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Coliphages Lytic; as a potential biological control agent against *E. coli* (O157:H7) in a mouse model.

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Abstract

The aim of this study was to isolate and characterize the phage for use as a potential biological control agent against Escherichia coli O157: H7. A standard strain of E coli O157: H7 (NCTC 12900, China) was used to isolate the lytic phage from bovine faeces and raw wastewater. The spot-assay test was used to check phage activity. Characterization of phage isolates included phage resistance to acidity and alkalinity, electron microscopy and PCR assay for stx1, stx2, and cI genes. Isolate phages were tested where a cocktail was administered to treat mice that was given a single oral dose (10^8 CFU) of Escherichia coli (O157: H7) (NTCC 12900). Three phages (P1, P3, P4) were successfully isolated from the sewage samples as they showed strong lytical activity against E. coli O157: H7 (NCTC 12900). The P1 and P4 phages had icosahedral head and a long, flexible, thin, inelastic tail with the tail fibers while the P3 phage had a less rigid, long and relatively thick tail with the tail fibers. All phages found are resistant to pH 4-9. The PCR results revealed the absence of genomic coding for stx1, stx2, and cI in the isolated phages. All mice treated with the phage gave a negative result for culture of Escherichia coli O157: H7.

Introduction

Bacteria can be attacked by bacterial viruses (bacteriophages, or simply phages). By nature, they are very specific such that an individual phage may only infect and subsequently kill a specific

bacterial strain within a species. This high degree of specificity permits phages to be utilitized against targeted bacteria in a miscellaneous population such as the ruminant gut without disturbing the microbial ecosystem. Bacteriophages are common natural members of microbial ecosystem in the gastrointestinal tract of food animals, including ruminants (Klieve & Bauchop, 1988). *Escherichia coli* O157:H7 is a causative agent of food-borne disease and this poses a global challenge to public health. Human disease due to *E. coli* (O157:H7) varies from hemorrhagic colitis to mild watery diarrhea. Cattle are an important domestic animal which are the primary reservoirs for *E coli* O157:H7 and are the most common sources for foodborne and direct human-animal contact infections (Chapman *et al*.,2001). The carrier cattle remain healthy, and *E. coli* serotype O157:H7 is a transient member of the gastrointestinal flora. Cattle are the major carrier of these pathogens (Nastasijevic *et al*.,2008), and contamination of meat with feces during slaughtering is the principal route for transmission to human beings (Kudva *et al.*,1999, Chase-Topping *et al*.,2008). Thus, elimination of *E. coli* O157:H7 from the bovine gastrointestinal tracts before the slaughtering would be the first barrier required to avoid the enterance of these pathogens into the food chain. Persistence carriage of *Escherichia coli* (O157:H7) in experimental and natural infection in animals may be continue for days or even months (Cray *et al.*,1995, Hancock *et al.*,1997, Besser *et al.*,1999, Sheng *et al.,* 2004). To reduce the chance of human exposure to *E. coli* (O157:H7) should be reduce and control the carriage and prevalence of this microorganism in live cattle. There is no animal vaccine or available method to eliminate of *Escherichia coli* (O157:H7) in living rumenants. A coliophage that lysis the *E. coli* (O157:H7) is a good method because bacteriophage therapies have been success in animal models against wide range of pathogenic bacteria such as enterotoxigenic and enteropathogenic *scherichia. coli* (Barrow *et al.*,1999, Jamalludeen *et al.*,2009), *Staphylococcus aureus* (Matsuzaki *et al.*,2003) and *Pseudomonas aeruginosa* (Soothill,1992). Therefore, the aim of this study was to apply phage therapy to control and treat the disease caused by *E. coli* O157:H7 and as a potential biocontrol agent against *E. coli* O157:H7 in rumenants. This might be reduced or replaced the need for antimicrobials in treatment and prevention of this possible infections.

Materials and methods

Phage isolation and titration. Standard strain of *E coli* O157:H7 (NCTC 12900, China) was used to isolate specific lytic phage from bovine feces and raw sewage. Phage was isolated by a standard enrichment procedure (Seeley *et al.*, 2001). Briefly, 15ml of raw sewage or bovine feces were centrifuged at speed 3,500 *g*, temperature at 10°C and time for 30 minute. Millipore filter (0.45 µm-pore-size) was used to filterate the supernatants, then adding of this filtrate to LB broth (10 ml), and 100 µl contain 10^8 CFU of *E. coli* (O157:H7) isolate was also added. This mixture was incubated for overnight at 37°C. This mixture was centrifuged at 10,000 *g* for 10 minutes to remove the bacteria and debris then 0.45 µm-pore-size filter was used to filterate the supernatants. Spot assay was used to test the coliphage activity of the supernatant was tested by put 5µl of coliphage on LB agar inoculated with a lawn of *Escherichia coli* (O157:H7). After 5

hours at 37°C incubation, the plates were tested for plaques formation. Serial dilution was done to supernatants which give lytic result, then by using a technique of top agar overlay with *Escherichia coli* (O157:H7) the plaques were isolated and purified (Sambrook and Russel,2001). The coliphage that gave plaques on all *E. coli* (O157:H7) isolates was selected for further studies.

Characterization of isolated phage

Resistance of the phage to acidity and alkalinity. lysates of each of phage were subjected to pH values range from (1-11) for a 16 hours period, and then tested for stability. A 100 µl volume of phage lysate (10^9 pfu/mL) was applied to 900 µl of saline set to a certain pH and the mixture was incubated for 16 hours at 37 C (Hazem, 2002). Control tube was made up from 100 µl of phage suspension and 900 µl of normal saline, pH 7.2, was also incubated at 37C for 16 hours. After the end of incubation period, a 100 µl volume of the phage suspension was diluted serially (ten fold), with 100 µl of *E. coli* (10^9 cfu/ml) and incubated at 37 C for 20 minutes then added to 7% top agar and spread(3 ml) over a plate of LB agar. The titers of the surviving phages were calculted by plaquing ten fold dilutions by the double agar overlay method.

Purification of Phage Lysates through Glycerol Gradient. Glycerol gradient protocol was used to yield phage lysate with good purity to be suitable for the subsequent electron microscopy and molecular analysis (Sambrook and Russel,2001).

Electron microscopy of coliophages. The morphology of coliphages was examined by transmission electron microscopy. A 10 µl drop of each phage suspension was negatively stained with 2% phosphotungstic acid and added on a copper grid surface then visaulized by transmission electron microscope. Phages have been classified depending on their respective families as set out in the International Committee on Virus Taxonomy guidelines (Walker *et al*., 2019).

Phage DNA Extraction. Phage DNA extraction was achieved by phenol chlorophorm protocol (Sambrook and Russel,2001)). Briefly, after phage had been propagated, bacterial debris was pelleted by centrifugation at 9,000 *g*, at 4°C for 20 min, , and then treatment of the supernatant with RNase A and DNase I (Thermo Fisher Scientific, USA) to discard any bacterial nucleic acid. The phage was pelleted by centrifugation at 25,000 rpm, at 4°C for 2 hours, and, then, resuspended in SM buffer; sodium dodecyl sulfate and proteinase K were applied to a final concentration of 0.5% and 50µg mL^{-1}, respectively. After incubation period for 10 min, at 65°C, the protein was removed from the solution. DNA precipitation was performed with sodium acetate and ethanol and then pelleted by a microcentrifuge. After washing with 70% ethanol, the DNA pellet was air dried and resuspended in a Tris-EDTA buffer.

Screening for *stx1, stx2,* and *cI* genes by PCR. Isolated phages were checked for the presence of *stx1* , *stx2* and *cI* genes in isolated phage genome before considered to be used for oral therapy. That was achieved by PCR according to a standard protocol mentioned by (Johansen *et al*., 2001) using primers designed by Fagan *et al*. (1999). *stx1* and *stx2* genes are responsible for shiga like toxin 1 and shiga like toxin 2 respectively while *cI* gene maintain the phage in lysogenic status.

Efficacy of Phage Therapy in Mice. Phage therapy experiments were designed as fulfilled by Tanji *et al.* (2005) with minor modification. Twelve mice were divided into two groups. Six mice in each group which were given 100 µl of 10^8 CFU *Escherichia coli* O157:H7 as a single oral dose (NCTC 12900, China) per animal on day 1. Mice in first group (phage group) were given 100 µl of 10^{10} PFU/ml from the isolated phages, on days, -1, 0, 1, and 2. Six mice in second group (control group) were not treated with phage.

Quantitative analysis of *E. coli* O157:H7 in faeces. Freshly voided faeces during 2 hrs were weighted and shattered in plain tube after adding of ten fold SM buffer. shattered faeces were centrifuged at 10000 xg for10 min at 4 C and then diluted serially with PBS, then varnished on sorbitol MacConkey agar plate for enumeration of *E. coli* O157:H7. Fecal samples from all the mice in experiments were tested for presence of *E. coli* O157:H7 or/and *E. coli* O157:H7 bacteriophage before the performance of experiment.

Results

Bacteriophage isolation. Phages were successfully isolated through classical amplification from samples of sewage after several trials (Figure 1). Initially six phages were isolated and of these about four phages were tested. Out of 4 phages only three phages (P1, P3, P4) were selected for further characterization (Phage P2 was not detected after storage). These phages were showing a strong lytic activity against *E. coli* O157:H7 (NCTC 12900). P1, P3 and P4 were purified by repeated plating and picking of single isolated plaques from the lawns of target host (*E. coli* O157:H7 ,NCTC 12900). The titre of the three phages P1, P3 and P4 was $2.2x10^{11}$, $1.8x10^{10}$ and $3x10^{10}$ respectively. A stock from three phages was prepared and purified. The P1 phage produced clear large to medium sized plaques (4-6 mm in diameter) while P3 and P4 formed tiny clear plaques with no halo on a lawn of *E. coli* O157:H7 (NCTC 12900). The isolated phages were tested against standard *E. coli* O157:H7 strain (NCTC 12900) by cultural methods. All three phages showed lytic activity and clear zone of lysis (Figure 1B).

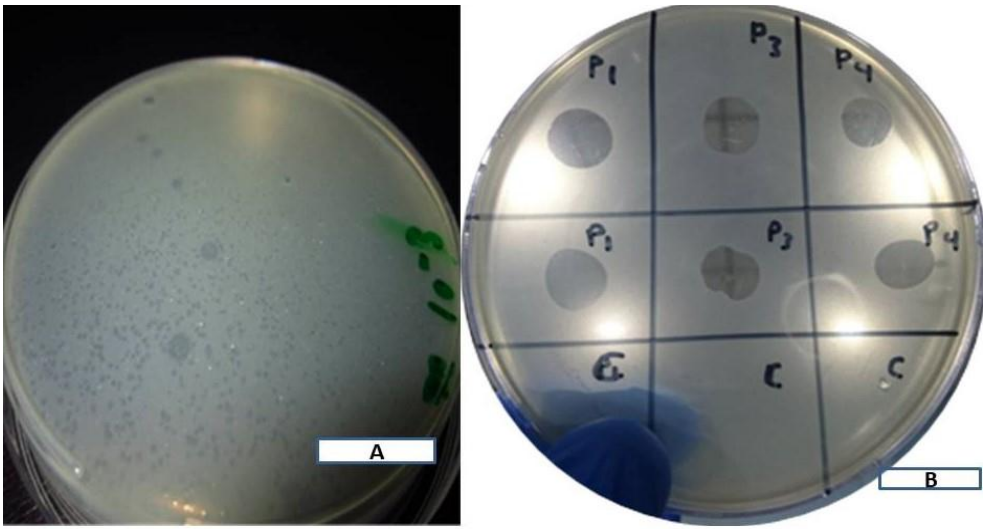

Figure 1 A. Bacteriophage isolation B. Spot test of the three phages (P1,P3,P4) against *E. coli* strain showing a clear lytic zone. C = control without phage.

Characterization of coliophage

Morphology of coliophage. The morphological characteristics of the three phages by transmission electron microscopy was revealed as in Figure 2. The phage P1 and P4 had icosahedral head and long thin flexible non-contractile tail with fibres. Depend on their morphological charcteristics, these phages under the family *Siphoviridae* (order *Caudovirales*), while Phage P3 had icosahedral head and less rigid, long and relatively thick tail with tail fibres suggesting that this phage under the family *Myoviridae* (order *Caudovirales*).

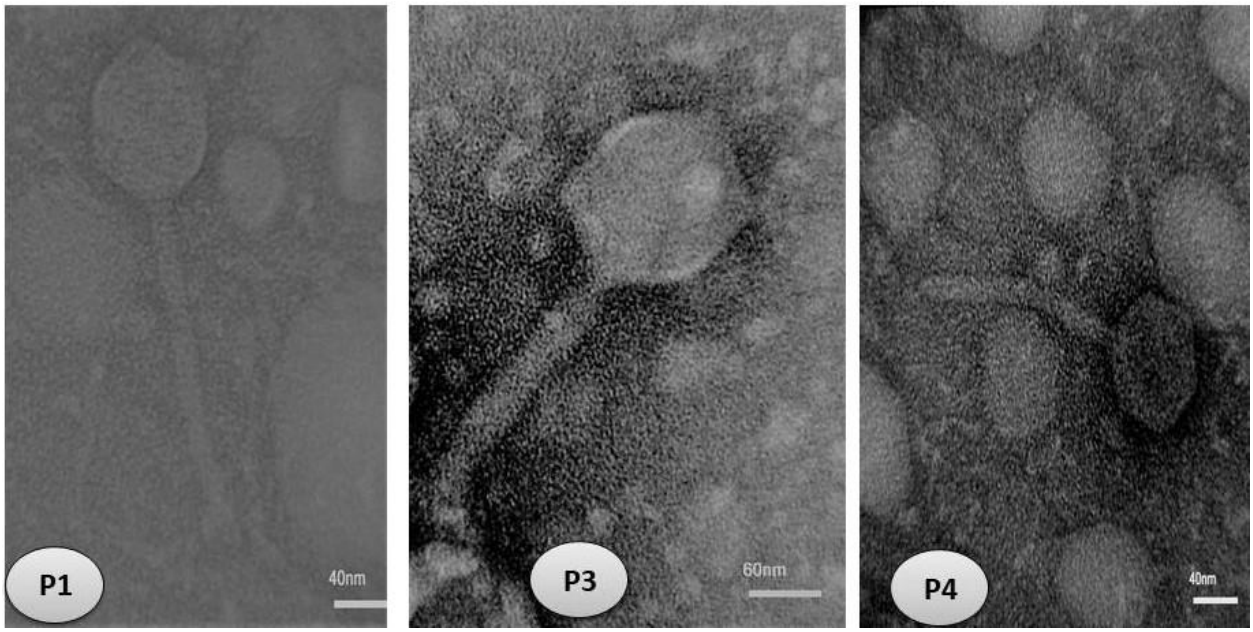

Figure 2. Electron micrograph images of three isolated phages (P1, P3 & P4), the phages P1 and P4 have long non contractile flexible of tail. Bar= 40nm, while P3 has less long, rigid, and relatively thick tail Bar=60.

The dimensions of head and tail of these three phages were revealed in Table 1. Five images of each phage were measured, and the mean values were recorded.

Table 1 Estimated dimensions of P1, P3 and P4. Each value was the mean of five independent measurements.

Phage name	Dimensions of head (nm)		Dimensions of tail (nm)	
	Length	Width	Length	Width
P1	77	70	176	12
P3	144	135	219	33
P4	74	65	158	15

Tolerance of coliophage to acidity and alkalinity. Minor variations in the titre among the three phages in the range of pH in which they were viable were noticed. All phages were found to be resistance at high survival rate to pH 4-9. P1 and P4 were found to be more resistant to pH ranging from (2-11) than P4, while all three phages were vulnerable to pH 12 (Table 2).

Table 2 Titre of P1.P3 and P4 after exposure to a range of pH (1-12).

pH	Titre of survived phages (pfu/ml)		
	P1	P3	P4
1	ND*	ND	ND
2	3×10^3	1×10^2	2×10^2
3	4×10^6	2×10^4	3×10^5
4-9	10^9	10^8	10^8
10	1×10^2	2×10^2	3×10^4
11	1×10^2	ND	1×10^1
12	ND	ND	ND
Control	10^9	10^9	10^9

*ND=not detected

Screening for *stx1*, *stx2*, and *cI* genes by PCR. Results of PCR revealing absence of gene encoding for *stx1*, *stx2*, and *cI* in isolated phages that make it suitable to be used as cocktail for eliminating the *E. coli* (O157:H7) in mice model.

The Efficacy of Phage Therapy in Mice. The isolated phages were tested as cocktail against *E. coli* O157:H7 in mice was given as single dose orally (10^8 CFU) of *Escherichia coli* O157:H7 (NTCC 12900). After bacterial challenge; mice in both experiments, the phage treatment and control (with no phage) groups remained healthy during the period of experiment. *Escherichia coli* O157:H7 count (CFU per faecal pellet) were high after oral dose of the *E. coli* then decrease slowly during the experiment period. *E. coli* O157:H7 count (CFU/faecal pellet) were reduced furthermore then were undetectable. However, the control mice (untreated) continued shedding 2 log *E. coli* (O157:H7) CFU/faecal pellet. All mice treated with phage gave negative result by *Escherichia coli* O157:H7 culture at day 6 (Figure 3)

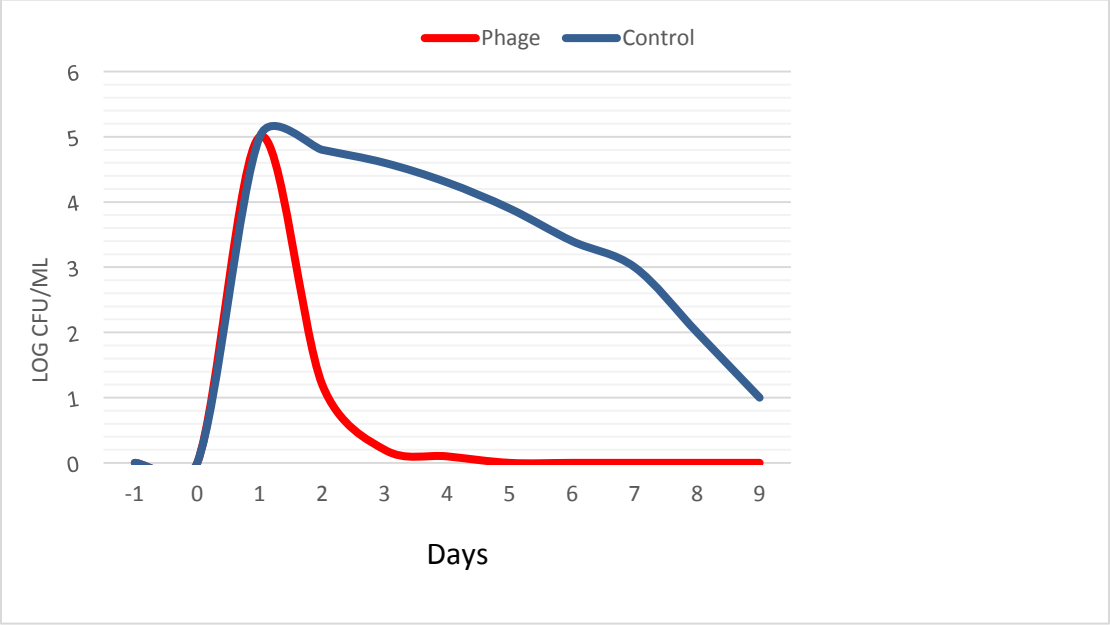

Figure 3 Oral therapy of coliphage against *E. coli* (O157:H7) in mice.

Discussion

Characterization of isolated phages was performed according to their spot size formation on their host bacteria in culture media. As spot size in soft agar might refer to phage replication and difuse in soft agar where bacterial hosts had grown (Abedon and Yin , 2008), phages with larg spot were chosen for phage treatment. The phages of *Myoviridae*, formed pinpoint plaques in all media assayed. Since *E coli* 's natural niche are ruminants, one of the first steps of the study phages is to isolate the phage from faecal feedlot and dairy cattle samples. This study was aimed on isolating EHEC-specific phages to eventually develop an efficient phage cocktail to be used as biocontrol of these pathogens in foods. Isolation of several phages unique to pathogenic *E. coli* provided a beginning point from which an antimicrobial cocktail could be created. Our study was to tested the lytic activity and host range of phages target *E. coli* O157:H7 strain isolated from sewage of human and faecal sample of cattle. From cattle faecal sample, the phages were isolated by Niu *et al*. (2009), in which they revealed that the prevalence of phages was high in fecal samples also recorded that the prevalence of phages fluctuated in a fashion like that described for *E. coli* O157:H7 (Barkocy-Gallagher *et al*., 2003). This manner of occurrence might be contributed to unsuccess of isolating phages from bovine fecal sample in our study. The phages morphology observed by transmission electronic microscopy (TEM) which revealed that P1 and P4 belong to *Siphoviridae* taxonomic family while P3 belong to *Myoviridae*, roughly like T4. All phages have icosahedral heads with tail. The same phage types which have same morphology have been isolated from human sewage by others (Ackermann *et al*.,1974, Ackermann and Nguyen,1983). Compared to the phages in other studies (Niu *et al.,* 2012), phages in our study were stable at a wide range of pH 2–11. Most phages are stable to pH 3 or 4 but the ability to withstand well in the range of pH 5–9 is a common feature to most phages (Ackermann, 1987). Decreasing of phage titers at pH 11 could be due to the detachment of the capsid protein, due to high concentrations of hydroxyl ion and hydrogen in the solution (Feng *et al*.,2003). Our study confers a better understanding of the infection kinetics of phages, specific to *E. coli* O157:H7, and their stability under acidic circumstances. This information will allow the applicability of phage therapy to be determined. Coliphages isolated in current study revealed a specificity and high lytic activity against *E. coli* O157:H7 with pH stability and could therefore be used in the food industry as biocontrol agents. Survival at low pH (1-5) make them a good candidat might be used to control foodborne pathogens that colonize in the animal gastrointestinal tract, through oral administration (Bach *et al*.,2003, Callaway ,2003, Andreatti Filho, *et al*.,2007). All phages carrying either *stx* or *cI* are contraindicated for any type of phage therapy, because they considered a hazard of possible horizontal transfer of genes (Brussow ,2005, Kropinski,2006, Parisien *et al*., 2008). In current work, all phages were negative for these genes. Our work confirm that phage therapy is successful to control experimental *Escherichia coli* O157:H7 infections in mice. Also, in other studies (Smith and Huggins, 1983,1987 Smith ,1987); they were found that a single oral dose of coliphage reduced the number of *Escherichia coli* colonize the gastrointestinal tract of calves, piglets and lambs. Coliphages that able to lysis the bacteria, they have antibacterial activity similar to antibiotics. Bacteriophages therapy is

more effective than antibiotics that have been reported in treating experimentally infected animals (Smith and Huggins, 1982, Jamalludeen *et al.*,2009) and certain infections in humans (Meladze *et al.*,1982, Kochetkova *et al.*,1989, Sakandelidze,1991). Despite there are many studies on bacteriophage therapy, there are little studies on the pharmacokinetics of phage therapy. Few studies are available on the pharmacokinetics (Bogovazova *et al*.,1991, 1992) suggest that after a single oral dose the bacteriophages reached into the bloodstream of lab animals within 2-4 hours and that they are reached to other organs (spleen, liver, kidney, etc.) in about 10 hours. However, other researches are needed to obtain more pharmacological data about bacteriophages. A more accurate description of pharmacokinetics would require the use of a single phage as an antimicrobial agent (Carlton *et al*.,2005). However, as observed *in vitro* (Kudva *et al*.,1999, Tanji *et al*.,2005) and *in vivo* (Rozema *et al*.,2009), *E coli* O157: H7 isolates can rapidly develop resistance to a single phage therapy. A cocktail of phages may prevent or delay the emergence of resistant bacterial strains. In addition, the use of a multi-phage cocktail will preserve therapeutic efficacy because of phageresistant *E coli* O157:H7 muta nts that remain susceptible to a particular phage in the cocktail (Yoichi *et al*., 2004). Cattle consider a major carrier of *Escherichia coli* (O157:H7). Foods origin from cattle are responsible for many outbreaks (Bell *et al*., 1994, Allerberger *et al*., 2001), therefor the reduce food-borne infection can occur by eliminate *Escherichia coli* (O157:H7) from cattle that lead to reduce the presence of this microorganism in the stockyard environments and also reduce animal contact and waterborne infections. Coliphage therapy of cattle may be a good strategy to control *E. coli* infection (Stevens *et al*.,2002). This phages collection can be grouped together and potentially used as an antimicrobial cocktail to control O157:H7 serotype and decrease their occurence in the food chain. However, sequencing and bioinformatic analysis of the phage genomes will be a next step in characterization of phage to determine to be safe before they would be recognized to control this serotype in foods (Viscardi *et al*.,2007). In conclusion, this study was a successful attempt to isolate an important phage which might be used as a therapeutic candidate as long with the encouraging results noticed in the experimental trail.

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