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Isolation and *In Vitro* Characterization of a Bacteriophage Targeting Antibiotic-Resistant *Helicobacter pylori*

Yasaman Zahra Mohseni, Ameneh Elikaei*, Bahareh Attaran, Mahsa Soltani

Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran

ABSTRACT

Background and Aim: The high occurrence rate of antibiotic-resistant *Helicobacter (H.) pylori* has led to consideration of novel therapeutic approaches. This study aimed to isolate and characterize a bacteriophage against antibiotic-resistant strains of *H. pylori* isolates from gastric biopsies of patients.

Materials and Methods: Untreated wastewater was collected from Imam Hossein, Taleghani, and Mofid Children's Hospitals and mixed together. The presence of bacteriophage was confirmed by double-layer agar technique. Transmission Electron Microscope (TEM) was used to determine the morphology of the isolated phage. In addition, stability tests were performed. The host range and multiplicity of infection (MOI) of bacteriophages were determined. Finally, the phage genome was extracted and its concentration was calculated using a Nanodrop spectrophotometer.

Results: TEM micrograph showed that the isolated phage belonged to the *Caudoviricetes* class and had Podoviridae-like morphology. It was effective against 9 clinical strains of *H. pylori* and the standard strain of *Campylobacter jejuni*. The optimal activity of this phage was at 37°C and pH 4 to 7. This phage was sensitive to chloroform, and was stable with increasing salt concentration. It was revealed that MOI=1 was optimal for this bacteriophage activity.

Conclusion: In conclusion, the isolated phage was effective against antibiotic-resistant strains of *H. pylori* isolates from gastric biopsies of patients. It can be considered a suitable candidate for further genomic and *in vivo* studies.

Keywords: Antibiotic Resistance, *Campylobacter jejuni*, *Caudoviricetes*, *H. pylori*, Phage Therapy, *Podoviridae*

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Corresponding Information: **Ameneh Elikaei**, *Department of Microbiology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran* & Email: A.elikaei@Alzahra.ac.ir

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1. Introduction

Helicobacter (H.) pylori is a Gram negative, spiral-shaped, and microaerophilic pathogenic bacterium considered a common global chronic infection in human **(1)**. It colonizes in the stomach and can lead to chronic gastritis, peptic ulcer, adenocarcinoma, mucosa associated lymphoid tissue lymphoma and gastric cancer in individuals **(2)**. It is reported that *H. pylori* infects approximately 4.4 billion people globally **(3)**, and its prevalence is higher in developing countries compared to developed countries. This higher prevalence is associated with personal hygiene, water quality, urbanization, socioeconomic status, and environmental sanitation **(3, 4)**.

The pathogenicity of *H. pylori* is driven by multiple virulence factors. The key factors are the Cag pathogenicity island, which delivers the CagA oncoprotein into epithelial cells **(5)**, and the VacA cytotoxin, which disrupts host cell function **(6)**. Additional traits such as urease activity **(7)**, flagella,

and helical shape support the bacterial survival and colonization within the gastric niches, contributing to persistent infection and disease progression **(8, 9)**. Eradication of *H. pylori* is crucial, as it is classified as Group I gastric carcinogen by the International Agency for Research on Cancer and the U.S. Department of Health and Human Services (HHS) **(10)**. Standard treatments with antibiotics and proton pump inhibitors (PPIs) **(11)** are compromised by declining the eradication rates, rising antibiotic resistance **(8, 12)**, and treatment-related side effects **(13)**. Consequently, the World Health Organization (WHO) has emphasized the urgent need for new therapeutic strategies, particularly against clarithromycin resistant strains **(14)**.

Bacteriophage therapy is considered an effective strategy to overcome multi-drug resistant (MDR) bacterial infections. They are safe, natural predators that can destroy bacteria with minimal disruption of normal flora **(15)**.

Bacteriophages are categorized into lytic (virulent), lysogenic (temperate), and pseudo-lysogenic phages. Lytic phages are treatment candidates for the phage therapy because they can attach to the target bacteria and inject their nucleic acids into the cell. Finally, they multiply, assemble, and destroy the host cell to release the progeny and infect other bacteria **(16)**. Temperate phages integrate their nucleic acids into the plasmid or chromosome of target bacteria, resulting in the formation of prophages that can promote bacterial evolution by mechanisms such as horizontal gene transfer and transduction **(17-19)**. Pseudo-lysogenic bacteriophages exist as episomes in the cell and can postpone the lysis process in target cells when nutrient depletion occurs **(20, 21)**. Bacteriophages offer potent advantages compared to antibiotics, including specificity of action, narrow spectrum of activity, higher safety, easy administration, higher tolerability, less expensive processes, localized effect at the site of infection, and the possibility of being engineered using genetic methods or technologies **(22)**.

There are different approaches in phage therapy, such as bacteriophage cocktail, the combination of bacteriophages and antibiotics, bacteriophage-derived enzymes, and bacteriophage engineering via clustered regularly interspaced short palindromic repeats associated (CRISPR-Cas) system **(23)**.

Despite increasing interest in phage therapy as a potential solution to antibiotic resistance, it remains a notable paucity of well-characterized lytic phages specifically targeting *H. pylori*. Most phage studies in *H. pylori* have focused on prophages or on phage with partial characterization such as morphology alone. For example, lytic phages such as ΦHPE1 and ΦHPE2 have been reported, but without comprehensive *in vitro* metrics critical for the therapeutic assessment **(20)**. The lack of comprehensive *in vitro* data on lytic *H. pylori* phages including host range, replication dynamics, stability in gastric-like conditions, and genomic safety hampers their evaluation as therapeutic candidates.

Due to the mentioned challenges of *H. pylori* treatments and the advantages of bacteriophages, the isolation and characterization of a bacteriophage against resistant strains of *H. pylori* isolates from gastric biopsies of patients was carried out in this study. We isolated a bacteriophage against antibiotic-resistant *H. pylori* from untreated hospital swage and characterized its morphology using Transmission Electron Microscope (TEM). We further assessed its physiochemical stability under varying environmental conditions and evaluated its lytic activity through host range and multiplicity of infection assays. The objective of this work was to determine whether the isolated phage exhibits the properties of a stable, lytic candidate with potential for future *in vivo* studies.

2. Materials and Methods

2.1 *H. pylori* Strains Growth Condition

The *H. pylori* clinical strains were isolated from gastric biopsies of children and adult patients with chronic infections. Their biofilm formation ability, and antibiotic resistance susceptibility were identified in the previous studies **(24)**. *H. pylori* strains resistant to different antibiotics such as amoxycillin, tetracycline, clarithromycin, and levofloxacin were selected as bacteriophage host ranges, which covers different combinations of antibiotic resistance. Moreover, most of them were classified as moderate biofilm formation strains. Strain 5, which exhibited resistance to amoxicillin, tetracycline, and levofloxacin with moderate biofilm-formation was selected as a bacteriophage host, as it represented a clinically relevant MDR phenotype. Moreover, its moderate biofilm-forming ability reflected the persistent infection characteristics commonly observed in patients. The *H. pylori* strains were cultured in Brucella agar medium supplemented with 5% sheep blood and incubated at 37°C for 72 hr under microaerophilic conditions using GasPak **(25)**. In addition, oxidase and urease tests, and Gram staining were performed to re-confirm the purity of the strains **(26)**.

2.2 Phage Isolation and Titration

Wastewater was collected from Taleghani, Imam Hossein, and Mofid Children's Hospitals in Tehran. The untreated wastewater samples were centrifuged for 10 min at 12000 g to remove solid contaminants. The

0.45 µm filter was then used to remove the remaining bacteria from the supernatants. A colony of *H. pylori* was removed from a 72-hr culture and inoculated into 5 mL of Brucella broth containing 10% FBS until its turbidity reached 2 McFarland (10^6 CFU/mL at OD 600 nm). Next, 40 mL of the centrifuged and filtered wastewater samples was mixed with 5 mL 2X Brucella broth, 5 mL target bacteria, and 10% FBS. The mixture was incubated at 37°C (100 RPM) for 24 hr under microaerophilic conditions using gas pack. Then, the sample was centrifuged again, and the supernatant was passed through 0.45 µm filter to separate the lysed bacterial remains. The supernatant was stored at 4°C, and a spot test was performed to confirm the presence of *H. pylori* specific phage **(27)**. For the spot test, 2 McFarland of the target bacteria (100 uL) was added to soft Brucella agar (3 mL) at 45°C (0.4% agar). The mixture was then poured onto a Brucella agar. Afterward, 10 uL of phage suspension was spotted on the middle of the plate and incubated at 37°C for 72 hr **(28)**.

2.3 Phage Propagation

This step was performed based on the previous protocol with some modifications. The phage suspension was 10-fold serially diluted in PBS (10^{-1} to 10^{-10}), with the last dilution serving as a negative control without phage suspension. Then, 100 uL of each diluted phage was added to 80 uL of the target bacteria in the logarithmic phase and incubated for 10 min at 37°C. Next, the mixture was added to the soft Brucella agar at 45°C (0.4% agar). The resulting mixture was poured onto a Brucella agar and incubated at 37°C for 72 hr for phage enumeration. Afterward, 5 mL of SM buffer was poured onto a plate with high number of plaques, and the plate was placed into a shaker incubator (50 RPM) for 6 hr at room temperature. After this period, the suspension was collected and used for further steps. This suspension was centrifuged and filtered as in previous steps and then added to a tube containing 10 mL Brucella broth with 10^6 CFU/mL of *H. pylori*. The mixture was incubated at 37°C for 72 hr. Finally, it was stored at 4°C for further studies **(29)**.

2.4 Phage Storage

To preserve the phage, 750 uL of phage-containing supernatant was mixed with 250 uL sterilized glycerol and stored at -70°C **(30)**.

2.5 Transmission Electron Microscopy (TEM)

A drop of high-titer phage (10^{10} CFU/mL) was placed onto a Formvar carbon-coated copper mesh and fixed with fixative (1% glutaraldehyde in PBS) for 1-3 min. The excess fixative was then removed with filter paper. After washing twice with distilled water, the sample was negatively stained with 2% uranyl acetate for 1-2 min. After removing the excess stain with filter paper and washing with water, the sample was dried at room temperature. Finally, the size and morphology of the phage was examined by TEM **(31, 32)**.

2.6 Host Spectrum of the Phage

The resistant *H. pylori* strains to amoxicillin, clarithromycin, levofloxacin, and tetracycline were obtained from Pasteur Institute of Iran, Tehran. Phage host range was evaluated on 8 resistant strains of *H. pylori* and a strain of *Campylobacter (C.) jejuni* using a spot test method. To check the sensitivity of bacterial strains, 100 uL of 2 McFarland of the *H. pylori* strains was added to 3 mL of soft Brucella agar at 45°C (0.4% agar). The mixture was then poured onto Brucella agar plates. Afterward, 10 uL of the phage suspension was spotted in the middle of each plate and incubated at 37°C for 72 hr **(28)**.

2.7 Stability Tests

The phage stability test was analyzed across different temperatures, pH levels, and concentrations of NaCl and chloroform. All tests were performed in triplicate. The sample with pH 7, and 37°C temperature was considered as a control.

2.7.1 Temperature Stability Test

The isolated phage (100 µL) was added to Brucella broth. The prepared solutions were incubated for 1 hr at different temperatures (-20, 4, 20, 37, 50, 60, and 70°C). The phage titer was then evaluated using double-layer agar method **(28, 33)**.

2.7.2 pH Stability Test

Brucella broth was prepared in different pH values (2, 4, 7, 10, and 14). Then, the isolated phage was added to Brucella broth with different pH values at 1:10 ratio and incubated at 37°C for 1 hr. The phage titer was evaluated using double-layer agar method **(28, 33)**.

2.7.3 NaCl Concentration Stability Test

Brucella broth containing different NaCl concentrations (5%, 10%, and 15%) was prepared. Next, the isolated phage was added to different NaCl concentrations of Brucella broth at 1:10 ratio and incubated at 37°C for 1 hr. The phage titer was evaluated using double-layer agar method **(28, 33)**.

2.7.4 Chloroform Stability Test

This test was performed based on the previous study with some modifications. The isolated phage was added to chloroform at 1:1 ratio in duplicate. One sample was incubated at 37°C for 1 hr, and the other was incubated at 37°C (100 RPM) for 5 hr. Finally, the

phage titer was determined by double-layer agar method **(34)**.

2.8 MOI of Phage

The phage MOI was defined as the average number of phages that infect each bacterial cell. To determine the optimal MOI for this phage, 4 tubes were prepared, each containing 10^6 CFU/mL of the target bacteria.

Tube 1: (control): No phage added (MOI 0), Tube 2: 10^6 PFU/mL (MOI 0.01) added, Tube 3:10^7 PFU/mL added (MOI 0.1), and Tube 4: 10^8 PFU/mL of phage added (MOI 1).

The tubes were then incubated in shaker incubator at 37°C. Light absorption was measured at 600 nm every 30 min for a total of 240 min to monitor bacterial growth **(35)**. The most significant reduction in bacterial absorbance was observed at a specific MOI, which was selected as the optimal MOI for our study due to its optimal lytic activity and killing bacterial cells.

2.9 Phage Genome Extraction

The phage nucleic acid was extracted according to previous protocols, with some modifications **(28, 33)**. A high-titer of phage stock (10^9 PFU/mL) was considered for the extraction. First, 450 uL of the phage stock was incubated with 1 uL DNase I and 1 uL RNase I at 37°C for 90 min to remove any contamination. To inactivate the DNase enzyme, the sample was then incubated at 75°C for 30 min. Then, EDTA (to a final concentration of 20 mM), proteinase K (2 uL), and 10% SDS (50 ul) were added to the mixture and incubated at 56°C for 1 hr to inactive the RNase enzyme and degrade the phage protein. After incubation, 375 uL of phenol-chloroform-isoamyl (25:24:1, pH 8) was added to the mixture. The solution was gently mixed and centrifuged at 13000 g for 10 min. The supernatant was carefully transferred to a new microtube.

The volume of the mixture was adjusted to 500 ulL by adding isoamyl alcohol-chloroform (1:24), and the sample was centrifuged at 13000 g for 5 min. To precipitate DNA, 40 uL of sodium acetate 3 M (pH 5.2) and 800 uL of ethanol 95% were added and mixed gently. The sample was incubated at -70°C for 30 min before being centrifuged at 13000 g for 10 min. The supernatant was discarded, and the resulting DNA pellet was washed with 1 mL of 70% ethanol and centrifuged at 13000 g for 20 min. The supernatant was carefully discarded, and the vial was allowed to dry. The DNA pellet was then re-suspended in 30 uL of deionized water and stored at -20°C. Finally, the concentration of the extracted genetic material was measured using Nanodrop spectrophotometer. To confirm the extracted genome, 5 uL sample was mixed with 1 uL loading dye and run on a 1% agarose gel (80 V, 45 min) alongside a control and a 3 Kb ladder. The gel was then observed using gel doc system. Moreover, to determine the type of bacteriophage nucleic acid, 10 ng of the extracted nucleic acid was incubated separately with 1 uL of DNase and RNase in two separate microtubes. After a 90 min incubation at 37°C, the samples were run on a gel electrophoresis to determine which enzyme degraded the nucleic acid **(36, 37)**. While the extracted DNA was quantified and assessed for purity, whole genome sequencing was not performed in this study; therefore, detailed genomic characterization, including gene content and the presence of lysogenic or virulence genes, was not determined.

2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9.0.0. Data were expressed as means ± standard division (SD). One-way ANOVA was performed and a value of $P<0.05$ was considered statistically significant.

3. Results

3.1 Phage Isolation and Titration Results

The isolated phage produced large, clear and amorphous plaques on the lawn of the *H. pylori* plates that showed phage lytic effect on *H. pylori* (Figure 1).

3.2 TEM Results

Based on TEM micrograph it was revealed that the isolated phage belonged to the *Caudoviricetes* class and had a *Podoviridae*-like morphology. This family is characterized by an envelope and a double-stranded DNA genome. Notably, no tail structure was observed in this phage (Figure 2).

3.3 Host Spectrum of the Phage

The bacteriophage was effective against all 8 clinical strains of *H. pylori* isolated from gastric biopsies (Table 1). Also, the phage exhibited lytic activity against the standard strain of *C. jejuni.*

3.4.1 Temperature Stability Test Results

The phage stability at different temperatures was shown in Figure 3. The optimal temperature for phage activity was 37°C. The phage titer remained stable at 4°C and -20°C with no significant decrease in lytic activity. Interestingly, lytic activity was retained even at 70°C. Furthermore, plaque size on *H. pylori* lawns increased at higher temperatures.

3.4.2 pH Stability Test Results

The phage demonstrated lytic activity within at pH range of 4 to 11, with optimal activity at pH 7 and 4. However, lytic activity decreased significantly in highly acidic and alkaline conditions (Figure 4) and was completely abolished at pH 14.

3.4.3 NaCl Concentration Stability Test Results

As shown in Figure 5, the bacteriophages titer increased with higher NaCl concentrations.

3.4.4 Chloroform Stability Test Results

The bacteriophage was sensitive to chloroform and phage count dropped to zero after 5 and 24 hr of exposure.

3.5 MOI Results

All three tested MOIs affected the host cells significantly compared to the control. However, the most significant reduction in bacterial absorbance or the highest lytic activity of the phage was observed at MOI=1 (Figure 6).

3.6 Phage Genome Extraction and Digestion Results

The nucleic acid concentration was 183 ng/uL. Enzyme digestion confirmed that genetic material was DNA (Figure 7).

Table 1. Antibiotic susceptibility and spot test results of isolated *H. pylori* phage.

Strain	Amx	CLR	LEV	TET	Spot test result
H. pylori 1	S	S	S	R	+
H. pylori 2	S	S	R	R	+
H. pylori 3	S	R	R	R	+
H. pylori 4	R	R	R	R	+
H. pylori 5	R	S	R	R	+
H. pylori 6	S	S	S	R	+
H. pylori 7	S	S	S	R	+
H. pylori 8	S	S	R	R	+

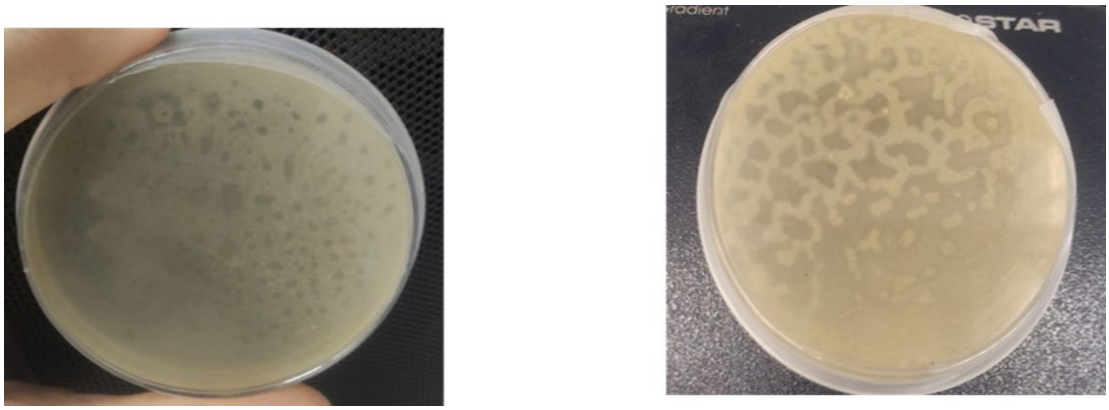

Figure 1. Isolated phage of *H. pylori*. Large, clear and amorphous plaques were formed on the plate **(Prepared by Authors, 2025)**.

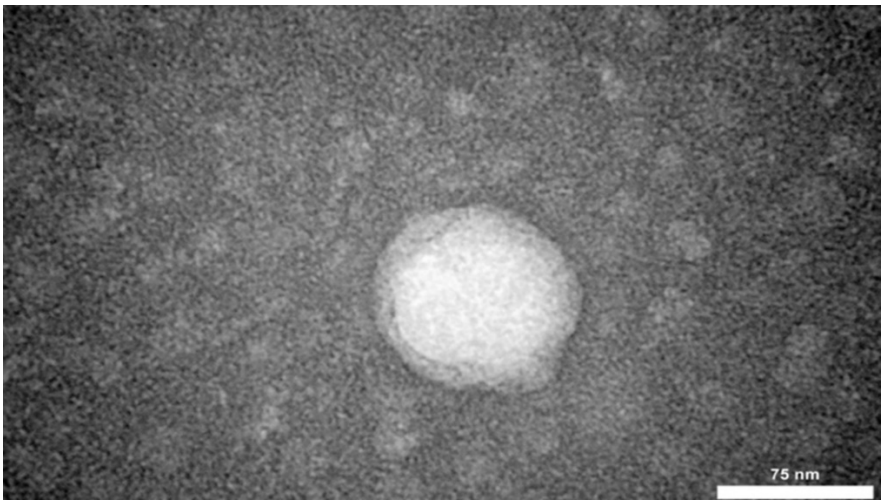

Figure 2. TEM micrograph of *H. pylori* isolated phage. A spherical, tailless virion with approximately 97.439 ± 5.858 nm in diameter. Scale: 75 nm **(Prepared by Authors, 2025)**.

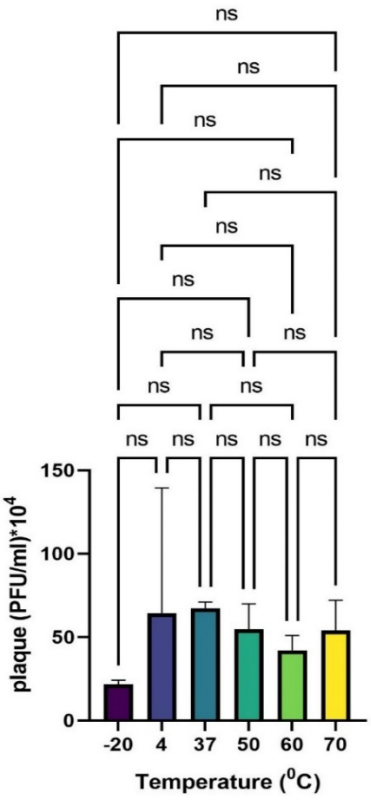

Figure 3. The temperature stability of *H. pylori* isolated phage. The phage titer did not reduce significantly (P>0.05) at different temperatures (-20, 4, 37, 50, 60 and 70°C). The test was performed in triplicate **(Prepared by Authors, 2025)**.

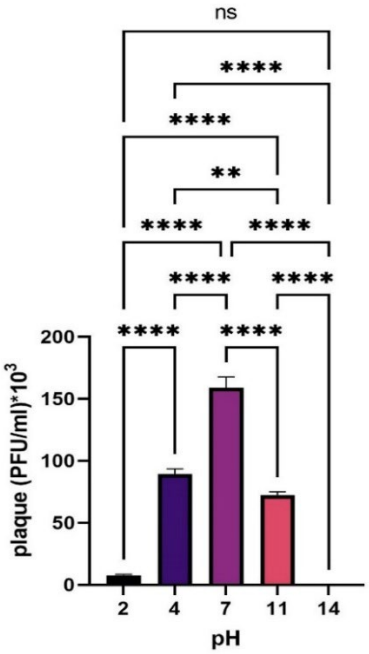

Figure 4. The pH stability of *H. pylori* isolated phage. The phage titer reduced significantly (****: *P*<0.0001) at different pH values (2, 4, 7, 11, and 14). The test was performed in triplicate **(Prepared by Authors, 2025)**.

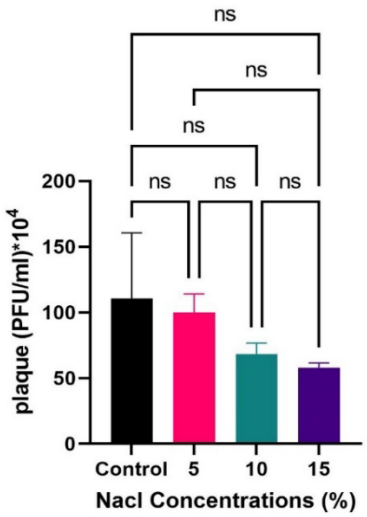

Figure 5. The NaCl concentration stability of *H. pylori* isolated phage. The phage titer did not decrease significantly (*P*>0.05) at different NaCl concentrations (0%, 5%, 10% and 15%). The test was performed in triplicate **(Prepared by Authors, 2025)**.

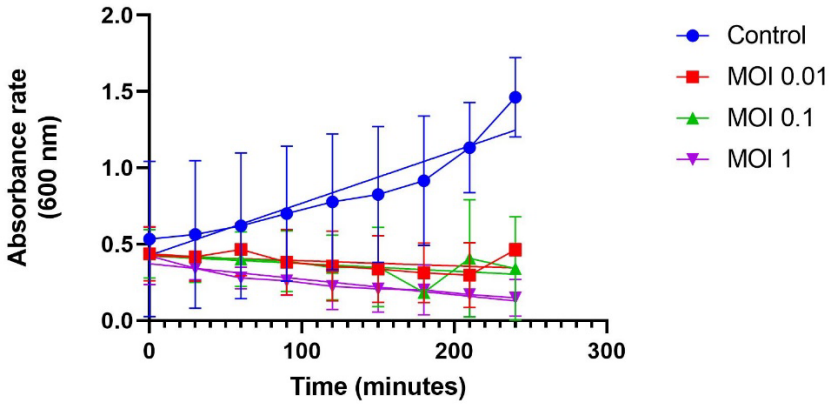

Figure 6. Optimal MOI of *H. pylori* isolated phage. Three different MOI (0.01, 0.1, and 1) were compared with control group (MOI 0). Absorption rate was measured at 600 nm every 30 min for a total of 240 min to monitor *H. pylori* growth. All three MOIs showed reduction in absorbance rate, but this reduction was more significant (P<0.05) at MOI 1. The test was performed in triplicate. **(Prepared by Authors, 2025)**.

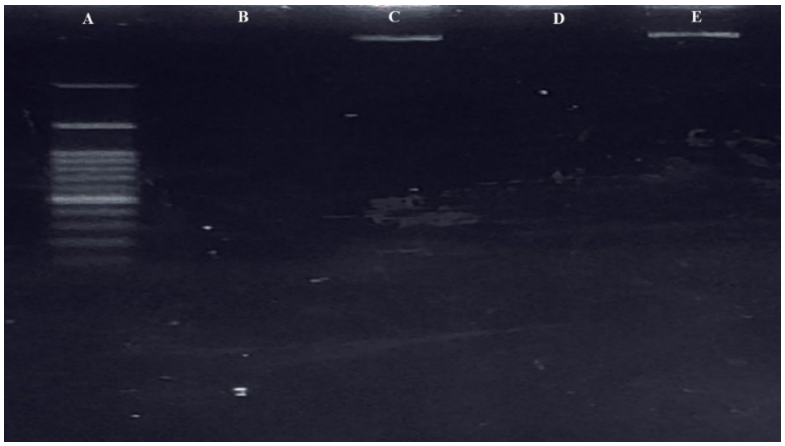

Figure 7. Genome extraction and digestion of *H. pylori* isolated phage. Ladder 3Kb (A); Negative control (B); Extracted nucleic acid (C), The bacteriophage nucleic acid treated with DNase enzyme (D); The bacteriophage nucleic acid treated with RNase enzyme (E). **(Prepared by Authors, 2025)**.

4. Discussion

H. pylori that is a spiral-shaped Gram-negative bacterium and is found in the pyloric region of the stomach causes chronic stomach infection. This bacterium infects more than 50% of the world's population. It is mostly transmitted through fecal-oral and oral-oral route by consuming contaminated water or food **(38)**. It causes 80% of gastric cancers and 5.5% of all malignant diseases globally. Its persistence in the host's stomach is responsible for the chronic inflammation, which is an important sign of carcinogenesis **(39)**.

Recently, drug-resistant bacteria have created a critical challenge for the treatment of clinical infectious diseases. Antibiotic resistance is increasing in all parts of the world and new resistance mechanisms have evolved. It is a major healthcare concern due to its potential to spread internationally, resulting in limited treatment options **(40)**. WHO reported that the number of people who die due to antibiotic resistance problems may reach 10 million by 2050 **(41)**.

The emergence of antibiotic-resistant *H. pylori* has made its treatment difficult, and novel treatment strategies are needed. Probiotics, prebiotics, and bacteriophages can be considered as alternative treatments against resistant *H. pylori* infections **(42)**. Bacteriophages are effective, natural, and safe strategy against bacterial infections. Some studies have examined the efficacy and safety of phage therapy as an alternative approach. To be considered for the therapeutic applications, phages must possess significant characteristics such as safety, multivalent, lytic, broad host range, stability, and ability to replicate in the host **(43)**.

Most of the bacteriophages isolated against *H. pylori* were prophage, and with a few exceptions, there is no available data about their family **(20)**. For example, the prophage PhiHp33 was classified in the *Siphoviridae* family, with a head size of 50-60 nm and a tail of 170*9.5 nm **(44)**. In addition, the phage named 1961P was reported in 2012 by Luo et al **(45)** with a head size of 68-74 nm and a tail size of 23*13.3 nm, and was classified in the *podoviridae* family. There are limited studies on lytic phages for *H. pylori*. In 2008, Vale et al **(46)** isolated a lytic phage from human feces against *H. pylori*. The TEM results showed that the phage was 100 nm, but no tail was observed. This research did not reveal any additional data about the phage characteristics **(46)**. In studies conducted in 2013, Abdel Haliem and Askora isolated two lytic phages against *H. pylori* bacteria, named ΦHPE1 and ΦHPE2. Based on older classification, the phages were reported to belong to the *Podoviridae* and *Siphoviridae*. However, this study did not report other data such as burst size, latent period, or the antimicrobial ability of these isolated phages **(47)**. Cuomo et al **(48)** isolated a novel *H. pylori* phage (HP Ψ) from gastric biopsies, though it's taxonomic family and genomic data were not provided. Notably, under the revised International Committee on Taxonomy of Viruses (ICTV) classification, major phage families such as *Siphoviridae, Myoviridae* and, *Podoviridae* have been abolished and reclassified as undefined taxa **(49)**.

In our study, a phage was isolated from untreated wastewater collected from Imam Hossein, Taleghani and Mofid Hospitals. TEM micrograph revealed a spherical, tailless virion approximately 97.439 ± 5.858 nm in diameter. It had a spherical capsid, and no tail was observed, consistent with previous studies. For instance, Vale et al **(46)** reported that their isolated *H. pylori* phage was also tailless and its size closely matched that phage in our study. Other studies also reported that phages of this family had capsids with short tail or no tail at all **(50, 51)**. Genome digestion analysis using DNase and RNase enzymes revealed fully digested DNase-treated sample, unlike the RNase-treated sample and the control group. This confirmed that the phage genome consisted of DNA. These results could indicate the possibility that this phage belonged to *Causoviricetes* class and had *Podoviridae*-like morphology.

Wide host range bacteriophages are generally more suitable for phage therapy. However, some studies suggest using a combination of narrow and broad host range phages in phage cocktails for the clinical applications **(52)**. For example, in a study on *H. pylori* phages, Hp1 and Hp2 exhibited host ranges of 20% and 4%, respectively **(47)**. Another 2013 study reported that the KHP30 had a 63% host range **(53)**. More recently, Ferreira et al **(29)** in 2022 found that bacteriophage HPY1R had a 6.6% host spectrum. In contrast, the phage isolated in our study had lytic activity against all 8 strains of *H. pylori* isolated from gastric biopsies of patients, as well as a standard strain of *C. jejuni* strain. It is acknowledged that a broader host range analysis would provide more comprehensive data on the phage lytic spectrum. Our main objective was to isolate a phage effective against the specific problem of antibiotic-resistant *H. pylori*, rather than to characterize a phage with a broad lytic spectrum against a general *H. pylori* population. However, this study focused on a representative set of clinically relevant strains. The 8 isolates of *H. pylori* were specifically chosen because they were all clinically isolated from gastric biopsies of patients and confirmed to be antibiotic-resistant. Future research could expand our findings by performing a host range analysis on a larger, more diverse collection of *H. pylori* strains, including those with different genotypes and from different geographical locations, to better assess the phage full therapeutic potential.

Storage stability is a critical factor in phage therapy. For industrial phage products, key requirements include low sensitivity to temperature fluctuations and the ability to maintain viability under various storage conditions **(30)**. Previous studies have evaluated phage stability under different temperatures. A study on Campylobacter strains phages vB-CjP and vBCcM found that their activity remained largely unaffected by short-term (10 min) temperature variations. It was revealed that the temperature changes had slight effects on these phages activities **(54)**. Steffan et al **(55)** reported that a *C. jejuni*-targeting phage retained a lytic activity up to 50°C but was completely inactivated at 70°C. In our study, the isolated phage exhibited optimal activity at 37°C. Notably, no significant reduction in phage titer was observed at 4°C or -20°C, confirming its stability under standard storage conditions. Surprisingly, unlike previous findings, this phage maintained good lytic activity even at 70°C, with only a minimal decrease in viable count and this reduction was not significant. These results align with the thermal stability reported by Al-Mohammai et al **(54)**, but demonstrated superior tolerance to prolonged temperature exposure compared to earlier studies. Given its robustness, this phage could show promise for targeting food-borne pathogens like *H. pylori* in food preservation applications, where temperature resilience is essential.

One of the current challenges in phage therapy is the lack of suitable pharmaceutical forms, particularly for treating intestinal and stomach diseases. The activity of oral phages is easily destroyed by stomach acidity, digestive enzymes, and bile salts **(56)**. Given the importance of bacteriophage stability under acidic

conditions, Ferreira et al **(29)** stimulated the stomach acidic condition in the laboratory using different buffers and enzymes. They reported that the activity of bacteriophage HPY1R did not significantly decrease under this conditions after 2 hr compared to the control group **(29)**. Previous studies on the *H. pylori* and *C. jejuni* phages found that these phages remained stable within pH ranges of 2.5 to 11 and 3 to 11, respectively **(29, 54)**. Similarly, Sabzali et al **(57)**, indicated that the *Salmonella enterica* phage had a broad pH stability range of 2 to 12, consistent with earlier findings. In our study, pH test revealed that this phage maintained a lytic effect at pH range of 2 to 11. Its activity declined significantly at extreme acidic and alkaline conditions, but it was not completely eliminated. However, the phage showed the most lytic activity at pH 7 and lost all activity at pH 14.

Bacteriophage activity was investigated in salt concentrations of 0, 5, 10, and 15%. By increase in NaCl concentration, a slight, non-significant decrease was observed in phage stability. The bacteriophage showed appropriate lytic activity in all three concentrations and the activity of phage did not reduce significantly. These results confirmed the findings of Ahmadi et al **(58)** and Cieplak et al **(59)**. The first study revealed *Escherichia (E.) coli* bacteriophage cocktail had appropriate stability in the presence of bile salts, while the second study reported similar results focusing on the phage PSE against Salmonella strains **(58, 59)**.

Chloroform and other stability tests were also assessed. A 2021 study on bacteriophage JN02, which was isolated for the control of *E. coli* and Shiga toxin, found that it was sensitive to chloroform **(60)**. Our study confirmed this finding; the isolated phage was also sensitive to chloroform, and its activity dropped zero after 5 and 24 hr exposure.

Determining the optimal MOI, which is the ideal ratio of phages to the host cell, is essential for maximizing phage production. Ferreira et al **(29)** reported the optimal MOI for a bacteriophage against *H. pylori* at 1 and our study result was similar. Three MOIs of 0.01, 0.1, and 1 showed lower absorption rates compared to the control and had a significant effect on the host cell. Additionally, the highest progenitor property was observed at an MOI 1.

Based on the isolated phage properties *in vitro* including host range, stability in different environmental conditions (such as temperature, pH, and salt concentrations), and lytic activity, it can be considered a good candidate to treat resistant *H. pylori* infections. However, further studies are needed. The major limitation of this work was the absence of a complete genome sequence of isolated phage. While our phenotypic characterization, such as host range and morphology, could provide a solid foundation, a full analysis of genome will be essential for comprehensive understanding. Genome sequencing step would enable a definitive taxonomic classification of this phage and help us for identification of genes related to its lytic life cycle, essential for its application as a therapeutic agent. Moreover, the genomic data would be crucial for screening the absence of undesirable genes, including virulence factors or lysogeny-related proteins, which is a key safety consideration. Therefore, a comprehensive genomic analysis is a priority for future work to completely evaluate the potential of this phage as a viable candidate for phage therapy against antibiotic-resistant *H. pylori*.

Furthermore, characterization of the phage lytic components, such as lysins and depolymerases, is a crucial next step in evaluating its therapeutic potential. The specific mechanisms of bacterial lysis are key elements to its efficacy and are critical parts of phage biology. Phage-encoded lysins (endolysins) are known to rapidly degrade bacterial cell wall peptidoglycan layer resulting in cell death and have been explored as a standalone antimicrobial therapy **(61)**. Similarly, depolymerases can degrade bacterial polysaccharide capsule, a common resistance mechanism, thereby enhancing phage access to the bacterial cell surface. For example, many bacteria, such as *H. pylori*, are surrounded by an exopolysaccharide (EPS) layer or capsule. Depolymerases are enzymes that can degrade this layer, allowing the phage to access and infect the bacterial cell. Understanding the presence and activity of a depolymerase would give you a significant advantage, as it would explain how your phage overcomes a potential barrier to infection **(62)**. Compared to phages for other pathogens like *E. coli* or *Staphylococcus aureus*, research on *H. pylori* phages is still in its early stages **(20)**. The number of well-characterized *H. pylori* phages is still very small. Therefore, isolating and characterizing new phages is essential to build a diverse library of potential therapeutic candidates, because *H. pylori* is a fastidious microorganism, which makes phage isolation and propagation challenging. This issue has historically limited the number of successful studies. Furthermore, many studies have found evidence of prophages in *H. pylori* rather than strictly lytic phages **(20)**. More research is required to isolate and characterize the obligate lytic phages that would be better suited for phage therapy.

5. Conclusion

This bacteriophage was concluded to be effective against the strains isolated from the patients' gastric biopsies. Due to its characteristics, such as the ability to

lyse the target bacteria, its host range, and stability in different physical and environmental conditions, it can be considered as an appropriate phage. These studies included morphological characteristics, but molecular characterization, such as a comprehensive genomic analysis, is a priority for future work to completely evaluate the potential of this phage as a viable candidate for phage therapy against antibiotic-resistant *H. pylori*. In addition, combination with antibiotics and animal model testing (*in vivo*) are also needed for further studies.

6. Declarations

6.1 Acknowledgment

Not applicable.

6.2 Ethical Considerations

This work was approved by the local ethics committee (Alzahra University) (IR.ALZAHRA.REC.1401.015).

6.3 Authors' Contributions

Ameneh Elikaei was responsible for this study. Acquisition of data was done by Yasaman Zahra Mohseni. The interpretation of data was implemented by Yasaman Zahra Mohseni, Ameneh Elikaei and Bahareh Attaran. Yasaman Zahra Mohseni prepared the draft of the manuscript. The critical revision of the manuscript as well as supervision of the study was performed by Ameneh Elikaei and Bahareh Attaran. Mahsa Soltani contributed to the article by participating in the editing and organization of its content. All participants agreed to be involved in this study.

6.4 Conflict of Interests

The authors declare no conflict of interest.

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This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

6.6 Using Artificial Intelligence Tools (AI Tools)

The authors were not utilized AI Tools.

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