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Controlling karish cheese contamination with *Escherichia coli* using lytic coliphages

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Abstract

Escherichia coli (E. coli) is considered a major foodborne pathogen. In this study, lytic coliphages were used to control contamination of Karish cheese with *E. coli*. Five bacteriophages specific to *E. coli* were isolated and characterized. The isolated phages showed a strong killing potential against *E. coli* and were selected for their thermal, pH, and UV stability. On the basis of their characteristics, the five isolated coliphages were found to belong to three phage types. These three coliphages were designated **∅Ec1**, **∅Ec2,** and **∅Ec3.** The *in vitro* experiments showed that coliphages were able to markedly reduce the numbers of *E. coli* in Karish cheese after 24 hrs. at room temperature and at 4°C. On the other hand, with a reduction in the number of *E. coli*, a higher number of coliphage was observed. These results support the possibility of using phages to control pathogenic bacteria in foods.

Key words: Coliphages, *Escherichia coli*, contamination, Bio-control.

INTRODUCTION

The genus *Escherichia* belongs to the family *Enterobacteriaceae. E. coli* is found in the normal bacterial flora of the digestive tract of humans. However, some strains of *E. coli* are pathogens that cause serious infections such as stomach cramps, bloody diarrhea, and vomiting (Mohamed and Habib, 2023). These infections can be caused by fecal contamination of food or water which it comes into contact, in addition to the lack of hygiene when handling food (Kehl, 2002). Fresh produce of fruits and vegetables can be contaminated with harmful bacteria, including multidrug-resistant pathogenic bacteria, posing a potential health risk. Contamination of fresh produce with pathogenic bacteria, especially those resistant to antibiotics, is a major public health concern. This is because fresh fruits and vegetables are often eaten raw, without cooking, which can lead to many people getting foodborne illnesses (Berger *et al*., 2010 & Luna-Guevara *et al*., 2019). Due to the extensive use of antibiotics, multidrug resistant mutants were developed. Therefore, efforts should be made to find out different agents to control food contamination with some pathogenic bacteria (*e.g.*, *E. coli*). Many researchers referred to using lytic bacteriophages to fight pathogenic bacteria in food. Where these phages can infect specific bacteria, replicate inside them, and then the infected bacteria was burst and release new phage particles. The use of bacteriophages a promising alternative for removing or reducing foodborne pathogens, due to their natural presence, widespread, eco-friendly, safe for humans, effectiveness in bacterial lysis, and being highly host-specific. (Litt *et al.*, 2020 & Jagannathan *et al.*, 2022). Thus, the major goal of this research is to isolate and characterize the bacteriophages which specific to *E. coli* (coliphages) as well as evaluate their effectiveness in controlling contamination of Karish cheese with *E. coli*.

MATERIALS AND METHODS

Karish cheese sample: Karish cheese was collected from a local market in Sohag City, Sohag Governorate, Egypt.

Bacterial isolate: *E. coli* isolate was obtained from the microbiological lab. department of Sohag University Hospital.

Pathogenicity test of *E. coli* isolate: To confirm the pathogenicity of *E. coli* isolate, Congo red agar and blood agar media were used. The *E. coli* isolate was streaked on a Congo red agar plate (Sharma *et al*., 2006) and incubated at 37º C for 48 hrs. The appearance of *E. coli* colonies in red confirms its pathogenicity. The blood agar medium also was used to confirmation the pathogenicity of the isolated *E. coli*. The *E. coli* isolate was streaked on a blood agar plate and incubated at 37 ºC for 48 hrs. The appearance of hemolysis (the destruction of red blood corpuscles) around the bacterial colonies confirms their pathogenicity.

Isolation of coliphages: Samples of sewage water collected from sewage treatment plant of El-kawther City, Sohag, Egypt, were used to isolate the bacteriophages specific to *E. coli*. The liquid enrichment technique of nutrient broth medium (Adams 1966 and Allen, 1959) with some modification by Hammad (1989) was used to isolate phages. Nutrient agar double layer plates were used for phage detection as described by Borrego (1987) using the spot test. Phages were purified using the technique of single plaque isolation.

High titer phage suspension preparation: To prepare phage suspension of high titer agar for each phage isolate, double agar layer plate method was used according to Maniatis *et al.* (1982).

Estimation Titer of Phage: the prepared phage titer was estimated as described by Kiraly *et al.* (1970).

Characterization of coliphage isolates:

- **Optimum pH for infection:** Eppendorf tubes were prepared where each containing one ml of SM medium with various pH (i.e. 3.0, 4.0, 5.0, 6.0 up to 12.0). Suspension of High titer phage from each phage isolate was added individually to Eppendorf tubes (200µl/tube). After incubation of the tubes at 30°C for 60 min,

5µl from each Eppendorf was spotted over double agar layer dishes (four replicates), containing the indicator bacterium (*E. coli*). The diameters of lysed spots were measured, then, calculated average values of the replicates.

● **Effect of UV radiation:** Suspension of High titer phage from each phage isolate was placed in Petri plates (5 ml/plate). The prepared plates were put at take away of 20 cm. from germicidal UV lamp (260 nm wave length). After 5, 10, 20, 30, up to 80 min. 10 µl from each irradiated isolate phage were spotted over petri plates which contain double agar layer, with *E.coli* as an indicator bacterium. The lysed spots were observed after incubation for 24 hrs. at 33°C.

●**Thermal inactivation point:** The suspension of high titer phage from each isolate was placed individually in Eppendorf tubes (1 ml/tube). Tubes were heated at 65, 70, 75, and 80 °C up to 100°C for 10 min. in water baths then cooled with tap water. Then 10µl from each tube were spotted over petri plates which contain double agar layer with *E. coli* as an indicator bacterium. The lysed spots in the plates were observed after incubation for 24-30 hrs. at 30-33°C,

●**Electron microscopy:** Formvar coated grids had 200 mesh were used to examination of the isolated phages. Uranyl acetate 0.5%, pH 4.5 was used to stain phage particles as described by Stacey *et al*. (1984). Transmission Electron Microscope (TEM) (jole,Model GEM 1010) at Assiut University, Assiut, Egypt, were used to examined The grids at 50 kV

●**Controlling contamination of Karish cheese with *E. coli* via the application of lytic coliphages:** 100 g of Karish cheese Samples were placed in sterile plastic plates (100 g/each) and washed with sterilized water. Plates were divided into groups and subjected to the following treatments:

1- Untreated sample as a control.

2- Mixed with 500µl of liquid bacterial culture (10^8 cfu/ml) of *E. coli.*

3- Mixed with 500µl of liquid bacterial culture of *E. coli* plus 2 ml of specific high titer phage suspension (10^9 pfu/ml).

All previous treatments were examined for total count of bacteria and the specific coliphages at zero time and after incubate for 24 hrs at 4ºC and at room temperature. Two replicates were involved for each treatment.

RESULTS AND DISCUSSION

The bacterial isolate was examined by a light microscope and was found to be rods in shape and Gram negative. Moreover, the bacterial isolate was identified as *E. coli* using the VITEK2 system in the Microbiological lab. department of Sohag University Hospitals.

Pathogenicity of *E. coli* isolate: Since the binding Congo red was used commonly as markers of virulence and pathogenicity of bacteria (Quadri *et al*., 1988), the bacterial isolate was streaked on Congo red agar medium. As shown in Figure (1), bacterial colonies appeared as red colored colonies after incubation at 37°C for 24 hrs. Such result may confirm the pathogenicity of the used *E. coil* isolate. Ishiguro *et al.* (1985); Agarwal *et al*. (1999); and Dubey *et al*. (2000) reported a positive correlation between Congo red binding and the pathogenicity of *E. coli*, and possibility using this character to confirm the pathogenicity of *E. coli*. Furthermore, blood agar medium was also used to confirm the pathogenicity of the used *E. coli* isolate. Blood agar is an enriched medium used for the cultivation of bacteria to detect and differentiate hemolytic bacteria. It is also a differential medium for detecting hemolysis (the destruction of red blood corpuscles) by cytolytic toxins secreted by certain bacteria (Murray *et al.*, 2022). As shown in Figure (2), the *E. coil* isolate, under study, was found to be a hemolytic bacterium**.** Such result may confirm the pathogenicity of this isolate.

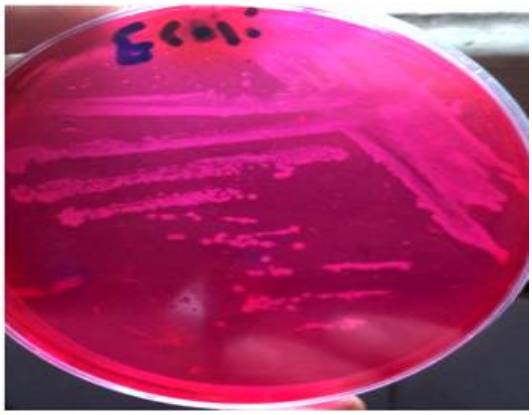

Figure (1): Congo red agar medium streaked with *E. coli* isolate.

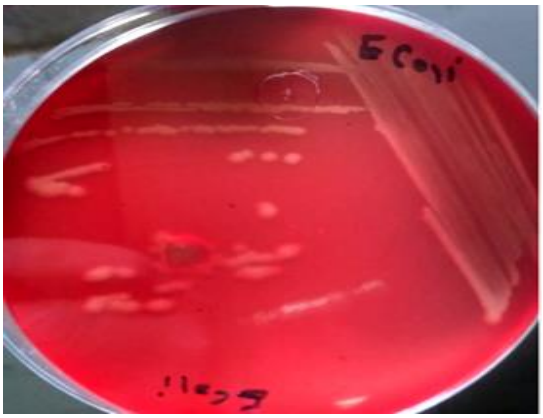

Figure (2): blood agar medium streaked with *E. coli* isolate.

Bacteriophages: Lytic coliphages were / was found to contain coliphages as indicated by spot test Figure (3). Similarly, El-Shibiny (2016) isolated coliphages from sewage sample to control *E. coli* in foods.

Purification of coliphages: Since plaque measurements and morphology are amongst the features used to differentiate phages, and it is well known that shape, size, and outline of the plaques using to characterize of the phage strain (Hammad, 1989), isolation of single plaques was used to purify coliphage isolates. As shown in Figure (4) various morphologies of single plaques from *E. coli* phages were formed. Five single plaques of different morphologies were picked and stored as pure isolates of phage. The single plaques of isolated phages were showed clear appearance and circular ranging from 1 to 3 mm in diameter.

Figure (3): A drop of phage lysate spotted on E. coli lawn and incubated for 30 hrs at 33°C.

Figure (4): Single plaques of coliphages with different morphologies

High titer phage suspensions:

High titer suspension was prepared for each of the five coliphages (50 ml each). The titer of the prepared suspensions ranged from $2.7x10^{10}$ to $3.2x10^{10}$ pfu/ml. It is well known that a single plaque about 2 mm in diameter may contain approximately 10^7 to 10^9 phage particles (Gunsalus and Stanier, 1960 & Adams, 1966). Therefore, such high concentration of coliphages in the prepared suspensions was not surprising.

Characteristics of coliphage isolates:

Effect of pH on infectivity of coliphages: At different pH levels (pH 3-12) the infectivity of coliphage isolates was tested. Data in Table (1) indicated that the five coliphage isolates were infectious at any tested pH level (pH 3-12). Similarly, the infectivity of different phages was observed at various pH levels (from pH 5 to 12) (Elsharouny *et al* 2022; Hammad and Ali, 1999; Challaghan *et al.*, 1969). And Roslycky *et al.*, 1962

Table (1): The infectivity of coliphages at different pH levels.

Phage group	Phage No.	pH level									
		3	4	5	6	7	8	9	10	11	12
		Diameter of the lysed spots (mm.)									
A	1	8.3	9.5	9.8	11.2	13.4	10.3	8.7	7.5	6.6	4.7
	2	9.3	9.8	10.4	11.0	12.9	10.0	9.1	8.2	7.5	5.0
	5	10.0	11,2	11.7	12.0	13.3	11.0	9.7	9.0	6.8	5.5
B	3	9.9	11.0	11.7	12.0	12.8	13.0	14.2	12.0	9.3	6.8
C	4	10.3	11.6	12.3	14.0	11.8	11.0	10.4	9.8	7.5	6.0

Moreover, isolates of coliphage No. 1, 2 and 5 were formed the widest spots at pH 7 with 13.4, 12.9 and 13.3 respectively. In addition, Isolates No. 3 and 4 were formed the widest spots at pH 9 and 6 with (14.2 and 14.0), respectively. These findings may indicate that the phages No. 1, 2 and 5 are similar in their optimum pH for infection. Therefore, these three phages (No. 1, 2 and 5) formed a group (Group A), whereas, phages No. 3 and 4 formed two groups (Groups B and C).

Effect of UV radiation on coliphage isolates:

The UV radiation of 260 nm wavelength lead to inactivate the phage isolates at different exposure times Table (2). Therefore, coliphages were divided into three groups. Group (A) included phages No. 1, 2 and 5 which were inactivated after 70 min. of exposure to UV. Whereas, coliphages 3 and 4 formed groups (B) and (C) since they inactivated after 80 and 60 min. exposure to UV, respectively. Interestingly, the phages of *E. coli*, which were divided in each group (A, B and C) based on the optimum pH, were found to have same sensitivity to UV. which may refer to that the phages of each group may be belonging to one phage type. Elsharouny (2007) stated that phage isolates of either Azospirillum or Azotobacter, which were belonging to one phage type, had the same sensitivity to UV.

Table (2): Effect of UV radiation (260 nm) on coliphage isolates.

Phage group	Phage No.	Exposure time (min.)							
		10	20	30	40	50	60	70	80
A	1	+	+	+	+	+	+	-	-
	2	+	+	+	+	+	+	-	-
	5	+	+	+	+	+	+	-	-
B	3	+	+	+	+	+	+	+	-
C	4	+	+	+	+	+	-	-	-

Thermal stability of coliphage isolates: Many researchers referred to that the thermal inactivation point of the phages can be using as a characteristic of bacteriophage isolates. Hammad (1993) and Hammad and Ali (1999) stated that the different bacteriophage types of *Bradyrhizobium japonicum* manifested various thermal inactivation points. As shown in Table (3) coliphage isolates No. 1, 2 and 5 were inactivated at 95°C after 10 min. Moreover, coliphages No. 3 and 4 lost their infectivity after 10 min at 100 and 90°C. respectively

Table (3): Thermal inactivation points of coliphages, exposed to 65-100°C for 10 min.

Phage group	Phage No.	Temperature (°C)							
		65	70	75	80	85	90	95	100
A	1	+	+	+	+	+	+	-	-
	2	+	+	+	+	+	+	-	-
	5	+	+	+	+	+	+	-	-
B	3	+	+	+	+	+	+	+	-
C	4	+	+	+	+	+	-	-	-

Obtained results may indicate that since, coliphages No 1, 2 and 5 which were divided in group (A) exhibited the same optimum pH, sensitivity to UV and thermal inactivation point it is likely for these three phages (1, 2 and 5) to be belonging to one phage type. In addition, coliphages No. 3 and 4 in groups (B) and (C) exhibited different optimum pH, sensitivity to UV and thermal inactivation points. Therefore, each of these two phage isolates (*i.e.* No. 3 and 4) may be belonging to two different phage types.

Electron microscopic study:

Coliphage isolates of each group (i.e., groups A, B and C) were examined after negatively stained, by electron microscope. The five isolates of coliphage were showed to be had head and tail. According to the classification of the International Committee on Taxonomy of Viruses (ICTV), these five phage types belong to the Order *Caudovirales*. As shown in Table (4), based on the phage particle measurements, the isolates of phage from each group were found to be similar in their diameters of head as well as tails length and width.

Table (4): Dimensions of phage particles specific to *E. coli*

Phage group & type	Phage No.	Head diameter (nm)	Tail	
			Length ± SD (nm)	Width ± SD (nm)
A ∅Ec1	1	79 ± 2	173 ± 3	8 ± 2
	2	82 ± 3	171 ± 4	7 ± 2
	5	81 ± 3	173 ± 2	8± 3
B ∅Ec2	3	94 ± 2	18 5 ± 3	11 ± 2
C ∅Ec3	4	89 ± 3	194 ± 3	14 ± 2

SD = Standard deviation

Generally, phages from each group showed similar particle dimensions. Accordingly, the isolates of phage from each group may represent one phage type. i.e. no doubt, the five phage isolates belong to three types. Phages No. 1, 2 and 5 of Group (A) were found to be one phage type of long contractile tail Figure (5) belongs to the Family *Myoviridae* and is designated ∅Ec1. The phages isolate No. 3 and 4 of groups B and C were different in their particle dimensions and morphology. Therefore, these two phage isolates belong to two phage types and designated ∅Ec2 and ∅Ec3. These two phages have long non-contractile tail and it could be classified under the Family *Siphoviridae*.

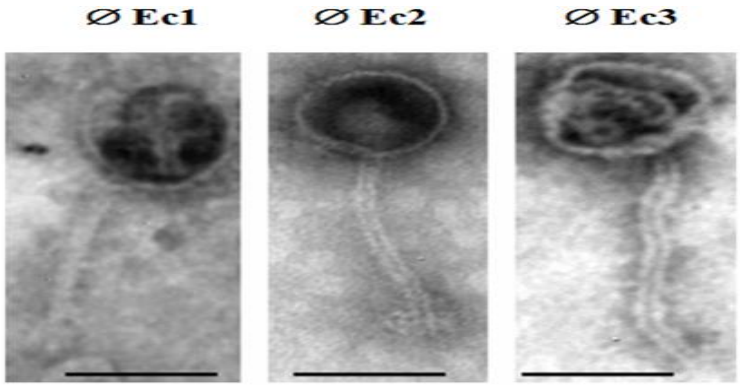

Figure (5): Electron micrographs of coliphage particles. Magnification bar = 100 nm

Francki (1973) pointed out that various unknown factors can effect on size of phage particles during preparation steps. Which may be lead to , difficulty to make true compare between morphometric data were published. According that, the differential morphological do not necessarily suggest differences in phages of each group, but because three phages of group-A showed the same sensitivity to UV, the same thermal inactivation point, moreover the same optimum pH, this may confirm that, these three phage isolates belong to one phage type. Moreover, the phage isolates of groups (B) or (C) each exhibited different characteristics (i.e. sensitivity to UV, thermal inactivation point, and the optimum pH). Therefore, each of these two phages belongs to a different phage type.

Controlling contamination of Karish cheese with *E. coli* via lytic coliphages:

Bacteriophages were used to controlled pathogenic bacteria, which can use as an important agent to controlling bacterial contamination in foods (Golkar, *et al*., 2014). Moreover, many previous studies referred to increasing in the antibiotic resistant bacteria, which resulted from the increase of using antibiotics as antibacterial agents, and other results indicated that phages were more effective against bacteria than antibiotics (Sulakvelidze, *et al*., 2001 and Park, *et al*., 2012). The using of bacteriophages as an effective anti-bacterial agents for get rid or at least reduction of food-borne pathogens is a promising alternative, due to their natural presence, being , safely to humans, environmental friendly, widely distributed, moreover the high specificity against bacterial host. (Litt *et al*., 2020; Jagannathan *et al*., 2022). In this study, a mixture of the isolated coliphages was used as antimicrobial agent to control contamination of Karish cheese with *E. coli*. As shown in Tables (5 and 6) the total count of bacteria markedly increased in Karish cheese treated with the liquid culture of *E. coli* from 4.20 $x10^5$ cfu/g. at zero time to 51.0 $x10^5$ cfu/g. and 47.1 $x10^5$ cfu/g. after 24 hrs at room temperature and at 4ºC., respectively. On the other hand, in the presence of coliphages total count of bacteria decreased from 4.2 x 10^5 to 0.60 $x10^3$ cfu/g. at zero time, then to 0.04 $x10^3$ cfu/g. and 0.52 $x10^3$ cfu/g. after 24 hrs at room temperature and at 4 ºC. in parallel with reducing the total count of bacteria in Karish cheese as a result of application of coliphages, the number of coliphages increased from 60.2 x 10^9 pfu/g. at zero time to 149.6 x 10^9 pfu/g. and 112.3 x 10^9 pfu/g. after 24 hrs at room temperature and at 4ºC, respectively.

Table (5): Effect of bacteriophages on density of *E. coli* in Karish cheese kept at room temperature for 24 hrs.

Sampling time	The used bacteria	Treatments			
		Count of bacteria (10^5 cfu/gm)		Treated with bacteria and bacteriophage	
		Untreated	Treated with bacteria	Count of bacteria (10^3 cfu/gm)	Count of bacteriophage (109 pfu/g)
Zero time	***E. coli***	0. 75	4.20	0.60	60.2
After 24 hrs	***E. coli***	3.70	51.0	0.04	149.6

Table (6): Effect of bacteriophages on density of *E. coli* in Karish cheese kept at 4°C for 24 hrs.

Sampling time	The used bacteria	Treatments			
		Count of bacteria (10^5 cfu/gm)		Treated with bacteria and bacteriophage	
		Untreated	Treated with bacteria	Count of bacteria (10^3 cfu/gm)	Count of bacteriophage (109 pfu/g)
Zero time	***E. coli***	0. 75	4.20	0.60	60.2
After 24 hrs	***E. coli***	1.98	47.1	0.52	112.3

Similar results were reported by El-Shibiny (2016), who found that the using of phages specific to pathogenic bacteria i.e. Salmonella and *E. coli* was very effective to reducing the numbers of these bacteria on the contaminated either eggs or cucumber surface, to below the limit of detection.

CONCLUSION

In conclusion, successfully isolate five bacteriophages (coliphages) specific to *E. coli* bacteria and characterized in this study. Based on the obtained results, it was concluded that the phages could be used to get rid or reduce harmful *E. coli* in foods. Accordingly bacteriophages can be beneficial agents to make food safer by getting rid of various foodborne pathogens.

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