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Evaluation of Bacteriophage to Reduce *Escherichia coli* Contamination in Different Food Samples

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ABSTRACT

Food security is critical in the foodstuffs industry since most bacterial pathogens may cause foodborne illnesses and harm general health. In food, *E. coli* infection was recognized as the major cause of many cases of foodborne diseases. Moreover, the hazards have increased remarkably as most bacteria have acquired resistance to many antibiotics. Bacteriophages (phages) are a natural alternative agent that can be used for food preservation; they are viruses that can target bacteria specifically without causing harm to the human, plant, or animal cells. This research aimed to evaluate the efficacy of phage isolates in suppressing *E. coli* strains isolated from food samples. The phage was collected from sewage water and tested against *E. coli* isolate *(EC/20) in vitro*. Phage ZCECO 5 genome size was assessed to be ~339.5 kbp, and electron microscopy examination revealed that it related to the *Myohoviridae* family. In addition, it was found to be tolerant to a wide range of temperatures and pH. At different Multiplicity of Infections (MOIs), it showed a decrease in bacterial counts, with more bacterial elimination at stronger MOIs. Additionally, at MOI 10, the phage inhibited *E. coli* optical density from 1.0 OD_{600} to 0.7 OD_{600} after 230 min, and following a four-hour incubation period at 37°C, the bacterial titer decreased. The results indicated that ZCECO 5 could lyse *E. coli* and inhibit its activity. As a result, phage ZCECO 5 is suggested to be a bio-control agent in food for *E. coli* control.

Keywords:
Antibiotic resistance, bacteriophage, *E. coli*, food safety, *Myoviridae*.

1. INTRODUCTION

Food safety difficulties caused by pathogenic bacteria contamination are considered the world's most challenging public health issues [1]. Foodborne infections are responsible for around 25% of all food loss

each year [2]. Previously, wide ranges of natural and chemical agents have been used to combat these foodborne diseases. However, many of these products have been documented to have low efficacy or harmful effects on human health [3], [4]. For example, antibiotics have lost their ability to combat bacterial illnesses as antibiotic resistance spreads [5]. New antibiotics are developed seldom, with the most recent class being announced in 2003 [5]. Bacteriophages, also called phages, are bacteria-infecting viruses with two life cycles resulting in the host cell's lysis [6], [7]. Phages were found in the early 1900s, and since, many studies have determined their bacteriolytic activities as medicinal agents. However, following the finding and general use of antibiotics, such as penicillin, bacteriophages have become better widely used as biomedical study agents [7]. In recent years, antibiotic overuse has resulted in a significant bacterial resistance problem and refocused researchers' interest in phages [7]. In addition, phages can combat foodborne infections, using whole phages without harming humans [7]. Experiments on the antibacterial efficiency of phages in foodstuffs settings have demonstrated that phages may be employed in pre and post-harvest therapies to effectively suppress the growth of target bacteria [7]. In addition, Domestic animals and crops could be fed or sprayed with phages. Fruit, meat, vegetables, and processed food can all be decontaminated with antibacterial agents by phages [8], [9]. Phages can actually prevent bacterial pathogen cross-contamination in food-contact items and products [9], [10]. Several commercial bacteriophage products have already been approved for food products since 2006 [11], [12]. *Escherichia coli* relates to the *Enterobacteriaceae* family and is a rod shape, facultative anaerobe and Gram-negative [13]. *E. coli* is separated into five categories based on its virulence: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), attaching and effacing *E. coli* (AEEC), and Shiga toxin-producing *E. coli* (STEC) [13], [14]. *E. coli* O157:H7 is a new *E. coli* strain that causes the majority of EHEC-related human illnesses [13]. *E.coli* has been associated with illnesses such as thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) [13], [15]. Following epidemics of hemorrhagic colitis connected to the eating of contaminated beef hamburgers, *E. coli* was first recognized as a human pathogen [13], [16]. In addition, *E. coli* can be found in both pathogenic and non-pathogenic populations in food, resulting in foodborne disease in consumers and substantial economic loss in the food industries [17], [18]. As a result, preventing or reducing *E. coli* growth is critical for food production. However, just a few types of research have looked at how non-pathogenic *E. coli* populations behave during food production [18]–[21]. *E. coli* infections have been associated to the consumption of contaminated fresh fruit and dairy products, particularly raw milk, and have been isolated from a range of animal-based foods. Many outbreaks have been linked to consuming contaminated fresh produce and dairy products [22]–[26]. *E. coli* infection usually results in self-limiting disease STEC, such as O157, can cause more severe symptoms, including hemolytic uremic syndrome in 10-15 percent of patients [26], [27].

The US Food and Drug Administration approved bacteriophages as antimicrobial food products for specific *Listeria* strain management in prepared chicken and beef in 2006 [28], [29]. Phage treatment is often utilized to lower *E. coli* in sheep and cattle animals, which serve as the bacteria's significant hosts [30], [31]. *E. coli* is an intestinal bacteria found in the intestines of warm-blooded living creatures. Infected persons may have abdominal pains and bloody diarrhea due to several pathogenic strains, such as *E. coli* O157:H7, despite the fact that they are normally harmless [32],[33]. *E. coli* causes 63,000 foodborne diseases and 61 fatalities in the United States every year [34]. Eco-ShieldTM (a cocktail that fights *E. coli* O157:H7) was also used to disinfect artificially infected raw beef in an earlier study, and *E. coli* was decreased by 94 per cent; phage was treated after 5 minutes [34]. Even though phages in the sample were at 4°C for a week, when the beef was re-infected with *E. coli* O157:H7; no permanent

resistance against the bacterium was identified. Another study examined the effectiveness of an *E. coli*-specific phage to control *E. coli* infections, and the findings indicated that ZCEC 1 was capable of decreasing *E. coli* by 6.45 log10 CFU/mL in only one hour after injection *in vitro* [32]. Also, when phage was directly applied to minced beef infected with *E. coli* strain, the number of bacteria in the samples was no longer detectable after 24 hours, showing that phage effectively reduces the abundance of this foodborne pathogen [31], [32]. This research aimed to isolate bacteriophage for *E. coli* that contaminates some food samples as a biocontrol for several foodborne contaminations.

2. MATERIALS AND METHODS

2.1. Samples Collection

One hundred food samples have been collected randomly in sterile falcons from various markets located in Port-Said and Cairo governorates, Egypt, including (Meat, dairy, canned food, vegetables, fruits, cooked, processed food and fish) products. The samples were preserved in an icebox until samples were prepared for the bacteriological examination and isolation of *E. coli*.

2.2. Isolation of *E. coli*

Ten grammes of each sample were weighed and grown in Tryptic Soy Broth (TSB; Oxoid, England) at 37°C using overnight shaking, followed by 1ml of each sample cultured on Eosin Methylene Blue Agar (EMB; Oxoid, England) at 37°C overnight. Stocks have been stored at -80°C in 20 percent (v/v) glycerol until they were utilized.

2.3. Purification and Identification

Depending on colonial macro morphology on EMB media, which gave metallic green shiny colonies, colonies were streaked on fresh plates repeatedly until pure cultures of *E. coli* were obtained. Identification was carried out by smearing the colonies and staining with Gram's stain, followed by microscopic examination for the staining reaction of bacteria and demonstrating the morphology, arrangement and staining reaction of bacteria [35]. Catalase test was carried out by using a small number of bacteria collected from a fresh-isolated 18-24 hour colony to a drop of 3% H_2O_2. Catalase positive results are evident by immediate effervescence and bubbles formation of O_2 [36][37]. A single pure colony from a freshly isolated bacterial culture was taken up and slowly mixed in droplets of distilled water for the Oxidase Test. When oxidase testing strips were loaded into the bacterial smears, there was no colour change [38].

2.4. Antibiotic Sensitivity Test

According to National Committee for Clinical Standards, antibacterial sensitivity test was done using disc diffusion techniques on 16 *E. coli* isolates. In brief**,** 100µL of the bacterial culture has been streaked over a tryptone soy agar (TSA) plate. Then, the antibiotic discs were placed and incubated for 24 hours at 37°C [39]. After reviewing the antibiotic discs ' bacterial growth inhibition zone, the effectiveness of nine various antibiotics against *E. coli* isolate *EC/20* from the *Enterobacteriaceae* family was investigated [40]. Nine different discs of the antibiotics (Oxoid, England.), Tetracycline (TE; 10 µg), Chloramphenicol (C; 30 µg), Gentamicin (CN; 10 µg), Cefotaxime (CTX; 30 µg), Amikacin (AK; 30 µg), Ceftriaxone

(CRO; 30 µg), Kanamycin (K; 30 µg), Amoxicillin (AML; 25 µg), and Rifampicin (RD; 5 µg) were tested against each *E. coli* isolate.

2.5. Polymerase Chain Reaction Thermal Cycle (PCR)

Three virulence genes (stx1, fimH and traT) and four antibiotic resistance genes (tetA, blaCTX, blaSHV, and blaTEM) (Table 1) were tested using the polymerase chain reaction (PCR) technique. Using PCR techniques, identify genes of antibiotic resistance and virulence in *E. coli* isolates [41]–[43]. Stx1 was tested in thirty-eight *E. coli* isolates, and other genes were tested only in two *E. coli* isolates *EC/20* and *EC/15*. The enzymatic procedure of PCR used 50 µL of the following; 25 µL Master-Mix 1× (Thermo Fisher Scientific, Waltham, MA, USA), 5 µL bacterial DNA (Single colonies), and 18 µL nuclease-free H_2O, 1 µL reverse primers and 1 µL forward primers. PCR criteria were; using an Applied Biosystems BIO-RAD thermal cycler, 35 PCR cycles were performed, each containing 1 minute of denaturation at 95°C, 2 minutes of annealing at 65°C for the first ten cycles, decrementing to 60°C by cycle 15, and 1.5 minutes of elongation at 72°C, increasing to 2.5 minutes from cycles 25 to 35 [13], [43]. Gel Electrophoresis Analysis: After 30 cycles, the PCR products have been run on a 1 per cent (w/v) agarose gel in 1x Tris-acetate-EDTA (TAE) buffer (acetic acid 20 mM; Tris-HCl Mm; EDTA 1 mM; pH 7.6) with 5 µL Ethidium bromide at 80 Volt for 50 minutes (Bio-rad) to determine their size [44]. Gel documentation system images were taken, and Gel Documenter advanced (version 2) software was used to analyze them. The gel was examined with Ethidium bromide after incubation and photographed by BioRad Chemidoc.

Table1. Primers, sequences, and parameters for amplification of virulence and antibiotic resistance genes in *E. coli* isolates by using polymerase chain reaction (PCR).

Virulence factor	Target gene	Primer's name	Length (bp)	Annealing temperature (°C)	Amplicon size (bp)	Sequence (5'to 3')	References
TraT	**traT**	traT-F traT-R	21 21	60	290	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	[42], [45]
FimH	**fimH**	fimH-F fimH-R	20 20	60	207	CATTCGCCTGTAAAACCGCC ATAACACGCCGCCATAAGCC	[42], [46]
stx1	**stx1**	stx1F stx1R	23 21	58	180	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	[13], [43]
blaCTX	**blaCTX**	blaCTX-F blaCTX-R	23 23	55	544	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCATA	[42], [46]
TetA	**tetA**	tetA-F tetA-R	20 20	60	494	TTGGCATTCTGCATTCACTC GTATAGCTTGCCGGAAGTCG	[41], [42]
blaTEM	**blaTEM**	TEM-F TEM-R	19 19	50	1150	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATCA	[42], [47]
blaSHV	**blaSHV**	SHV-F SHV-R	19 19	50	885	CACTCAAGGATGTATTGTG TTAGCGTTGCCAGTGCTCG	[42], [48]

2.6. Phage Isolation, Selection, Purification, and Amplification

Ten different phages were collected in clean falcons from Giza and Port Said, Egypt sewage water. The supernatants were filtered from other microorganisms using 0.22 µm syringe filters (Chromtech, Taiwan) after centrifugation at 4000 rpm [49], [50]. The bacteriophages were chosen after actions based on their capability to inhibit a variety of *E. coli* isolates and generate consistent, clear zones of lysis and their replication power to create significant titers on the chosen host concerning the time [44]. A double-layer agar technique and a spotting assay were used to evaluate the antibacterial efficacy of isolated phages [50], [51]. Finally, considering *E. coli* isolates as a bacterial host, a pure plaquing phage was chosen for investigation. Enrichment was achieved to extract the phages, and later 10% (v/v) of chloroform (CHCl3) was added to lyses of infected bacterial cells for phage filtration [50], [52]. To achieve high-titer stocks, all isolated phages have been amplified as described in the following: 100 mL of the bacterial host (10^7 CFU/mL) was inoculated by phages at a multiplicity of infection of (MOI 0.1) and let to lyse bacteria for 4-6 hours in TSB (Oxoid, United Kingdom) at 37°C on a shaker incubator at 120 rpm [50], [53], [54]. After that 1 ml of chloroform (CHCl3) was added so the infected cells are completely lysing [50]. Then, a four-hour calibration investigation was conducted on a smaller scale to determine the appropriate multiplicity of infection MOI for propagation [55]. The pure culture was centrifuged at 5000x g and 4°C for 20 minutes [50], [56], [57]. Phage plaques have been purified by continuous individual plaque extraction using sterilized micropipette tips at least three times to create pure phage stocks[54], [58]. Finally, the phage-containing supernatant was centrifuged for 1 hour at 15,300× g at 4°C [44], [50], [54]. SM buffer (10 mM NaCl; 50 mM Tris-HCl; 100 mM MgSO4/7H2O; pH 7.5) was utilized for resuspending the phage pellets and then filtered using 0.22 µm syringes filters [44], [54]. Also, the titer of the phages was determined using a double-agar overlay plaque assay. [50], [59]. In a 96-well plate, the titer of phage solution has been diluted ten times, with every lane containing 180 µl of SM buffer and 20 µl of phage suspension. As a result, just 10µL aliquots have been spotted on bacterial lawn in triplicate [50], [60]. Shortly, a single colony of bacteria was collected by a sterile inoculating loop, cultured in TSB, and then incubated at 37°C for many hours with shaking. Next, 100 µL of this culture was mixed with 3 ml of 0.3 % Bacto top agar in TSB (~55°C) before being poured on TSA plates. Following solidification, 10 µl aliquots of serially diluted phage were cultured in triplicate on the bacteria. A phage titer was determined after 24 hours of incubation at 37°C [54]. Double-agar overlay plaque technique was used to detect bacteriophage titer [44], [59].

2.7. Host Range Determination of Isolated Phages

The host range of ten various phages collected from sewage water was examined on sixteen *E. coli* isolates. In addition, the double agar overlay plaque test was operated to identify phages with a broad spectrum of lysis against *E. coli* isolates and the capability to generate prominent plaques on the host bacteria [54]. Primary phage titer injected into lawns was not under 10^9 plaque-forming unit (PFU)/mL in 10 µL for every spot, equal to a 10^7 PFU standard test dilution [54]. Therefore, the lytic power of phages was evaluated by observing prominent lysis zones [54]. For the spot test, 100 µL of freshly grown bacteria aliquot was combined with 4 ml of 0.3 % top agar and spread on 1.5 per cent TSA agar plates [50]. Just after the solidification of the top agar, 10 µL of phage dilution was cultured on the plates at 37°C overnight [50], [61].

2.8. Detection of the Frequency of Bacteriophage Insensitive Mutants (BIM)

The frequency of BIM's emergence was determined, as previously stated. At MOI of 100, phage ZCECO 5 with a titer of 10^9 PFU/ml was injected to bacterial host strain *EC/20* with a titer of 10^6 CFU/ml

that was verified to be sensitive to the phage [50]. Following a 10-minute incubation at 37°C, the mixture was spotted after serial dilution in triplicate by a double-agar overlay plaque experiment; the plates were then incubated for 24 hours [50]. BIM has been measured by dividing the number of viable bacteria after phage infection by the number of viable bacteria before infection [62].

2.9. Temperature, UV, and pH Stability of Phage.

After one hour of incubation, the temperature stability of ZCECO 5 (10^{10} PFU/mL) was evaluated at 4°C, 37°C, 50°C, 60°C, 70°C, 80°C and -20°C [50]. Serial dilution of the phage has been spotted in triplets way on host strain *EC/20* by a double-layer standard method rapidly after incubation for phage titer detection [50], [63]. Furthermore, ZCECO 5's UV stability was studied at 10, 20, 30, 40, 50, and 60 minutes. After each duration, successive dilutions of phage were spotted in triplicate on host *EC/20* by a double-layer procedure to quantify phage titer [50]. The phage titer was calculated after a 24-hour incubation period at 4°C of *EC/20* with ZCECO 5 at pH values of 2, 3, 4, 5, 7, 9, 10, 11 and 12 [50], [64]. Different pH levels were established in the SM buffer for comparable conditions [50], [65]. HCl or NaOH was used to adjust the pH range of the SM buffer [54].

2.10. Transmission Electron Microscopy Examination of Phage Morphology (TEM)

The phage ZCECO 5 was centrifuged for half an hour at 12,000 rpm and resuspended in 100 µL SM buffer at a 10^{10} PFU/ml titer [54]. Morphology was examined on glow discharged (1 minute under vacuum) by TEM at the Faculty of Science, Alexandria University, Egypt [44], [50], [66]. The phage solution was placed on Formvar/carbon-coated copper grid (Pelco International) and fixated with glutaraldehyde (2.5 per cent v/v), rinsed, and dyed with 2 per cent phosphotungstic acid. A high-resolution transmission electron microscope (HR-TEM) (JEOL-1230, JEM-2100, Tokyo, Japan) was utilized to examine grids after they had dried, with images taken at various magnifications.

2.11. Pulsed-Field Gel Electrophoresis (PFGE)

The genome size of ZCECO 5 phage (10^{10} PFU/mL) DNA was estimated using pulsed-field gel electrophoresis [50], [67]. First, plugs of phage were made as previously reported, [67] by putting 200 µL of phage in a 200 µL agarose gel made from TE buffer 1x (Tris-HCl, Loba Chemie, India, EDTA, Fisher chemical, Pittsburgh, PA, USA; pH 8) and 1.4 per cent agarose (Lonza, Switzerland), and drying for 15 minutes [66]. The phage was then placed into agarose plugs and digested by lysis buffer (1 per cent *w*/*v N*Lauryl sarcosine [Sigma Aldrich, Gillingham, UK]; 0.2 per cent *w*/*v* SDS [Sigma Aldrich, Gillingham, UK]; 100 mM EDTA; 1 mg/ml Proteinase K [ThermoFischer Scientific], Waltham, MA, USA; pH 8), for 18 hours at 55°C with slight shaking for lysis of the phage capsid and digesting protein parts [50]. After the incubation, the plugs were moved to a 5 ml washing solution and incubated for 1 hour at 37°C, followed by two 20-minute washing stages in 1 ml washing buffer [62]. After being rinsed by washing buffer, two slices (2 mm) of agarose possessing DNA have been put into the wells containing 1 per cent *w*/*v* agarose gel [50,54]. The gel had been prepared with Tris-borate-EDTA buffer 0.5x (TBE buffer) (Tris-HCl, boric acid, Fisher chemical, USA, EDTA; pH8), and the run was done with 2700 ml of 0.5x TBE buffer [66]. Using a Bio-Rad CHEF DRII system, the phage plugs were placed into the gel and then ran for 18 hours at 14°C at 200 Volts (6 V/cm) with a switching time of 3s start to 15s end [50,54,66]. Standard concatenated lambda DNA markers (Sigma Aldrich, Gillingham, UK) were also used to estimate the genome size [50], [54]. The gel was then examined with ethidium bromide and photographed using the BioRad Chemidoc.

2.12. The Time-Killing Curve

The bacterial destruction activity of phage ZCECO 5 against *E. coli* isolate *EC/20* was tested separately at various MOIs **(**0.001, 0.01, 0.1, 1 and 10) as earlier published with minor changes [39,66,68]. A 20 µL aliquot of every phage at titers of 10^5, 10^6, 10^7, 10^8 and 10^9 PFU/ml was combined individually with 180 µL of *E. coli* isolate *EC/20* at a density of 10^7 CFU/L, producing MOIs of 0.001, 0.01, 0.1, 1 and 10, respectively. After that, the combinations were incubated at 37°C, and the bacterial growth was measured using a microplate-reader to estimate OD_{600} (BMG-LABTECH, FLUOstar-Omega, Germany). The MARS Data Analysis Software programme (version 3.42), collected data at 10-minute intervals for four hours. As a control, bacterial culture without phage injection was performed. The procedures were carried out three times [39].

3. RESULTS

3.1. Sampling, Bacterial Isolation and Biochemical Tests Identification

Fifty-one presumptive *E. coli* isolates were isolated from one hundred food samples. They were gathered randomly in sterile falcons from various markets located in Port Said and Cairo governorates, Egypt. Depending on colonial macro morphology on EMB media, which gave metallic green shiny colonies, then streaked on fresh plates repeatedly until pure cultures were obtained. The pure colonies were recognized by their morphology, Gram staining, and biochemical tests, which revealed negative oxidase and positive results.

3.2. Antibiotic Sensitivity Profile

The sensitivity of bacteria to antibiotics similar to those used in humans is divided into resistant, intermediate, and sensitive. The results showed that nine antibiotics from six different classes produced various sensitivity responses in sixteen *E. coli* isolates (Table 2). According to data, nine isolates showed resistance to Gentamicin, and three isolates were resistant to Tetracycline. There was only one resistant isolate to Chloramphenicol. On the contrary, there were no resistant isolates to Cefotaxime and Ceftriaxone. In addition, the results indicated that ten, fourteen and six isolates showed resistance to Amikacin, Amoxicillin and Kanamycin, respectively. Furthermore, the data stated that sixteen isolates gave resistance to Rifampicin. *E. coli* isolate *EC/20* is the most resistant bacteria since it resists six different antibiotics out of nine and used as the primary host.

3.3. Polymerase Chain Reaction Thermal Cycle (PCR)

Three virulence (Stx1, fimH, and traT) and four antibiotic resistance genes (tetA, blaSHV, blaCTX, and blaTEM) were tested using PCR. Stx1 was tested in thirty-eight *E. coli* isolates, and other genes were tested only in two *E. coli* isolates *EC/15* and *EC/20*. The data showed that traT and fimH genes were founded only in *EC/20*, at 290 bp and 207 bp, respectively (Figure 1), while the other genes were absent (Table 3). In addition, Table 4 shows that Stx1 was founded in twenty-eight *E. coli* isolates at 180 bp (Figure 2).

Table2. Antibiotic sensitivity profile of *E. coli* isolate

	Antibiotic	Symbol	Reference of CSLI	*E. coli* Isolates															
				EC/ 11	*EC/ 32*	*EC/ 08*	*EC/ 24*	*EC/ 29*	*EC/ 36*	*EC/ 05*	*EC/ 13*	*EC/ 02*	*EC/ 20*	*EC/ 01*	*EC/ 27*	*EC/ 21*	*EC/ 10*	*EC/ 38*	*EC/ 15*
1	**Gentamicin**	**CN 10**	**S ≥ 15 13–14 ≤ 12 R**	14	12	14	12	14	12	20	12	12	6	12	14	12	14	14	12
	Class: Aminoglycoside			I	R	I	R	I	R	S	R	R	R	R	I	R	I	I	R
2	**Tetracycline**	**TE 10**	**≥ 15 12–14 ≤ 11**	18	18	18	8	18	20	20	22	18	6	16	16	18	16	6	20
	Class: Tetracyclines			**S**	S	S	R	S	S	S	S	S	R	S	S	S	S	R	S
3	**Chloramphenicol**	**C 30**	**≥ 18 13–17 ≤ 12**	22	22	20	24	24	24	24	24	22	6	24	24	22	18	24	24
	Class: Phenicols			S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
4	**Cefotaxime**	**CTX 30**	**≥23 15–22 ≤ 14**	24	22	26	26	30	26	28	24	22	18	22	26	22	20	22	22
	Class: Third generation cephalosporins			S	I	S	S	S	S	S	S	I	I	I	S	I	I	I	I
5	**Amikacin**	**AK 30**	**≥ 17 15–16 ≤ 14**	16	14	14	26	14	14	14	16	14	18	14	16	14	16	14	14
	Class: Aminoglycoside			I	R	R	S	R	R	R	I	R	S	R	I	R	I	R	R
6	**Kanamycin**	**K 30**	**≥ 18 14–17 ≤ 13**	12	12	14	12	12	12	12	12	10	12	12	14	12	12	12	12
	Class: Aminoglycoside			R	R	I	R	R	R	R	R	R	R	R	I	R	R	R	R
7	**Amoxicillin**	**AML 25**	**≥ 18 14–17 ≤ 13**	18	6	6	18	18	18	20	18	6	6	18	18	20	26	8	6
	Class: Aminopenicillins			S	R	R	S	S	S	S	S	R	R	S	S	S	S	R	R
8	**Ceftriaxone**	**CRO 30**	**≥ 23 20–22 ≤ 19**	26	24	22	26	28	28	28	28	26	28	28	28	28	20	24	28
	Class: Third generation cephalosporins			S	S	I	S	S	S	S	S	S	S	S	S	S	I	S	S
9	**Rifampicin**	**RD 5**	**≥20 17–19 ≤16**	6	6	6	10	6	6	6	6	6	6	6	6	8	6	6	6
	Class: Rifamycins			R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

*I Intermediate, *R Resistant, *S Sensitive.

Table3. PCR data for resistance and virulence gene detection

E. coli isolates	Genes of Virulence		Genes of Resistance			
	fimH	traT	blaTEM	blaSHV	tetA	blaCTX
EC/15	--	--	--	--	--	--
EC/20	+	+	--	--	--	--

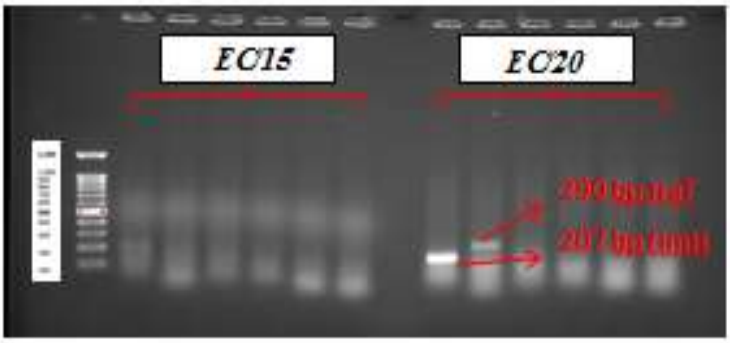

Figure1. PCR detection results in an agarose gel electrophoresis of (fimH, traT, blaTEM, SHV, tetA and CTX) respictively genes in *EC/15* and *EC/20* isolates Lane1: 100 bp ladder (BioLabs)

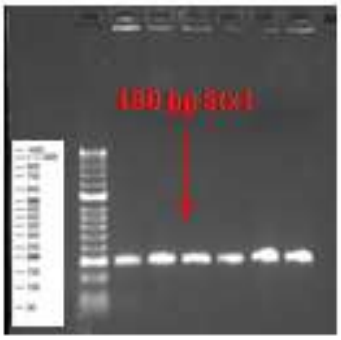

Figure2. PCR detection results in an agarose gel electrophoresis of Stx1 in *E. coli* isolates, Lane1: 50 bp ladder (BioLabs), lanes 2 to 7: positive samples.

Table4. Sources of *E. coli* isolates and polymerase chain reaction (PCR) results for Stx1 gene

Isolate	Source	*E. coli* PCR Results for Stx1
EC/01	Commercial fried chicken 1	Positive
EC/02	Mango juice	Positive
EC/03	Whey "mesh"	Negative
EC/04	Pumpkin	Negative
EC/05	Commercial fried chicken 2	Positive
EC/06	Cooked pasta	Positive
EC/07	Cooked meat	Positive
EC/08	Cheese	Positive
EC/09	Tomato	Positive
EC/10	Commercial fried chicken 3	Positive
EC/11	Cow's colon	Positive
EC/12	Armenian cucumber	Positive
EC/13	Raw milk	Positive
EC/14	Pickles	Negative
EC/15	Lanchon	Positive
EC/16	Commercial fried chicken 4	Negative
EC/17	Leafy vegtebles 1	Negative
EC/18	Canned beef	Negative
EC/19	Cucumber	Positive
EC/20	Leafy vegtebles 2	Positive
EC/21	Green pepper	Positive
EC/22	Raw Burger	Negative
EC/23	Lupine	Positive
EC/24	Basterma	Positive
EC/25	Garlic sauce "Thomia"	Negative

EC/26	Sugar Can juice	Negative
EC/27	Raw Cow's Liver	Positive
EC/28	Shrimps	Negative
EC/29	Commercial chicken soup	Positive
EC/30	Grilled fish1	Positive
EC/31	Plum	Positive
EC/32	Yoghurt	Positive
EC/33	Cow's colon 2	Not tested
EC/34	Raw meat	Not tested
EC/35	Spinach	Not tested
EC/36	Bechamel sauce	Positive
EC/37	Pomegranate	Positive
EC/38	Basterma 2	Positive
EC/39	Canned tomato sauce	Not tested
EC/40	Whey "mesh" 2	Not tested
EC/41	Raw fish	Not tested
EC/42	Grilled fish 2	Positive
EC/43	Basterma	Positive
EC/44	Commercial pasteurized milk	Not tested
EC/45	Raw oyster	Not tested
EC/46	Banana	Not tested
EC/47	Sepia	Not tested
EC/48	Egg	Not tested
EC/49	Mozarella	Not tested
EC/50	Canned tuna	Not tested
EC/51	Crab	Positive

3.4. Phage Isolation and Host Range of Isolated Phages

Ten phages were collected from sewage water in Giza and Port Said, Egypt, against *E. coli* isolates. In addition, ten phages were tested against the isolated host strains that showed positive results with Stx1. Sixteen *E. coli* isolates out of twenty-eight demonstrated susceptibility to the multiple phages, whereas the other isolates showed no susceptibility. Phage ZCECO 5 showed the most antibacterial activity against 14 isolates out of 16 (Table 5).

Table5. Host range of isolated phages against *E. coli* isolates

Phages	***E. coli* isolates**															
	EC/ 15	***EC/ 01***	***EC/ 13***	***EC/ 38***	***EC/ 05***	***EC/ 36***	***EC/ 27***	***EC/ 08***	***EC/ 21***	***EC/ 11***	***EC/ 10***	***EC/ 20***	***EC/ 32***	***EC/ 29***	***EC/ 02***	***EC/ 24***
ZCECO ١	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+
ZCECO ٢	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	-
ZCECO ٣	+	-	+	+	+	+	+	-	+	-	+	+	+	+	+	+
ZCECO ٤	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+
ZCECO ٥	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+
ZCECO ٦	+	+	+	+	+	+	-	-	+	-	+	+	+	+	+	+
ZCECO ٧	+	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+
ZCECO ٨	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	+
ZCECO ٩	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
ZCECO ١٠	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-

3.6. Bacteriophage Insensitive Mutant Frequency (BIM)

BIMs were done *in vitro* to test the long-term efficacy of phage ZCECO 5 by a Multiplicity of Infection (MOI) 100 of bacterial host *EC/20* with phage ZCECO 5 at 37°C, with BIM frequencies of 0.133± 0.65.

3.7. Phage pH, Temperature, and UV Stability

The stability of phage ZCECO 5 was tested for an hour at various pH, UV and temperature levels (Figure 3). At -20°C, 4°C, 37°C and 40°C, phage titers were constant at around 10^{10} PFU/mL for 60 minutes. When incubated at 50°C, the phage's titer declined to 10^{9} PFU/mL, and when incubated at 60°C, the titer reduced to 10^{7} PFU/ml. When incubated at 70°C, the phage titer rapidly reduced until inactivity. These results suggest that the phage might survive in normal temperatures (Figure 3A). At pH 5.0, 9.0, and 10.0, phage ZCECO 5 achieved high titers of around 10^{10} PFU/mL, but at pH 4.0, 7.0, and 11.0, it had about 10^{8} PFU/ml titer. Titers decreased dramatically to around 10^{6} PFU/mL when incubated at pH 2.0 and 3.0, and the phage was completely inactive at pH 12. As a result, phage ZCECO 5's optimal pH range was 4.0–11.0. (Figure 3B). The UV stability of phage ZCECO 5 was maintained even though it was slightly reduced from 10^{10} PFU/mL after 40 minutes to about 10^{9} PFU/mL, and the phage remained stable within 40 minutes. The phage titer slowly reduced after 45 minutes, reaching 10^{8} PFU/mL at 50

minutes. After 60 minutes, the phage titer had dropped to around 10^6 PFU/ml. Therefore, the phage had the highest UV stability around 40 minutes and was already active after an hour (Figure 3C).

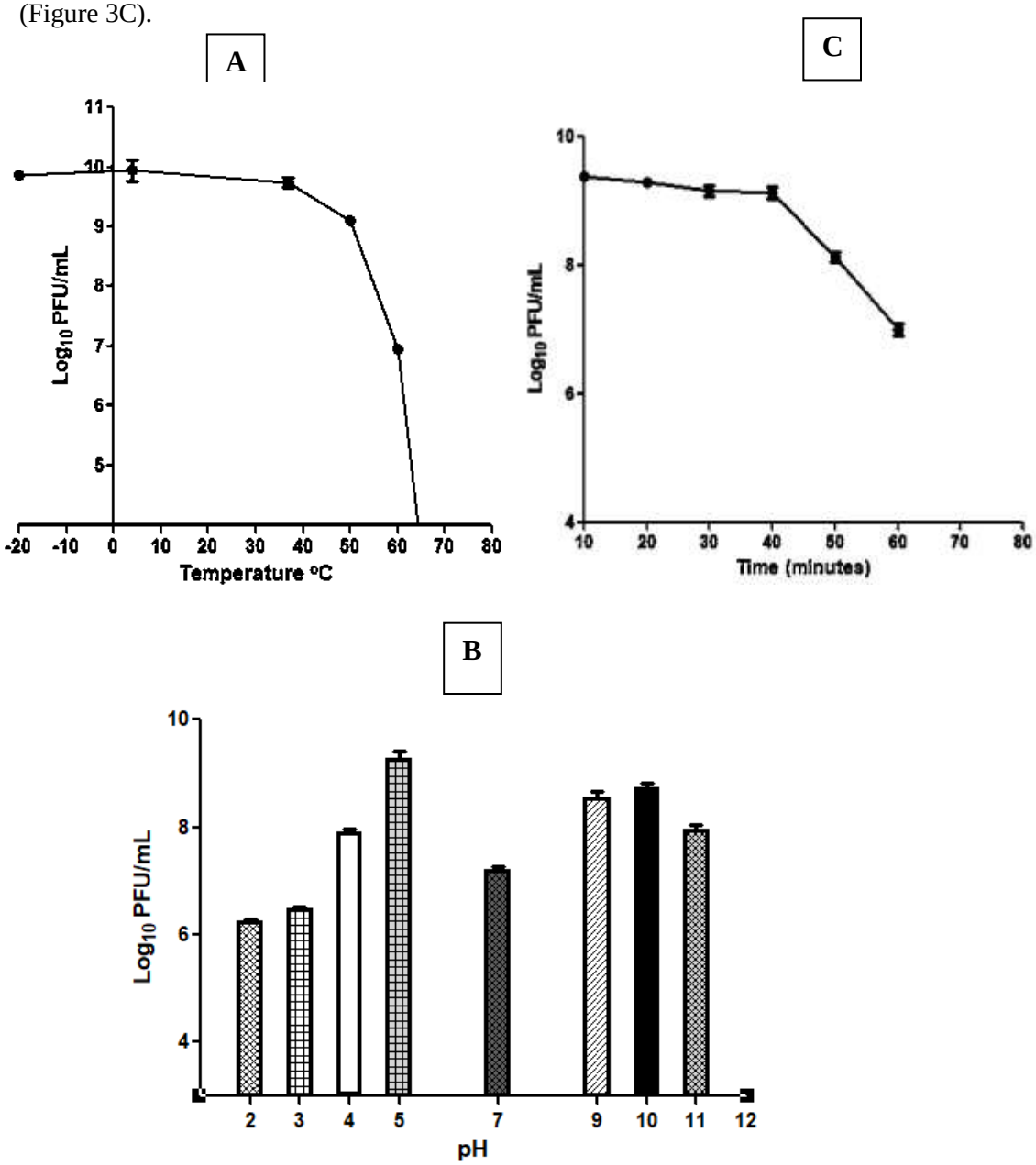

Figure3. The stability of phage ZCECO 5 is illustrated at various temperatures (A), pH levels (B), and under UV light (C). PFU stands for a plaque-forming unit.

3.8. Phage morphology by Transmission Electron Microscopy (TEM) and Pulsed-Field Gel Electrophoresis (PFGE)

The phage ZCECO 5 morphology relates to the *Myoviridae* family, having a short tail and an icosahedral head. The phage's head measures ~133.88 nm. The tail length is ~117.71 nm, similar to the actual values reported by the International Committee on Taxonomy of Viruses (ICTV) for phages in

the *Myoviridae* family (Figure 4C). PFGE estimated the double-stranded DNA genome of phage ZCECO 5 to be ~ 339.5 kbp (Figure 4D).

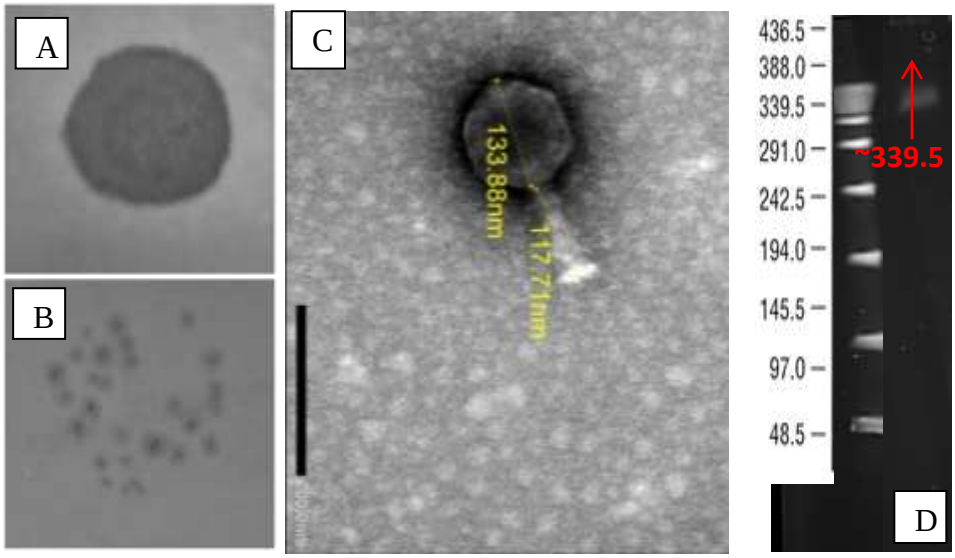

Figure4. Characterization of the phage ZCECO 5. (A, B) Plaques on a plate with bacterial overlay; (C) Transmission electron microscope (TEM) image of phage showing its morphology with a scale bar of 200 nm; and (D) Pulsed-field gel electrophoreses (PFGE) of phage ZCECO 5, with the red arrow indicating the location of the genome band in agarose.

3.9. Time-Killing Curve

To assess the phage's bacterial activity, *E. coli* isolate *EC/20* was grown in TSB and treated with ZCECO 5 at different MOI values **(**0.001, 0.01, 0.1, 1 and 10). Optical densities at OD_{600} then were utilized to monitor the growth of bacteria. In every case, phage infection inhibited bacterial growth and became more effective as the MOI grew higher. The phage's lysis mechanism was studied on the *EC/20* isolate. The optical density of the culture decreased about 230 minutes after infection at all MOI levels. The bacterial growth rate was significantly slowed by phage infection (MOI of 10) at high phage titers. At lower phage titers (MOIs of 0.01 and 0.001), bacterial growth was higher than infected cells at MOIs of 0.1, 1, and 10 but stayed lower than the control (Figure 5). The non-infected culture exhibits the *EC/20* isolate's typical bacterial activity over time.

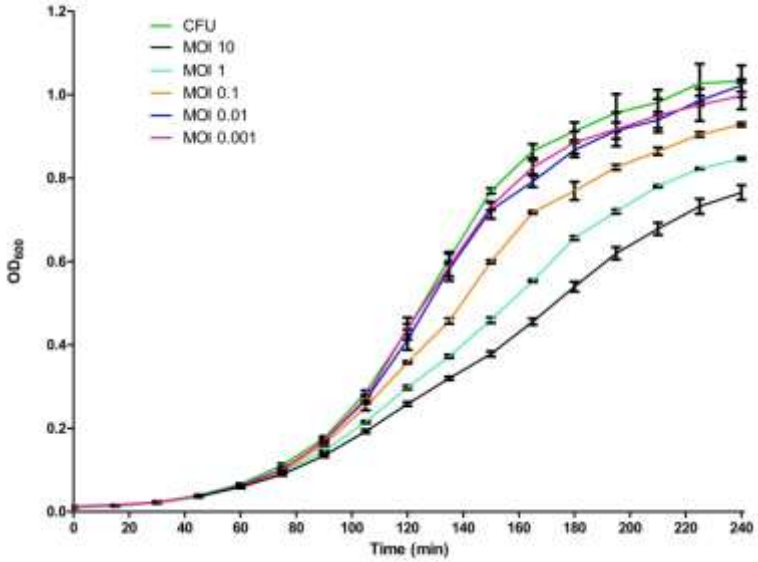

Figure5. Time-killing curve of phage against *E/C 20* isolate at 37°C. The bacterial density of *E/C 20* isolate as control and bacterial viability infected with phages ZCECO 5 at various MOIs (1, 0.1, 0.01, 0.001 and 10). For up to 4 hours, optical density at 600 nm was recorded every 10 minutes. MOI is for Multiplicity of Infection, while OD stands for Optical Density.

4. DISCUSSION

The post-antibiotic revolution is starting as a result of extreme and unwise overuse of antibiotics in animals and humans, once all of the known antibiotics are outplayed by multidrug-resistant bacterial strains (MDR) [39], [69]. As a result, resistance to antibiotics, which is now the world's second major cause of death, murdering 700.000 individuals per year, is predicted to hit ten million in 2050, exceeding cancer [39], [69]. *E. coli* has already been identified as the primary cause of several food-borne diseases. Food conservation is generally done using a physical or chemical strategy to avoid food-borne illness. These procedures, moreover, may degrade the food's value and aroma quality. In addition, chemicals, such as antibiotics, are already a risk since they can lead to antibiotic-resistant pathogens [70], [71]. This demands a coordinated effort to discover alternative antibiotic medicines in a short time. For example, the phage is the most potential antibiotic-free treatment for diseases caused by superbugs [32], [39], [72], [73]. This research study obtained fifty-one *E. coli* isolates from one hundred different food samples with an isolation rate of 51% (51/100). All fifty-one *E. coli* isolates were observed as Gram-negative rods, negative oxidase and positive catalase. Molecular characterization of the Shiga toxin gene (Stx1) in 38 *E. coli* isolates by the PCR technique revealed that 28 of the 38 isolates were positive for this gene, with a rate of 73.68 per cent (28/38). In contrast, two virulence genes (fimH and traT) and four antibiotic-resistance genes(blaCTX, tetA, blaSHV, and blaTEM) were tested in only two isolates *EC/20* and *EC/15* (most resistant isolates) and the results showed that traT and fimH genes were founded only in *EC/ 20* isolate, while the other genes were absent. The phage ZCECO 5 was collected from sewage samples to eradicate *EC/20* isolate as the primary host. The phage was categorized as a *Myoviridae* member with a short tail and icosahedral head. The phage's double-stranded DNA genome is predicted to be ~339.5 kbp. In a previous study, lytic phages T6 and T4 seemed to have a restricted host range, contrary to our isolated phage that exhibited lytic behavior against various hosts [74]. ZCECO 5 demonstrated lytic

efficacy toward pathogenic isolates of *E. coli* in this research, with *EC/20* serving as the primary host. In addition, the antibacterial property of ZCECO 5 was investigated in this study using *E. coli* isolates that had various sensitivity reactions to nine different antibiotics. *EC/20* is found to be the most resistant bacteria since it was resist to six different antibiotics out of nine and used as the main host, which is resistant to multiple antibiotics, including Gentamicin (CN; 10 µg), Tetracycline (TE; 10 µg), Rifampicin (RD; 5 µg), Kanamycin (K; 30 µg), Amoxicillin (AML; 25 µg) and Chloramphenicol (C; 30 µg). The phage's stability and a more wide pH and temperature range are necessary for preserving and medicinal uses [75]. Several investigations have documented that phages may vary in temperature, pH and UV stability [76]. At physiological circumstances, phage ZCECO 5 achieved significant viability in the pH range of 4.0–11.0, similar to earlier research [39], [50], [77], [78]. However, the titer of the VB-EcoS-Golestan phage was stable only at pH levels of 7.0 and 8.0 in another investigation [39], [79]. Gratefully, phage ZCECO 5 showed stability at pH 2.0 in which could be promising in phage therapy application to reduce *E. coli* because it will withstand stomach acidity. Interestingly, phage ZCECO 5 demonstrated great thermostability from -20°C to 60°C. In contrast, remaining slight active at 65°C and deactivated at 70°C, similar to earlier research [80,81]. Because the phages were tested after the incubation time, these results indicated that all phages have high thermal stability [39], [82]. Earlier investigations indicated that any rise in temperature lowers a phage's titer [39], [83]. However, several phages that have been investigated so far can withstand high temperatures [39], [84]. Phage ZCECO 5 maintained an excellent activity (~10^9 PFU/mL) after exposure to UV light for 40 min, but the phage was still active with titer approximately 10^6 PFU/mL after 60 min. However, in this investigation, the phage ZCKP 8 exhibited a high UV stability of around 10^8 PFU/mL after 15 minutes. On the other hand, the phage's titer rapidly decreased until it was utterly inactive after 60 minutes. The multiplicity of infection is a fundamental factor when using the phage to combat bacteria [39]. The bacterial density (OD_{600}) rapidly rose when bacteria were injected with phage at relatively low MOIs (0.01 and 0.001), but the bacterial density (OD_{600}) effectively reduced when bacteria were injected at higher MOIs (0.1, 1.0 and 10). Additionally, because of the decline in bacterial density, a comparison of the isolated phage versus the host at the evaluated MOIs confirmed that the optimized MOI had been 10. The results indicated that phage efficiency in controlling the bacterial host *E. coli* isolate *EC/20* is concentration-dependent, similar to earlier research [39]. In a previous research study, both phages vB-KpnS-Kp 13 and ZCKP 8 were infected with *K. pneumoniae* in TSB and LB broth in a concentration-dependent way over several hours [50], [85].

5. CONCLUSION AND RECOMMENDATION

Finally, this research focuses on the isolation and characterization of new phage from sewage water. *E. coli* bacteria have been confirmed to be susceptible to the phage ZCECO 5. The phage's lytic ability was proven in various conditions and bacterial densities. To reduce *E. coli* contamination, phage ZCECO 5 is highly recommended as a food preservative. More investigations are required to confirm ZCECO 5's bio-control effectiveness and lysis capacity in other *E. coli*-contaminated foodstuffs under various production techniques.

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