucial parameter in understanding the life cycle of Vb-Ecm1 and its potential therapeutic implications (**Abedon, 2011**). Also, the one-step growth curve revealed a 10-minute latent period followed by a burst size of 128, vital parameters in understanding phage dynamics in the bacterial host. Phages are often quite sensitive to protein denaturation in an acidic environment, which may result in a loss of viability of the phage(**Jamalludeen *et al*., 2007**) .The ability to survive well over the pH range 5–9 is a feature common to most phages but many phages are stable to pH 3 or 4 (**Ackermann *et al* ., 1987**). The thermal resilience and pH tolerance of Vb-Ecm1 imply its potential use in a variety of environments. The observed stability up to 50°C and across a broad pH range (4-11) is consistent with earlier research reporting bacteriophages' survival under varying environmental conditions **(Jończyk, *et al*., 2011**). However, this study does not explore the potential for bacterial resistance against Vb-Ecm1 or its broader applicability against other pathogenic strains. Future research needs to address these points for comprehensive understanding and application. In conclusion, the findings provide new insights into the potential of bacteriophages as biocontrol agents against harmful bacterial infections, particularly STEC O157:H7, and contribute to the broader effort to develop alternative or complementary strategies to traditional antibiotics.

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