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Research article

Kinetic and lytic in vitro properties of newly isolated bacteriophages against Escherichia coli O157:H7

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Abstract

Bacteriophages, recovered from sewage samples target E. coli O157:H7, were tested for their biokinetic and morphological characteristics. Bacterial lysis profile of isolated phages was performed using Escherichia coli O157:H7 (NCTC) 12900 as host bacterial cell. Morphological characteristics of phages was visualized by transmission electron microscopy. Multiplicity of infection (MOI) and biokinetic properties, particularly adsorption rate, rise period, latent period, and burst size were also calculated. Electron microscopy revealed that the isolated phages P1and P3 belongs to Siphoviridae family while the P4 phage belongs to Myoviridae; similar to other E. coli O157:H7-specific phages previously isolated. MOI of P1 was 0.01, while P3 and P4 was 0.1 phage/bacterium. These typical MOI yielding the higher titer of phage were used in next phage tests. The adsorption curve of P1 phage were reached to 100% adsorption rate after 25 min of host exposure . Whereas P3 and P4 were taken 35 and 30 minutes, respectively to achieve the same adsorption rate. An implication of this is used in efficient biocontrol strategies for elimination of this bacterial pathogen from their reservoirs (cattle) as well as in the food industry.

Keywords: Escherichia coli O157:H7, Phage, Biokinetics, Plaque

Introduction

Enterohemorrhagic E. coli (EHEC) O157:H7 has been reported as a major foodborne pathogen which responsible for frequent gastroenteritis outbreaks in human. Healthy cattle are primary reservoir of such pathogens (1), and the most common mode of transmission to humans is fecal contamination of meat during slaughter (2). Thus, elimination of enterohemorrhagic E. coli (EHEC) from gastrointestinal tracts of cattle before the slaughtering that form the first blockade necessary to avoid entrance of the pathogens into the food chains. In human, E. coli O157:H7 causes 2,801,000 acute diseases, resulting in 3,890 cases of haemolytic uremic syndrome (HUS) and 230 deaths worldwide annually. (3)

Bacteriophages, or simply phages, are viruses which invade bacteria as host cells. They are the most plenteous biological entities on the earth, the overall phage multitude in the biosphere had been evaluated $\geq 10^{30}$ (4, 5). The high host specificity of phages to infect its bacterial host is important trait which is usually at

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level of the species or strain. This property of phage reduce the damage of the normal microbiota as the phage have recognition receptors for target host only, unlike antibiotics that minimize normal flora and may result in severe infection and complications (6).

Efficient approaches for removal or reduction of E. coli O157:H7 carriage and prevalence in ruminants may decrease the hazard of transmission this serotype to human. There is actually no effective therapy or animal vaccine obtainable to prevent transit of E. coli O157:H7. A lytic phage that particularly direct against this serotype is promising strategy as therapy with phage had been effective in animal testing toward many bacterial pathogens such as enteropathogenic E. coli (7,8), Staphylococcus aureus (9) and Pseudomonas aeruginosa (10).

Understanding the interactions between phage and host is very critical for programming effective biological controls of bacterial pathogens. When lysis happens too soon it does not create sufficient new bacteriophages to kill adjacent bacteria. while it is too slow, the phage lacks a chance for further replication to attack new bacterial cells, may due to allow the bacteria time to adapt to the phages and turn into resistant. This bacterial alteration, supported by the effect of environmental factors (11), can also lead to the lysogenic cycle, enabling the incorporation of phage DNA into host DNA. If extracellular phage concentrations in this regard are too high, some lytic Phages can act in a process known as lysis inhibition, where entire phage progeny does not instantly lysis out of the cell. This mechanism is not synonymous with the dormant temperate phage mechanism, and is usually temporary.

Several critical factors play a role for in successful interaction of phage with its host. Precise calculation of the optimum MOI is one of the first and significant factors (12). Multiplicity of infection is a significant criterion when bacteriophages are used to defy bacteria

Adsorption of phage to its bacterial host is another important factor influencing the burgeoning interaction of phage with its host. It is of utmost importance to carefully assess the duration required for the phages to adsorb the bacterial host (13).

The study of one step growth curve is of great importance as phages with long latent periods can be less efficient to be used in phage therapy. The burst size can also be determined if the treatment requires less phage particles than all the bacteria infecting the patient can infect immediately (14).

In this study, we describe characterization of phages target E. coli O157:H7 recovered from sewage, including virion morphology, biokinetics, and specific characteristics of this bacteriophages, especially a very short latent period and the ability to lyse its host, suggest that they can be used (after DNA genomic analysis) in food protection / medicine.

Materials and methods

Phage isolation and titration

Standard strain of E coli O157:H7 (NCTC 12900,HKM company, China) was used to isolate specific lytic phage from bovine feces and raw sewage. Phage was isolated by a standard enrichment procedure (15). Briefly, 15ml of raw sewage or bovine feces were centrifuged at speed 3,500 g for 30 minute, under temperature10°C and time. Millipore filter (0.45 µm-pore-size) was used to filterate the supernatants, and add this filtrate to 10 ml of LB broth , as well as100 µl of 10^8 CFU of E. coli (O157:H7) isolate. This mixture was incubated for overnight at 37°C. This mixture was centrifuged at

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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 determinate the coliphage activity of the supernatant. This test was performed by added 5µl of coliphage to LB agar inoculated with a lawn of Escherichia coli (O157:H7). After 5 hours at 37°C incubation, the plates were inspected for plaques formation. Supernatants which give lytic was serially diluted, then by using a technique of top agar overlay with Escherichia coli (O157:H7), the plaques were isolated and purified (16). The coliphage that gave clear plaques on all E. coli (O157:H7) isolates was selected for further studies.

Characterization of isolated phage

Optimal Multiplicity of Infection

Multiplicity of infection (MOI) is the proportion of bacteriophage particles to bacterial host. It is estimated the number of added phage (volume in ml x pfu/ml) being divided by the number of added bacteria (volume in ml x cfu/ml). In brief, bacterial cells were challenged with various MOI (0.0001,0.001,0.01, 0.1, 0.5, 1, 5 and 10 pfu/ml), and incubated for 1 hr at 37°C. The mixture was centrifuged at 8000 x g for 10 min after the incubation period, and supernatant was filtered through a 0.45µm Millipore filter. The lysate was then tested using the method of double agar overlay to estimate the phage titre. Phage-free cultures (which only contain bacteria) and host-free cultures (which contain only phage) were included as controls. All tests were carried out in triplicates. The maximum yield MOI was regarded an optimal MOI (17).

Phage adsorption

Phages attachment to susceptible bacteria is the first stage of infection. This process is commonly referred to as adsorption. The adsorption experiments were performed according to (18).Log phase of host culture was infected with at the optimal MOI and incubated at 37°C. 5 ml were collected after infection at time intervals of 0, 5, 10 , 15, 20, 25 , 30, 35, 40, 45, 50, 55- and 60-min. All samples drawn were filtered directly through 0.45µm Millipore filter. After suitable dilutions the phage titre was calculated by method of double agar overlay. All plates were performed in triplicates and suitable controls were included. The percentages of adsorption rate were determined as follows: [(control titre - residual titre)/control titre] X100% (19). The phage titre at time (zero) was regarded as the control titre.

One-Step Growth Curve

The one step growth curve experiment was carried out in accordance with the protocol (20). Culture of the host (100 mL) at mid log phase was centrifugated at 10000 xg for 10 min and resuspended in 20% of the starting volume (20 mL) of prewarmed LB broth. Phages added at the optimal MOI, permitting for adsorption for 15 min at 37°C and harvesting of phage is performed by centrifugation at 10000 x for 5 min and resuspended in 100 ml LB broth and incubated at 37°C. Aliquots were obtained at intervals of 10 min (up to 2 h) and titration was performed directly by method of double agar overlay. Experiments were performed in triplicates and suitable controls were involved. The graph marked against time, using pfu/ml log. The latent period, the rise period and the phage burst size were determined from the obtained one-step growth curve.

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 (16).

Electron microscopy of coliophages. The morphological characteristics of coliphages was visualized by transmission electron microscopy (Zeiss EM10C electron microscopy located in Khajeh Nasir Toosi University of Technology,IRAN). A 10µl drop from each phage suspension was

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stained negatively with 3% phosphotungstic acid and added on a copper grid surface then visualized by transmission electron microscope. Phages have been classified depending on their relevant families as set out in the International Committee on Virus Taxonomy guidelines (21).

Results

Bacteriophage isolation. Phages were successfully isolated through classical amplification from samples of sewage after several trials (Figure 1). Firstly, six phages had been isolated and of these about 4 phages were tested. Out of 4 phages only 3 phages (P1, P3, P4) were selected based on their plaque morphology for further characterization (Phage P2 was not detected after storage). These phages were showing a potential lytic activity toward E. coli O157:H7 (NCTC 12900). Purification of P1, P3 and P4 were made by multiple plating and picking of single clear separated plaques from the bacterial lawns of target host. The titre of the three phages P1, P3 and P4 was $2.2x10^{11}$, $1.8x10^{10}$ and $3x10^{10}$ respectively. The P1 phage yielded clear medium to large 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). All three phages showed lytic activity and clear zone of lysis (Figure 1).

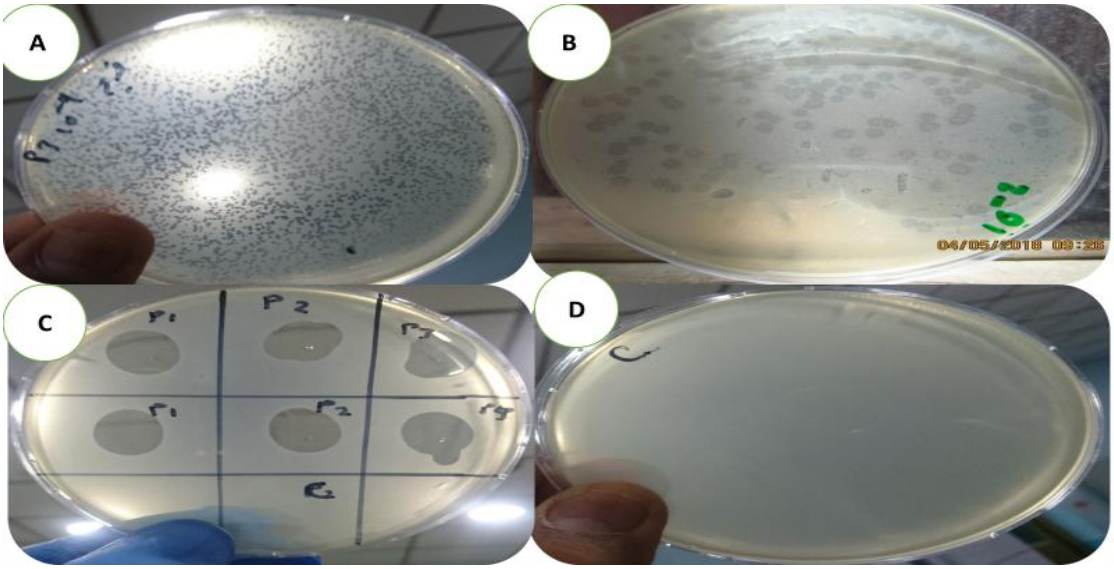

Figure 1. A: P3 & P4 phages showing tiny clear plaques on double agar overlay, B: P1phage showing medium to large clear plaques on double agar overlay . C= Spot test of the three phages (P1, P3, P4) against E. coli strain showing a clear inhibition zone. D = control (Bacterial lawn) without phage.

Multiplicity of Infection

Estimation of optimal multiplicity of infection **(**MOI) is the proportion of phage particles to the host. It is important because it has effect on phage titre. The optimal MOI of P1 against E. coli O157:H7 strain as host was 0.01, while MOI of P3 and P4 was 0.1 phages per bacterium. In all subsequent phage experiments, these optimal MOIs that yield higher phage titre under standard conditions were used (Fig. 2).

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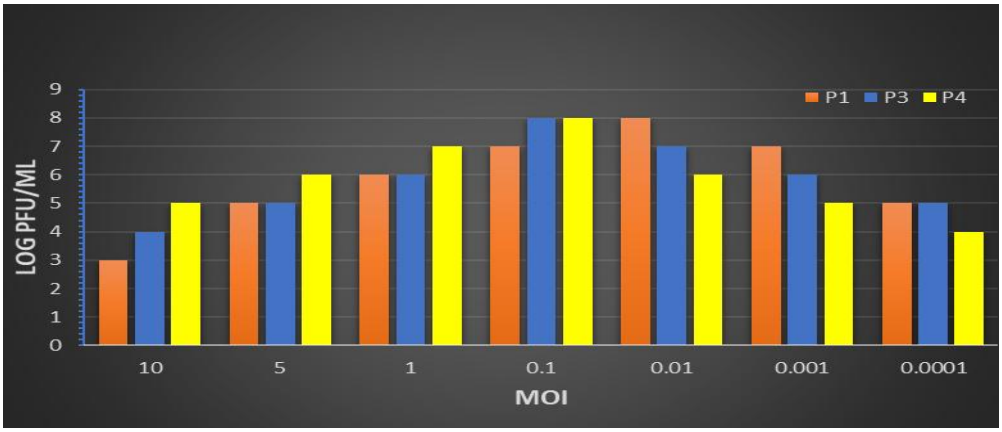

Figure. 2: Multiplicity of Infection of the studied P1, P3 and P4 phages

Phage adsorption

The curve of adsorption for P1, P3 and P4 are illustrated in Figure. 3. P1 phage were reached 100% adsorption rate after 25 min of exposure to the host. Whereas P3 and P4 were taken 35 and 30 minute, respectively to reach the same adsorption rate.

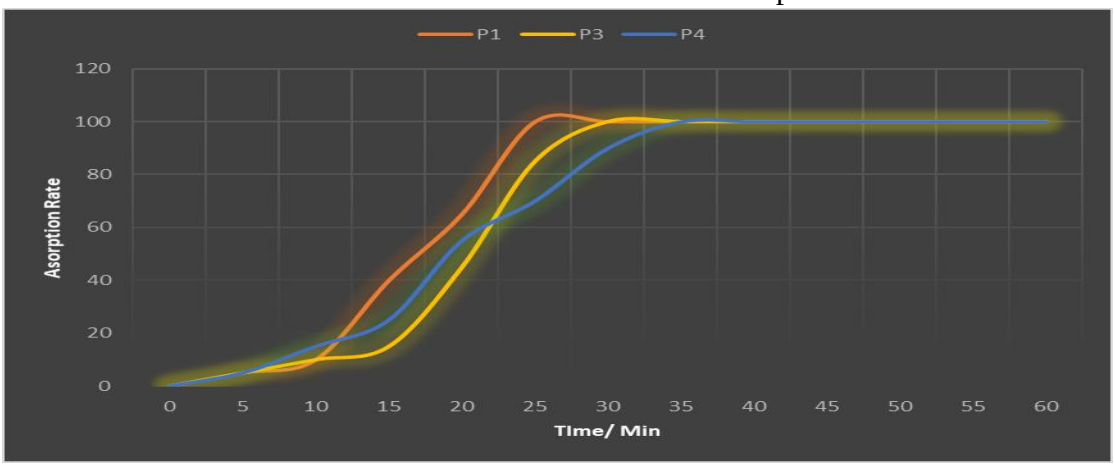

Figure. (3). Adsorption rate of P1, P3 and P4 phages

One step growth curve

The one step growth curve plays a role in providing insight to biokinetics criteria of the growth. Figure (4) shown the one step growth curve of P1, P2 and P4, where MOI of 0.001 for P1 and 0.01 for P3 and P4.

Latent period of P1 and P3 was about ≈30 min, the rise period was as 60 min and the burst size estimated 114 phages particle/bacterium for P1 and 108 phages for P3. The period of multiplication attained a plateau at approximately 70 min after challenge with P1and P3. (Figure. 4).The one step growth curve of P4 revealed the latent period was approximately 20 min however, rise period was 70 min and burst size was determined as 137 phages per bacterium.

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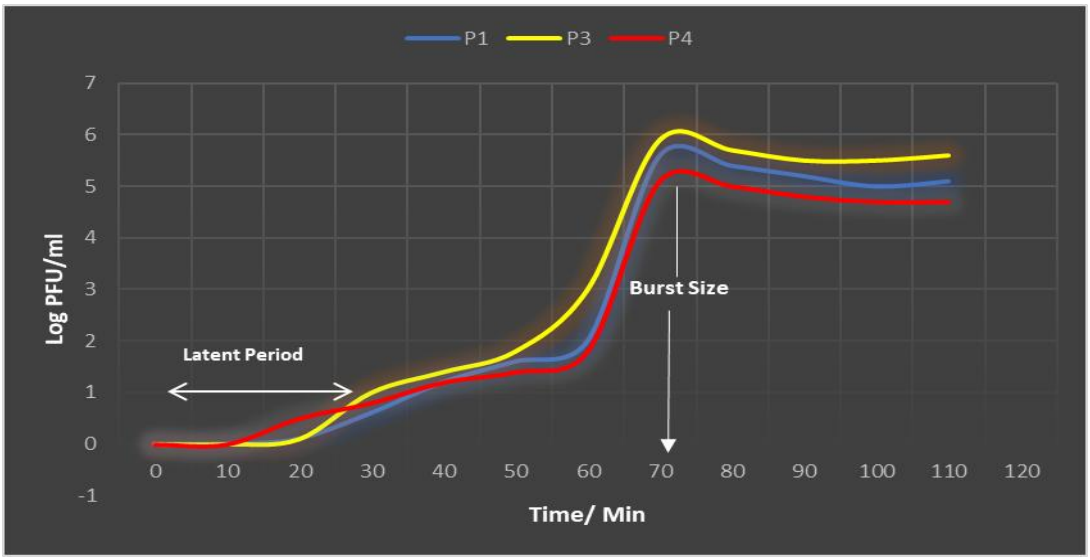

Figure (4): One step curve shows the burst size and latent periods of P1,P3 and P4 phages

Morphology of coliophage

The morphological characteristics of the three phages by transmission electron microscopy was revealed as in Figure 5. The phage P1 and P4 had icosahedral head and long thin flexible non-contractile tail with fibres. Depend on their charcteristics of head and tail morphology, these phages under Siphoviridae (the family,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).

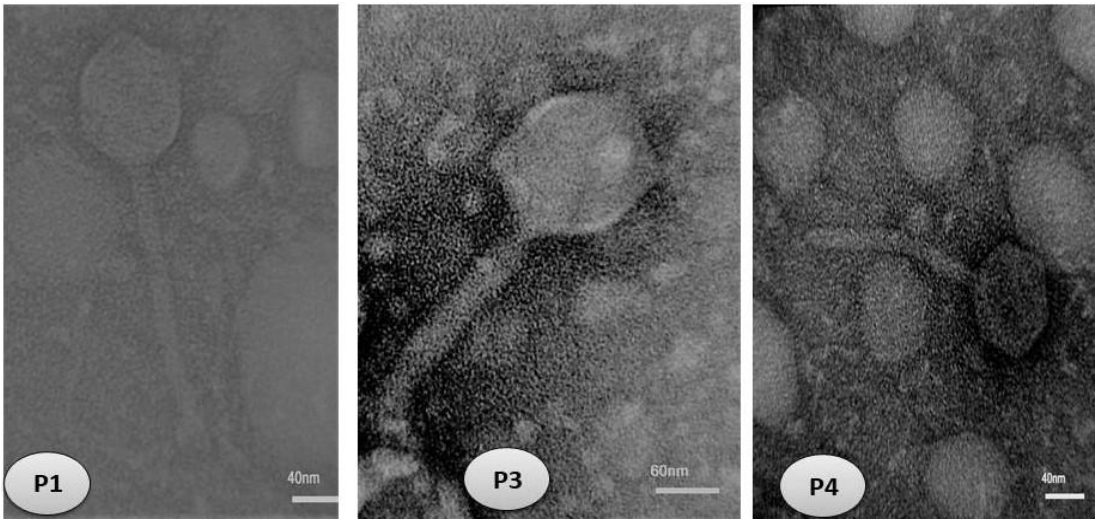

Figure 5. Electron micrograph images of three isolated phages (P1, P3 & P4), the phages P1 and P4 have long non contractile flexible of tail. Bar= 40nm, while P3 has less long, rigid, and relatively thick tail Bar=60.

The dimensions of head and tail of the studied phages were summarized in Table 1.

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Table 1: Estimated dimensions of P1, P3 and P4.

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

Each value was the mean of five independent measurements.

Discussion

Characterization of isolated phages was performed according to size formatted spot in culture media. As spot size in soft agar might refer to phage replication and difuse in soft agar where bacterial hosts had grown (22), phages with larg spot were selected for phage treatment. The phages of Myoviridae, produced pinpoint plaques in all assayed media. Since E coli 's natural niche are intestinal tract of human and animals. For studying phages, the initial step is to isolate the phage from sewage and bovine fecal samples. This study was aimed to isolating phages specific to E.coli O157:H7 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 conferred a first step from which efficient cocktail of phages might be created. Our study was to tested the lytic activity of phages target E. coli O157:H7 strain isolated from sewage. From cattle faecal sample, the phages were isolated by Niu et al.,(23), they revealed that the isolation of phages was high in fecal samples also recorded that the presence of phages fluctuated in a pattern like that for E. coli O157:H7 (24). This manner of occurrence might be attributed to unsuccess of isolation of phages from bovine fecal sample in our study. The phages morphology examined by transmission electronic microscopy which revealed that P1 and P4 under Siphoviridae family while P3 belong to Myoviridae, roughly like T4. All phages have icosahedral heads with tail. The same phage types with same morphology have been recovered from human sewage by others (25, 26)

Several critical factors require simultaneous entering into play for a good interaction of phage with its host. Precise estimation of the optimum MOI is one of the first and significant factors (12). Multiplicity of infection is a significant criterion when phages are used to target host. The optimal MOI for P1 was 0.01 phage/bacterium, while MOI of P3 and P4 was 0.1 phages/bacterium.

Infected with higher MOIs (10 and 1), the bacterial density (OD600) was suddenly elevated as the bacterial cells were inactivated earlier than those with lower MOIs (0.1 and 0.01). Estimation of typical MOI is critical because too many bacteriophages adhere to one bacterium that may lead to lysis of bacterial cell, even earlier to the replication cycle can produce phage progeny. This finding also indicates that heavy phage loads are likely to cause bacteria to become resistant to the phage infection. (27), when exposed to lytic phage selection pressure, the bacterial population will quickly transition from the predominance of phage sensitive clones to phage-resistant clones (27;28). This knowledge indicates high phage loads can be a selection burden and should be had an attention in phage therapy.

Phage adsorption to its target bacteria is another important criterion influencing the burgeoning interaction of phage with its host. It is of utmost importance to carefully assess

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the time required for the phages to adsorb the bacterial cell (13), as it can serve for the accurate characterisation of the phage in later experiments. It took 25 min for 100% adsorption by P1, whereas for P3 it took 35 min and P4 took 30 to achieve the same.

The biokinetics of virus growth had been studied for many phages (20,29,30). The one step growth curve in this study of the growth curve of P1,P3 and P4, used host cells at log phase. The phages P1 and P3 revealed a parallel latent time of 30 minutes. Also, the rise period of P1 and P3 was the same at 60 minutes and the burst size for P1 was estimated to be 114 phages/bacterium, that was larger for P3, which were 108 phage particles/bacterium. The one step growth curve of P4 revealed the latent period was about 20 minutes , however, rise period was 70 minutes. The estimated burst size was larger than P1 and P3 as 137 phages per bacterium.

The results of P1 and P3 show similar patterns to the other results reported by Kim et al., (31) where the burst times of O157 phages were at 45-65 min, and the burst sizes were ranged from 54-172 pfu/bacterium.

Park et al., (32) recorded that the burst size of SFP10 100 PFU/bacterium and latent period was 25 min which were shorter latent period and smaller than our results for three phages. while, Li et al., (33) recorded that the burst size of EEP was calculated at 375 ± 43 PFU/bacterium, that was higher than those of our study.

In corresponding to their morphology, the latent period of P4 longer than other Myoviridae phages which were characterized by other studies (34) that was calculated only15 min that is less than the optimal latent periods (21–120min) for many phages under Myoviridae. The burst size of P4 was approximately 137 phage particles/bacterium, which is larger than 50-100 PFU/cell for most Myoviridae phages (35,36,37). A few phages under Myoviridae family have very large burst sizes, phage PhaxI (another phage of O157:H7) has bursts size 420 PFU/bacterium (38).

These three isolated bacteriophages can be collected together for using as potential an antimicrobial cocktail to control O157:H7 serotype and decrease their occurence in the food chain. However, whole sequencing, bioinformatic analysis of the genomes of isolated phages will be a subsequent stage in characterization of phage to determine to be safe before they would be recognized to control this serotype in reservoir animals and foods (39). 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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