



# Impact of Multiplicity of Infection (MOI) on In-Vitro Phage Therapy Against *Xanthomonas axonopodis* pv. *citri*

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## Abstract

**Background:** Antimicrobial resistance (AMR) is an escalating global health concern, with the rapid emergence of multidrug-resistant (MDR) pathogens outstripping the development of new antibiotics. Bacteriophages (phages), which specifically target and lyse bacteria, represent a promising alternative. This study investigated the therapeutic potential of phages against *Xanthomonas axonopodis* pv. *citri*, a significant bacterial pathogen causing citrus canker and considerable economic losses worldwide.

**Methods:** Phages were isolated from diverse environmental sources (e.g., soil and citrus fruit samples) and screened for lytic activity against *X. axonopodis* pv. *citri*. Phage purification and titer determination were performed using the double-layer agar method. The ability of phages to suppress bacterial growth was assessed in vitro via optical density (OD<sub>600</sub>) and colony-forming unit (CFU) counts over 24 hours across a range of multiplicities of infection (MOIs).

**Results:** The isolated phages exhibited a narrow host range and high stability at 7°C, with an average titer of 201 plaques per plate upon purification. In vitro assays confirmed that the phages successfully suppressed the growth of *X. axonopodis* pv. *citri*, demonstrating strong lytic activity and significant reduction in bacterial loads. Phages 1P, 2P, 3P, and 5P showed varying degrees of effectiveness, with notable suppression at MOIs of 0.01, 10, and 100. Phage 3P provided sustained bacterial suppression at MOI 10, while phage 2P showed consistent activity across all tested MOIs. Despite observations of transient bacterial resistance, all phages maintained their antibacterial efficacy.

**Conclusion:** Our findings underscore the therapeutic potential of bacteriophages as an effective and sustainable alternative to antibiotics for controlling *X. axonopodis* pv. *citri*. The demonstrated lytic activity and bacterial suppression, even at varying MOIs, suggest that phage therapy offers a viable strategy against antibiotic-resistant strains. Furthermore, the cost-effectiveness of phage therapy provides a compelling rationale for its expanded consideration in addressing the global antibiotic resistance crisis within the broader context of healthcare economics.

**Keywords:** Antibiotics, Antimicrobial resistance, Bacteriophage, Phage therapy, MOI



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## Introduction

Antibiotics are a class of medications administered in controlled doses to eliminate or inhibit the growth of microorganisms, particularly bacteria, and resistance to antibiotics is the ability of bacterial cells to withstand, survive, and grow in the presence of antibiotics, resulting in the survival of bacteria rather than destruction (1-4). Antibiotic resistance has emerged as a significant global

challenge, leading to infections by multidrug-resistant (MDR) pathogens that are increasingly difficult to treat. Bacterial cells acquire resistance through various mechanisms, including genetic alterations, such as DNA mutations, and the acquisition of drug-resistance genes, often exacerbated by the improper use of antibiotics. Consequently, bacteria employ diverse strategies to counteract the effects of these therapeutic agents (5-8).



It is a fact that the number of MDR bacteria has been rising as a result of the widespread and excessive use of antibiotics. Misuse and overuse of antibiotics have also increased the emergence of MDR bacteria (6,9). Annually, a considerable number of individuals are admitted to hospitals worldwide, and this figure continues to rise. Patients in intensive care units (ICUs) face a particularly high risk of infections. These infections are frequently caused by MDR microorganisms, posing a significant threat to patient outcomes and public health (10,11).

The use of antibiotics in agriculture and animal husbandry, alongside human medicine, is linked to the rising concern over antimicrobial resistance (AMR). High levels of antibiotic consumption in animal husbandry are particularly notable in countries like China, the United States, and Brazil (12-14). Citrus canker, caused by *Xanthomonas axonopodis* pv. *citri*, poses a significant threat to the citrus industry. The disease, which has become more severe in the last decade due to resistant *Xanthomonas* strains, leads to major crop losses worldwide (15). Therefore, there is an urgent need to solve this global problem.

Bacteriophages (phages) are viruses that specifically infect and lyse bacteria. Their high host-specificity and lack of threat to eukaryotic cells make them a promising therapeutic alternative (16, 17). The therapeutic application of bacteriophages was first proposed by Twort and d'Herelle in the early 20th century (18). Evidence suggests that phages and their encoded proteins can be effectively utilized to treat bacterial infections, especially in scenarios where conventional antibiotic therapy is ineffective (19). The phages are highly host-specific. They replicate inside the host bacterium and multiply in large numbers to maintain their population for critical times (20). For almost a century, phages have been used in therapeutic settings to treat bacterial diseases (21,22). They appeared to be effective in the treatment of chronic infections (23,24), wound infections, or lung-associated infections in various studies (25,26). According to the WHO report on bacteriophage therapy, only in the Eliava Phage Therapy Center in Tbilisi, Georgia, 5256 patients were treated using bacteriophage therapy across various medical specialties between 2019 and 2023 (27-30). Phage therapy is a century-old treatment targeting bacterial infection, which was used to treat over 6300 patients in various clinical specialties from 1922 to 2022 and was implemented across the world in Europe, America, Asia, and Africa (31).

Bacteriophage therapy, despite its potential, faces challenges that limit its efficacy, such as the need for an optimal multiplicity of infection (MOI) to avoid poor eradication or undesirable immune responses. However, phages offer key advantages, including their ability to replicate at the infection site and their high host specificity. Therefore, this study aimed to isolate and characterize lytic bacteriophages specific to *Xanthomonas axonopodis* pv. *citri* and to identify appropriate therapeutic doses.

This was achieved by determining the optimal MOI for these phages, aiming for effective bacterial clearance while maintaining phage stability and minimizing potential drawbacks.

## Materials and Methods

### Collection of Bacterial Strain

An environmental isolate of *X. axonopodis* pv. *citri* (accession number: MH106434) was provided by Dr. Mujaddad-ur-Rehman (Department of Microbiology, Abbottabad University of Science and Technology, Pakistan). The bacterial strain was utilized as a host for bacteriophage isolation and characterization.

### Bacteriophage Isolation and Propagation

To isolate bacteriophages, soil samples were collected from Khanpur region in Haripur. The collection and processing were performed using a previously reported method with slight modification (32,33). Through this refined procedure, a bacteriophage was successfully isolated from these soil samples. For this purpose, 10 g soil samples were collected from citrus orchards exhibiting citrus canker symptoms. These samples were transferred to conical flasks and left undisturbed for 24 hours to facilitate the sedimentation of heavy particles. Then, the soil was suspended in 90 mL of SM buffer [100 mM NaCl, 8 mM MgSO<sub>4</sub>, 50 mM Tris-HCl (pH 7.5)] and thoroughly vortexed for 10 minutes to release phages into the solution. After 24 hours, the clear supernatant was filtered using a 0.22 µm sterile syringe filter and collected in a separate sterile Falcon tube. For phage enrichment, 10 mL of the filtered supernatant was combined with 10 mL of an overnight culture of *X. axonopodis* pv. *citri*. The resulting enriched filtrate was subsequently assessed using the spot assay.

### Assessment of Bacteriophage by Spot Assay

Bacteriophage presence was confirmed using the spot assay, as previously described (34,35). Phages showing robust lytic activity on the most susceptible host strains were then selected. These active filtered phage isolates were subsequently advanced for further analysis.

### Purification of Bacteriophages

Initially, serial dilutions of the phage suspension were prepared for purification. Each dilution received an addition of 100 µL of an overnight bacterial culture (*X. axonopodis* pv. *citri*). The resulting mixtures were subsequently incubated at 37 °C for 5 minutes. After pouring the mixture onto an LB agar plate, 3-4 mL of semi-solid LB medium was added to form an overlay. Each Petri plate was then gently rotated to ensure thorough and even distribution of the overlay. Afterwards, the plates were incubated at 37 °C for 24 hours. After overnight incubation, small clear plaques, indicative of phage formation, were observed on the LB agar plates. Using a sterile micropipette tip, an isolated plaque was picked

from the LB agar plate. This plaque-picking process was repeated two to three times to ensure the purification of the phage isolate. The purified phages were then stored in SM buffer supplemented with 0.002% (w/v) gelatin for short-term storage at 4 °C. For long-term preservation, phages were stored at -80 °C with the addition of 7% (v/v) dimethyl sulfoxide (DMSO) (32).

### Phage Titer Determination

Titration of phages was done using the double-layer agar method described by Adams (36). The phage stock solution was serially diluted. Briefly, 100 µL of each dilution was transferred into different sterile test tubes. Then, 100 µL of bacterial culture was added to each test tube and kept at room temperature for 10 minutes. Afterwards, 3 mL of 0.7% top agar was added. The mixture was carefully poured on fresh nutrient agar plates and kept for 10-15 minutes to allow the top agar layer to solidify. Then, the mixture was incubated at 37 °C for 24 hours, and the plates were checked for plaque formation. Finally, the plates with clear plaques were selected for phage titer determination.

(PFU/mL) = number of plaques /  $D \times V$

D = dilution factor

V = volume of diluted virus

### Determination of Bacterial Growth Curve

Briefly, 50 mL of LB broth was aliquoted into two 250 mL Erlenmeyer flasks. One flask was inoculated with a fresh culture of *X. axonopodis* pv. *citri*, while the other served as an uninoculated control. Both flasks were incubated at 37 °C. Optical density measurements at 600 nm ( $OD_{600}$ ) were performed every 30 minutes using a spectrophotometer, commencing after two hours of incubation. After 24 hours of incubation, samples were serially diluted and plated on agar to determine colony-forming units (CFUs). Both CFU counts and  $OD_{600}$  measurements were subsequently plotted over time to characterize bacterial growth (37).

### Determination of Bacteriophage MOI for Bacterial Growth Reduction

To assess bacterial growth and evaluate the effectiveness of phage infection, a bacterial reduction assay was performed, as described previously (38). This assay quantifies bacterial growth by measuring bacterial viability over time. For this assay, a standardized overnight culture of *X. axonopodis* pv. *citri* at a concentration of  $3 \times 10^7$  CFU/mL was utilized. Four selected bacteriophage isolates (1P, 2P, 3P, and 5P) were individually tested. Experiments were conducted in a 96-well plate format with varying MOIs, defined as the initial ratio of phages to bacterial cells.

The plate setup was as follows:

Column 1: Contained 100 µL of LB broth and served as a sterile negative control.

Column 2: Inoculated with  $3 \times 10^7$  CFU/mL of bacterial culture alone, serving as a positive control for bacterial

growth.

Columns 3 to 8: Contained bacterial culture mixed with phages at MOI values of 0.001, 0.01, 0.1, 1, 10, and 100, respectively, to investigate the effect of varying phage concentrations on bacterial growth.

Upon completion of the plate setup, the plates were incubated at 37 °C. At 2, 4, 8, 12, 16, 20, and 24 hours post-incubation, 10 µL samples were collected from each well at its respective MOI. These samples were then serially diluted in 90 µL of LB broth. Following incubation of the dilution plates, colonies were counted in each dilution, and the bacterial concentration was computed as colony-forming units per milliliter (CFU/mL). These CFU/mL values were subsequently plotted graphically to illustrate the bacterial concentration dynamics over time at varying MOIs, enabling the comparison of bacterial reduction across different MOI values and time intervals (33).

## Results

### Isolation and Purification of Bacteriophage

Bacteriophages were successfully isolated from soil samples using the soft agar overlay method, and *X. axonopodis* pv. *citri* served as the host bacteria. The spot test indicated the presence of bacteriophages showing the clear plaques on the lawn of host bacteria. The isolated phage, specific to *X. axonopodis* pv. *citri*, produced several small circular plaques with a diameter ranging from 3-5 mm on the LB agar plates. These plaques were further analyzed to get uniform bacteriophage. A double-layer agar assay was performed for the purification of bacteriophages. The results are summarized in Table 1 and illustrated in Figure 1.

### Determination of Bacteriophage Titer

Purification and bacteriophage titer determination were done using a double-layer agar test. This approach enabled accurate quantification of plaque-forming units (PFU), confirming the presence and quantification of active bacteriophages. The PFUs are confirmed via Table 2 and Figure 2.

### Determination of Bacterial Growth Curve

Antibacterial activity was assessed using a bacterial growth reduction assay. The optical density method was used to develop a growth curve. *Xanthomonas axonopodis* pv. *citri* culture was inoculated in LB broth and optical density at 600 nm ( $OD_{600}$ ) was measured hourly to monitor bacterial growth dynamics. Simultaneously, aliquots of culture, prepared using the spreading technique, were

**Table 1.** Results of the Spot Assay against *Xanthomonas axonopodis* pv. *citri*

S. No.	Bacteriophages	Area	Result
1	1 P	Khanpur	Positive (+)
2	2 P	Khanpur	Positive (+)
3	3 P	Khanpur	Positive (+)
4	5 p	Khanpur	Positive (+)

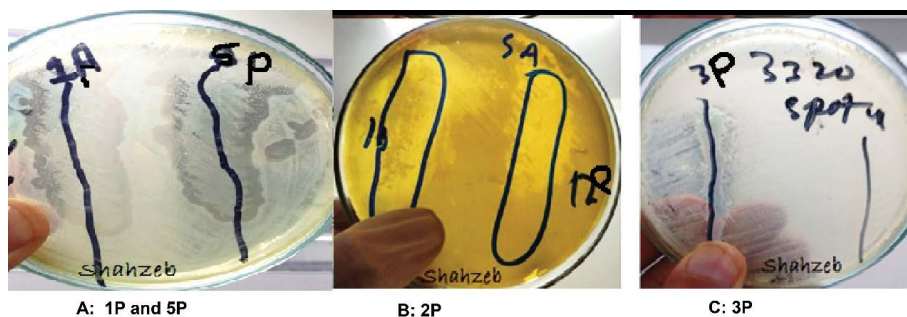


Figure 1. Spot test confirming phage lysis on *Xanthomonas axonopodis* pv. citri



Figure 2. Plaques of Different Titrations

Table 2. Result of Bacteriophage Titer Targeting *Xanthomonas axonopodis* pv. citri

S. No.	Bacteriophages	Titer (PFUs/mL)
1	1P	$3.0 \times 10^8$
2	2P	$2.7 \times 10^8$
3	3P	$3.5 \times 10^9$
4	5P	$4.0 \times 10^9$

plated on LB agar plates to measure the impact of bacterial proliferation. The recorded OD600 values are presented in Figure 3).

**Growth of *Xanthomonas* pv. citri at 7 °C**

*Xanthomonas axonopodis* pv. citri was streaked on LB agar plates and incubated at 7 °C to assess the effect of temperature on its growth. The colonies developed a yellow pigment, characteristic of *X. axonopodis*, indicating active synthesis. These characteristics suggest that the bacteria continue to grow even at 7 °C and metabolize effectively, showing that low temperature does not affect the growth of *X. axonopodis* pv. citri (Figure 4).

**The Activity of Isolated Bacteriophages at 7 °C against *Xanthomonas axonopodis* pv. citri**

The activity of isolated bacteriophages specific to *X. axonopodis* pv. citri was observed at 7 °C using a double-layer technique. The phage produced several tiny asymmetrical elongated circular plaques on the LB Agar plates, indicating successful infection and lysis of bacterial cells. Our results revealed that phages retained their viability at low temperatures with an average of 201 plaques observed on each plate, suggesting that low temperature conditions did not affect the activity of

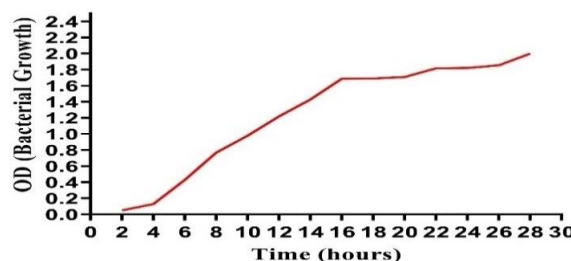


Figure 3. Bacterial Growth Curve until 28 Hours

bacteriophage (Figure 5).

**Bacterial Growth Reduction by Bacteriophages**

The isolated bacteriophages demonstrated a strong ability to reduce bacterial cell counts. To establish their lytic function, the phages were tested against *X. axonopodis* pv. citri in liquid medium using the bacterial reduction assay. During a 24-hour in vitro incubation period at 37 °C, efficient bacterial lysis by the selected bacteriophages was observed. The results of this phage-mediated bacterial reduction assay were compared to those of control samples. Compared to the controls, phage-infected samples exhibited a significant decrease in bacterial concentrations (Figure 6).

**Effect of Different Bacteriophages on Bacterial Growth at Different MOIs**

The in vitro therapeutic efficacy of bacteriophage 1P against *X. axonopodis* pv. citri was evaluated using the bacterial growth reduction assay. Compared to control samples, phage infections resulted in a significant reduction in bacterial colonies. Bacteriophage 1P effectively inhibited *X. axonopodis* pv. citri growth for 24 hours at MOIs of 0.01, 10, and 100 (Figure 7). In contrast, at lower MOIs

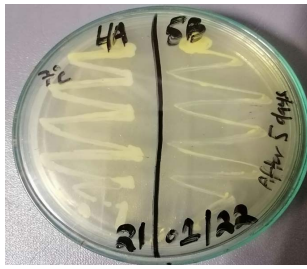


Figure 4. Growth of *Xanthomonas axonopodis* pv. *citri* at 7 °C

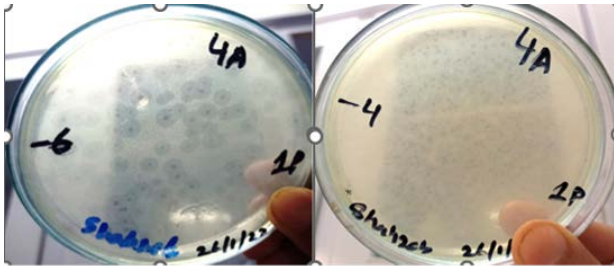


Figure 5. Activity of Isolated Bacteriophages at 7 °C against *X. axonopodis* pv. *citri*

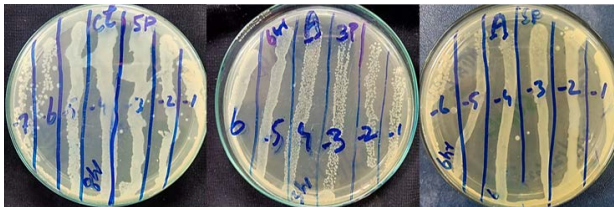


Figure 6. In Vitro Effect of Bacteriophage on the Respective Bacteria: Reduction of Bacterial Cells by Corresponding Phages Compared with Control

of 0.001 and 1, bacterial populations exhibited some resistance or regrowth after 20 hours. The considerable reduction in bacterial growth observed at MOI values of 0.01, 10, and 100 after 24 hours of incubation highlights the potential of phage 1P as a therapeutic agent against *X. axonopodis* pv. *citri* (Figure 7).

Phage 2P demonstrated a decrease in bacterial viability across all tested MOIs (0.001, 0.01, 0.1, 1, and 100) during a 24-hour incubation (Figure 8). While a slight increase in bacterial resistance was noted at an MOI of 10 after 20 hours, phage 2P maintained maximum lytic activity between 20 and 24 hours. This sustained efficacy further indicates its therapeutic potential as an effective antibacterial agent (Figure 8).

Phage 3P demonstrated maximum lytic activity at 37 °C by effectively controlling the growth of *X. axonopodis* pv. *citri*. Phage 3P was found to effectively control bacterial growth until 24 hours. At MOIs of 0.01, 0.1, 1, 10, and 100, the bacteriophage 3P inhibited *X. axonopodis* pv. *citri* growth until 24 hours compared with the control, specifically at MOI 10, bacteriophages maintained bacterial suppression throughout the 24 hours, while at the lowest MOI (0.01), some bacterial resistance was observed after 16 hours. Overall, bacterial growth in the 3P-treated bacterial sample was greatly reduced, which also indicates the therapeutic potential of 3P (Figure 9).

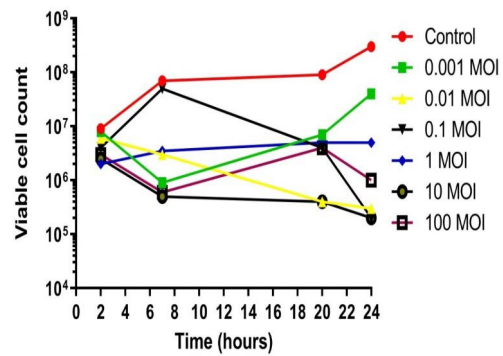


Figure 7. In Vitro Effect of Phage 1P on Bacterial Growth: Reduction Compared to the Control. Viable cell count (PFUs/mL)

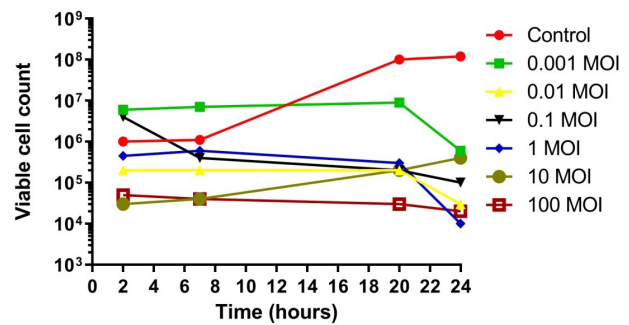


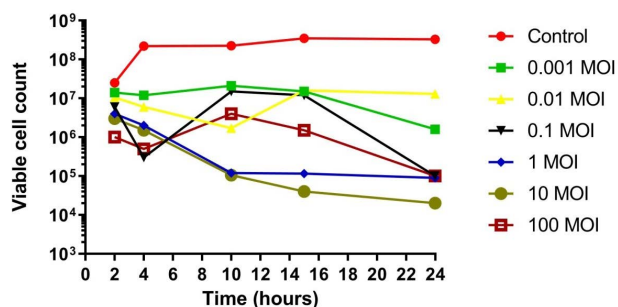
Figure 8. In Vitro Effect of Bacteriophage 2P on Bacterial Growth: Reduction Compared to Control. Viable cell count (PFUs/mL)

Phage 5P served as a notable example of effective antibacterial action; infections by phage 5P led to a significant decrease in bacterial colonies compared to controls. Despite observing transient bacterial resistance during the initial two hours, this phage subsequently maintained inhibition of bacterial growth for the remainder of the 24-hour period, indicating promising potential for phage therapy (Figure 10). A general decline in bacterial populations was observed from 4 hours to 24 hours across all four tested phages. Nevertheless, transient increases in bacterial growth between 12 and 16 hours of incubation were attributed to the emergence of phage-resistant bacteria or the inactivation of phages.

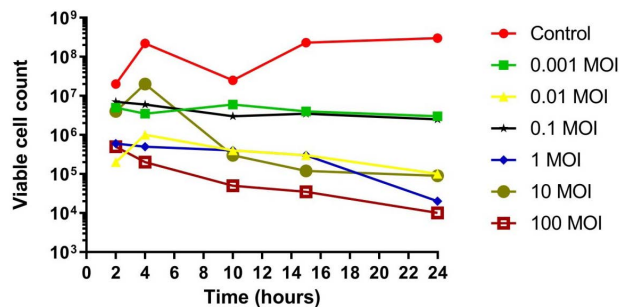
Overall, this in vitro study demonstrated that bacteriophages isolated from soil samples possessed potent lytic effectiveness against *X. axonopodis* pv. *citri*, leading to a significant reduction in bacterial load. These findings suggest that these bacteriophages present a viable alternative to antibiotics for treating bacterial infections, positioning them as promising candidates for subsequent research and therapeutic development. These bacteriophages were thus selected for further investigation.

## Discussion

AMR constitutes a formidable global health crisis, originating in the 20th century but now reaching alarmingly high levels worldwide, necessitating urgent intervention. Phage therapy offers a compelling strategy to combat this challenge. Economic impacts are



**Figure 9.** In Vitro Effect of Bacteriophage 3P on Bacterial Growth: Reduction Compared to Control. Viable cell count (PFUs/mL)



**Figure 10.** In Vitro Effect of Bacteriophage 5P on Bacterial Growth: Reduction Compared to Control. Viable cell count (PFUs/mL)

substantial; an Organization for Economic Co-operation and Development (OECD) report indicates that high-income countries incur average annual costs of US10000 to US40000 for hospitalizations related to antibiotic-resistant bacterial infections. For middle-income and low-income countries, these costs approximate US12476 and US1025, respectively (39). Consequently, phage therapy has recently experienced a resurgence of interest (40). This work aims to contribute to this critical need by focusing on the isolation and characterization of effective phages. The therapeutic application of bacteriophages dates back approximately a century. Felix d'Herelle's initial medical report on the use of bacteriophages to treat patients at the Hôpital des Enfants Malades in Paris was published in 1919 (41).

In this study, citrus fruits and soil served as primary sources for isolating *X. axonopodis* pv. *citri*-specific bacteriophages. Phage isolation and confirmation of lytic activity were performed using the spot test method. Four bacteriophages were subsequently selected for experiments and tested for their lytic activity and effectiveness against *X. axonopodis* pv. *citri*. Soil samples were initially collected from Khanpur region and transported to the laboratory for analysis. Of the 10 soil samples collected, only 4 yielded *X. axonopodis* pv. *citri*-specific bacteriophages, as detected by the spot assay technique. No lytic zones were observed in the remaining samples, even after enrichment.

Bacteriophages are obligate intracellular parasites, and their replication is entirely dependent on the growth and metabolism of their host bacteria (42). Consequently, phage recovery and amplification tend to be more successful in environments where the density of susceptible host bacteria is higher (43). Several studies suggest that the abundance of bacterial hosts directly influences the effectiveness of bacteriophages in altering microbial community composition (44, 45). Since bacteriophages rely on specific bacterial hosts for replication (46), this observation can be attributed to variations in the concentration of *X. axonopodis* pv. *citri* at the sample collection sites, which could influence the availability of host bacteria for phage proliferation. When host bacteria are present in lower densities in the source soil, host-specific phages are less likely to have contact with each other. Most bacteriophages require a minimum

bacterial density of approximately  $10^3$ – $10^4$  CFU/mL to sustain successful multiplication (47).

Only clear plaques were chosen for further analysis based on plaque morphology, as clear zones indicate complete lysis of host bacteria *X. axonopodis* pv. *citri*. The isolated phage produced circular transparent plaques on double agar plates against the host bacteria. However, it was also observed that all the clear plaques were not the same size, which shows the presence of more than one phage in the soil. The plaque is made up of two circular layers, with a completely transparent inner center surrounded by a halo. The halo surrounding the plaque indicates that phage-produced soluble enzymes, such as depolymerase, degrade the bacterial cell walls (48). This observation is consistent with previous studies that have documented a wide range of plaque sizes even within a single phage collection, reflecting inherent genetic and functional diversity among phages (49). The presence of the depolymerase enzyme was shown by the halo, which diffused through the agar layer and broke down the bacterial capsular polysaccharide into distinct oligosaccharide components (50). Rahimzadeh et al achieved a titer of  $20 \times 10^8$  PFU/mL using the double-layer agar assay (51). In an in vitro study, bacteriophages isolated from soil samples displayed lytic effectiveness against bacterial isolates from citrus fruits. These phages displayed lytic activity and successfully reduced the bacterial loads, indicating their potential to serve as a natural alternative to antibiotics. Various studies have been conducted to investigate antibacterial activity utilizing growth reduction assay against *X. axonopodis* pv. *citri*. For example, a study conducted on *X. axonopodis* pv. *manihotis* discussed methods for visualizing bacterial movement and growth in plants, emphasizing the relevance of growth dynamics in understanding pathogenicity (52). Another study focused on the growth dynamics of *X. axonopodis* pv. *punicae* using optical density measurements to evaluate the efficiency of antagonistic strains against this disease, stressing the importance of CFU counts in determining bacterial proliferation (53).

Our observations indicate a significant inhibitory effect of bacteriophages on the growth of *X. axonopodis* pv. *citri*, particularly within the initial 2 to 18 hours of treatment. At MOIs of both 0.001 and 1, we observed the emergence of some bacterial resistance to the bacteriophages after

20 hours. Specifically, *X. axonopodis* pv. *citri* populations were almost entirely suppressed until 18 hours. While a slight increase in bacterial count was observed after 20 hours, likely attributable to the emergence of phage-resistant mutants, this increase was still considerably lower compared to the control group. This phenomenon aligns with findings of Wang et al, who reported a continuous reduction in bacterial count over an 8-hour period with the novel bacteriophage vB\_SauS\_SA2, followed by the development of phage resistance (54).

Initially, bacteria showed some resistance to phage infection during the first two hours; however, phage activity could overcome this resistance effectively, suppressing bacterial growth up to 24 hours, which indicates promising potentialities of bacteriophage therapy. From 4 to 24 hours, all four tested phages showed a continuous decrease in bacterial populations. This delayed but effective lytic response aligns with previous reports suggesting that early-phase resistance may be due to host stress responses, adsorption inefficiency, or population heterogeneity (21). Our in vitro investigation revealed that bacteriophages isolated from soil exhibited potent lytic efficacy against citrus-associated bacteria, significantly reducing the bacterial load and underscoring their potential as alternatives to conventional antibiotics. A study by Lerdittikul et al discovered a new bacteriophage, VL10, which demonstrated strong lytic activity against MRSA. Their results revealed a dramatic reduction in bacterial counts within the first 20 hours post-infection, consistent with our current findings. However, a subsequent resurgence in bacterial growth was observed after this period due to the development of resistance, which correlates with our own observation of higher bacterial counts after 20 hours (55).

Tuomala et al (2021) explored the application of phage therapy for the eradication of LA-MRSA in carrier pigs. They reported initial reductions in bacterial counts, but they also noted the evolution of resistance over time. These findings parallel our own observations regarding the emergence of resistance following sustained exposure at MOIs of 0.001 and 1. Such results suggest that phages have the potential to serve as an alternative to antibiotics for treating bacterial infections (16).

The outcomes of the current study are highly significant, as the isolated phage has the capability to lyse *X. axonopodis* pv. *citri*. By leveraging phage therapy as an alternative, this approach could pave the way for sustainable and targeted bacterial control in agricultural settings, ultimately enhancing crop protection while minimizing negative effects on beneficial microbes and the environment and serving as an important step toward phage therapy. This study resulted in the identification of *X. axonopodis* pv. *citri*-specific phage, which is a valued finding associated with microbiology and other fields.

## Conclusion

A novel bacteriophage with high specificity for *X.*

*axonopodis* pv. *citri* was successfully isolated and characterized. The isolated phage exhibited significant lytic activity, effectively inhibiting the growth of *X. axonopodis* pv. *citri* for over 24 hours. These results underscore the strong potential of this bacteriophage as a valuable biocontrol agent for citrus canker. It could be developed as an effective tool for disease management, providing both preventive and curative strategies against infections caused by *X. axonopodis* pv. *citri*.

## Authors' Contribution

**Conceptualization:** Mohammad Irfan Khan, Shahzeb Javed, Mujaddad Ur Rehman, Muhammad Rizwan, Amir Sasan Mozaffari Nejad.

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**Formal analysis:** Mohammad Irfan Khan, Shahzeb Javed, Mujaddad Ur Rehman, Muhammad Rizwan, Amir Sasan Mozaffari Nejad.

**Funding acquisition:** Mujaddad Ur Rehman.

**Investigation:** Mohammad Irfan Khan, Shahzeb Javed, Mujaddad Ur Rehman, Muhammad Rizwan, and Amir Sasan Mozaffari Nejad.

**Project administration:** Farjana Islam Aovi, Turki M. Dawoud, Atraw Asrat Mengistie.

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## Competing Interests

The authors declare that they have no competing interests.

## Consent for Publication

Not applicable.

## Data Availability Statement

The data will be made available when requested.

## Ethical Approval

Not applicable.

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