Isolation and characterization of phage specific to *E. coli* O157:H7

Siti Fariza Juharul Zaman and Yahya Mat Arip*

School of Biological Sciences, Universiti Sains Malaysia, 11800, Minden, Penang Malaysia. Email: ctfariza87@gmail.com. * Corresponding author: ymarip@usm.my

Abstract. Phage therapy is not new because it has been practiced since the early 1900s to cure diseases such as shigellosis, typhus and dysentery that caused by bacteria. However, when antibiotics were discovered in 1941, this antibacterial therapy was quickly abandoned by most western scientists. Antibiotic treatment is usually prescribed to treat pathogenic bacteria *E. coli* O157:H7. However, since 1950s, the widespread resistance to antibiotics has raised public concern on development of alternatives to antibiotics. As a result of this resistance, there becomes a need for an alternative treatment to work synergistically with antibiotic to deal with bacterial infections. This has led to a new approach by the use of bacteriophage which is phage therapy. Once again, this treatment has received attention as an alternative method against bacterial infection. Phage therapy is the treatment on pathogenic bacterial infections by using bacteriophage which capable of invading bacterial cells and disrupting bacterial metabolism before lysing it. Phages are extremely host-specific and the natural enemies of bacteria. It soon becomes clear that there are huge numbers of bacteriophages existed and waiting to be isolated. Phage specific to *E. coli* O157:H7 was successfully isolated from sewage treatment sample. The appearance of plaques on lawn of *E. coli* O157:H7 plate indicates phage capabilities to infect and lyses the host cells. The isolated phage was further characterized based on its morphology, genomic profile, physicochemical attributes, and host specificity to assess their potentials to be developed as phage therapy against *E. coli* O157:H7.
Keywords: Isolation, characterization, E. coli

Introduction

The spread of infectious diseases caused by foodborne pathogens such as *Campylobacter, Salmonella, E. coli* and *Listeria* leads to huge impact on public health. Nevertheless, the numbers of cases of some foodborne diseases have been increased continuously including diseases caused by *Escherichia coli* O157:H7 (Hagens & Loessner, 2007). *E. coli* O157:H7 is an enterohemorrhagic strain of the bacterium and the most important enterohemorrhagic *Escherichia coli* (EHEC) to cause diseases include abdominal cramps and diarrhea that may in some cases develop to bloody diarrhea (Snyder, 1998). It was considered as a rare serotype when it was first described in 1983 but today it has become one of the major causes of foodborne diseases in industrial countries (Hagens & Loessner, 2007). The recent outbreaks of *E. coli* O157:H7 which is related to the consumption of contaminated meat and fresh vegetables emphasizes the importance of developing new and more effective antimicrobial approaches (Viazis *et al*. 2010).

Meanwhile, the usage of antibiotic within medicine and veterinary medicine leads to the prevalence of antibiotic resistant bacteria. This is due to the fact that, risk of the development of resistance rises when duration of exposure to antibiotics increases (Gupta, 2011). In addition, the continuous overuse or misuse of effective antibiotics has also led to the constant development of new antibiotic-resistant pathogenic bacteria (Bruttin & Brussow, 2005). Therefore, when resistance towards antibiotics becomes *more* common, there is a greater *need* for alternative treatments (Gupta, 2011). The resistance to antibiotics of some pathogens illness has caused a growing demand for more natural control strategies, such as the use of bacteriophage (Coffey *et al*. 2011)**.** In fact, the interest in alternative remedies against *Escherichia coli* diarrhea rises as well when the antibiotic treatment of the disease is said to be problematic.

The idea of using phages was formerly discovered by Felix d'He'relle who exploit the lytic effect of phages on bacteria for therapeutic purposes (Bruttin & Brussow, 2005). Bacteriophages, also known as phages are the most prevalent entities on Earth (Fenton *et al*. 2010). On a global scale, it was estimated that 10^{31} bacteriophages living in the environment which are ten times more numerous than bacteria making them the most abundant life forms on Earth (Hanlon, 2007)**.** Out of this estimation, only 5100 phages have been identified and reported towards the end of the last century. Moreover, 4950 out of 5100 phages discovered are isocahedral tailed phages, double-stranded genome and lytic (Courchesne *et al*. 2009). Therefore, there are enormous numbers of phages waiting to be identified (Goodsell, 2010). Bacteriophages are extremely host-specific viruses that specifically infect and lyse their bacterial hosts (Nishikawa *et al*. 2008)**.** Like all viruses, phages are obligate intracellular parasites and lack their own

metabolisms which require the metabolic machinery of the host cell to support their reproduction (Withey *et al*. 2005)**.** Two categories of bacteriophages are recognized as temperate and virulent. Virulent phages have more value for the purpose of phage therapy as they cause lysis and death of bacterial cell by injecting their nucleic acid into the host cell following attachment before synthesizing new phage capsules (Hanlon, 2007).

Materials and Methods

Isolation and Purification

Bacterial host *E. coli* O157:H7 was cultured in Luria-Bertani (LB) Broth and incubated overnight at 37°C with shaking at 180 rpm. Bacterial cultures was mixed together with the raw sewage sample and allowed for incubation overnight. Next day, the incubated culture was filtered with filter paper, centrifuged at 10000 rpm and followed by filtration using bacteria filter with pore size of 0.45µm and sterile syringe filters. The filtrate was considered as raw phage stock. Next, the filtrate was serially diluted in LB Broth. 100µl of each dilution was mixed with 100µl of culture *E. coli* O157:H7 and plated on LB agar media, overlaid with 3ml of LB top agar onto the plate. The plates were allowed for overnight incubation at 37°C. The next morning, the formation of clear zones or plaques was observed on the plate indicated lysis of *E. coli* O157:H7 infected by phage. For purification part, well isolated plaques were transferred onto new LB plate containing host *E. coli* O157:H7, overlaid with LB top agar. The transfer was carried out using sterile tooth picks. Once again, the transfer plates were incubated overnight for same plaques formation. Top agar which contained the plaques was scrapped gently without scraping the LB agar and eluted in TMS buffer overnight at 4°C. Later, the eluted sample was centrifuged and filtered through 0.45µm filter and sterile syringe filter to collect phage stock. Finally, the filtrate was added with 0.1ml chloroform and stored at 4°C. For phage enrichment purposes, certain volume of host was cultured with equal volume of phage stock overnight. Then, the culture was centrifuged and filtered again to be stored at 4°C in chloroform as enriched phage stock. The virus titer of the enriched phage stock was tested by performing plaque assay before the characterization steps were carried out.

Electron Microscopy

A drop of purified phage sample (10^{10} pfu/mL) was applied to the surface of a carbon-coated grid (400 mesh copper grid). Then, 2% methyltungstate was used to negatively stain the sample. The morphology of phage was identified using transmission electron microscope (Philips CM12 equipped with analysis system, Philips Electron Optics).

Genomic profiling

This part involved genome determination, whether the isolated phage was DNA or RNA virus. The phage genome was extracted using phenol/chloroform and ethanol precipitation (Sambrook & Russel, 2001). For genome identification, the extracted genome was digested with either DNase or RNase. Next, restriction enzyme digestion was carried out using a restriction enzyme, *Dra*I to estimate the genome size.

Physicochemical analysis

The ability of bacteriophage to survive and infect the host cells was tested under two conditions; different pH value and temperature, before the number of phages surviving was determined by the plaque assay. The isolated bacteriophage was exposed to a certain pH value adjusted from 1-12 in one hour incubation and overnight incubation of temperature range from 10°C to 80°C.

Results and Discussion

One phage was successfully isolated from the raw sewage treatment. Figure 1 (A), (B) and (C) show the images of isolated phage which is typical head and tail phage. This phage possesses elongated icosahedral head of approximately 86 nm in diameter with a tail of 142 nm in length. The head is a hexagonal with contractile sheath and tail fiber. Based on the observed physical morphologies, it was suggested that this phage could be assigned to *Myoviridae* family as the morphology is similar to bacteriophage T4 which also has icosahedral head and contractile tail that falls into the same family (Miller *et al*. 2003).

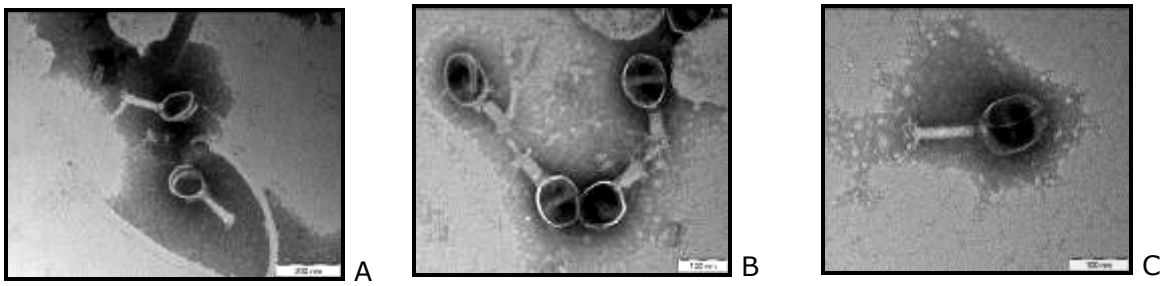

Figure 1. Negatively stained electron micrographic of isolated phage.

Based on DNase, RNase and restriction enzyme digestion, the isolated phage was DNA virus as shown in Figure 2. For genome profiling, the phage genome was digested with few restriction enzymes to identify the best restrictions patterns and numbers of restriction sites. Nevertheless, among several types of restriction enzymes used to digest the genome extract of the isolated phage, only *Dra*I was able to cut the genome and produced multiple bands (Figure 3). Since *Dra*I gave the best digestion, it was chosen for further molecular work. The phage genome was digested with *Dra*I and compared with virus from *Myoviridae* family, bacteriophage T4 and also bacteriophage T7 which belongs to Podoviridae family that capable of infecting *E. coli* 0157:H7 as well. Based on the numbers of cutting site, it shows that the isolated bacteriophage is different from T4 and T7 phages; two common phages.

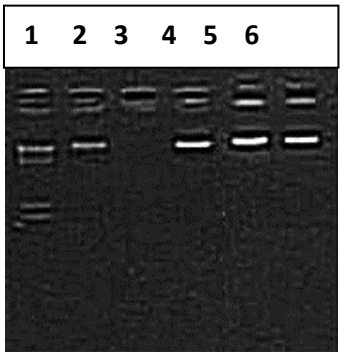

Figure 2. Genome identification on 0.8% agarose gel. Lane 1: Lambda *Hind*III Marker. Lane 2: Phage genome treated with RNase A. Lane 3: Phage genome treated with DNase. Lane 4: Untreated phage genome (control). Lane 5: Untreated T4 bacteriophage genome. Lane 5: Untreated T7 bacteriophage genome.

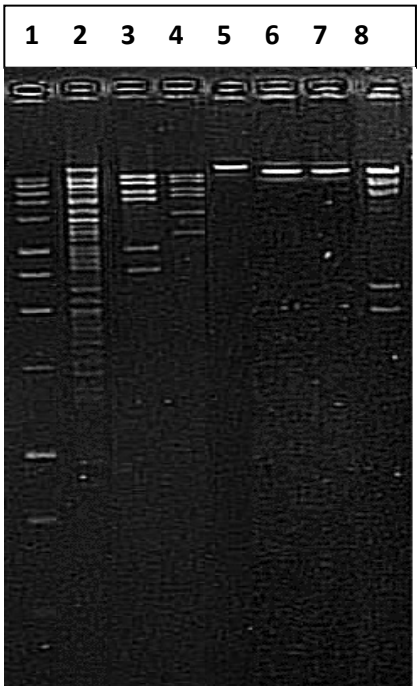

Figure 3. 1.2% agarose gel electrophoresis of phage genomes digested with restriction enzyme. Lane 1: 1kb DNA Ladder. Lane 2: Phage genome digested with *Dra*I. Lane 3: Bacteriophage T4 genome digested with *Dra*I. Lane 4: Bacteriophage T7 genome digested with *Dra*I. Lane 5: Uncut phage genome (control). Lane 6: Uncut bacteriophage T4 genome (control). Lane 7: Uncut bacteriophage T7 genome (control). Lane 8: Lambda *Hind*III Marker.

Figure 4 shows the effect of different pH-values and temperature on the isolated phage. The pH stability of the isolated phage with respect to infectious capacity is shown in Figure 4(A). From the figure, it was apparent that the infectivity of the isolated phage was stable in the range of pH 5-11. In the acid region, the phage showed lytic capability from pH 5 to pH 6. However, the isolated phage was very unstable below pH 4. This is because; phages are sensitive to protein denaturation in acidic condition (Hazem, 2002). While on the alkaline side, the isolated phage was unable to survive above pH 11. The maximum stability seemed to be at pH 6 followed by a

decline in infectivity. This result is consistent with the previous study that survival of most phages is within pH 5 to 9 at physiological conditions which maintain the native virion structure and stability (Jamalludeen *et al*. 2007). Results from Figure 4(B) shows the exposure of isolated phage to varying temperatures ranging from 10°C to 80°C. The isolated phage was stable in the range of 10°C to 37°C and became less stable when the numbers of phage count were significant reduced following exposure to 40°C and 50°C. The region of optimum stability was at close to body temperature 37°C as the phage was appeared to be stable and survive in this range. The isolated phage was unable to grow well in temperature that is higher than body temperature and most phages do not have the ability to survive at temperature above than 50°C. Thus, it can be seen from the graph that the isolated phage was rapidly inactivated at this temperature range. This result could show that extreme condition might affect the phage structure and cellular functions (Coffey *et al*. 2011).

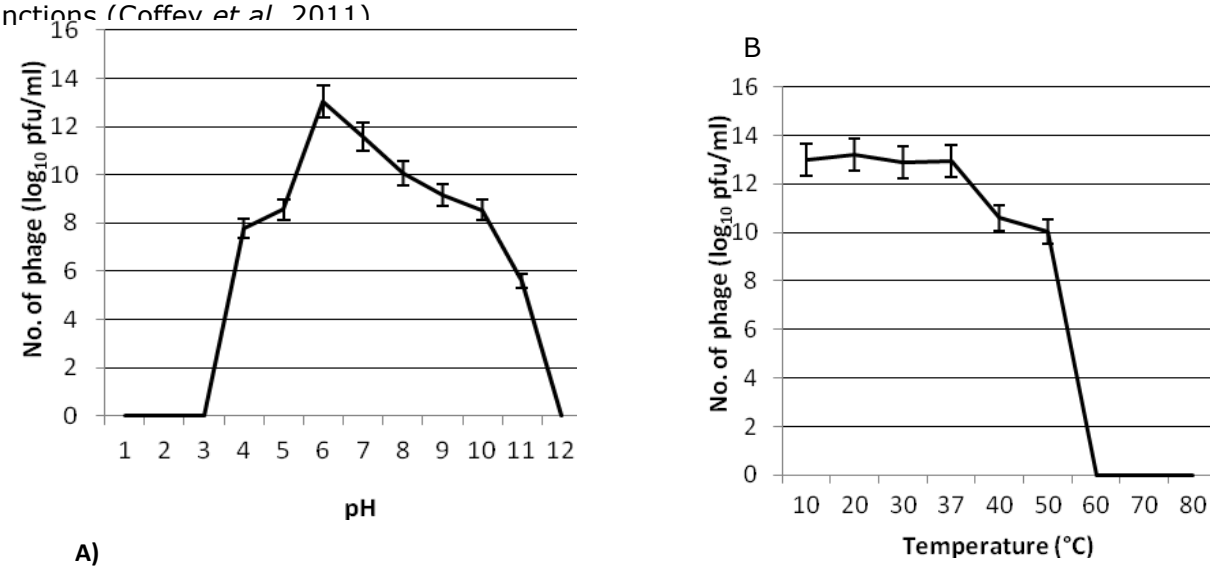

Figure 4. Infectivity of phage following exposure to A) pH and B) temperature. Phage counts (log_{10} pfu/ml) was obtained after performing plaque assay.

Conclusions

To sum up, this research showed that phage specific to *E. coli* O157:H7 was successfully isolated. Based on morphological and molecular studies, the isolated bacteriophage might belong to *Myoviridae* family. The bacteriophage was able to survive in pH region of pH 5 to 11 while the temperature range of stability was from 10°C to 37°C. Besides, further characterizations are still in progress. Since the isolated phage is specific to *E. coli* O157:H7, then, it has the potential to be developed into phage therapy.

Acknowledgements

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