Exploring phage diversity and potential: Development of phage therapy for bacterial infection

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Abstract. The recent increase in drug-resistance bacteria has become a very serious threat to the treatment of infectious diseases. Over recent decades, a growing numbers of literatures have validated the application of bacteriophages for therapy against antibiotic-resistance bacteria. With rapid dissemination of these resistant bacteria pathogens, the interest in alternative remedies to antibiotics using bacteriophages therapy is gaining new ground. Based on the recent studies of bacteriophage applications against bacterial infections in countries where this alternative therapy has been approved, many scientists and companies believed that phages have the ability to treat and prevent diseases caused by bacteria. Malaysia, being well known as one of the megadiverse countries could promise the potential new phages that could be applied in phage therapy. The overall objectives of this research including isolation and purification of potentially new phages from environments, characterization of the isolated phages based on morphological study, physicochemical attributes, genomic and proteomic analysis. Lastly, the isolated phages would be developed into phage therapy against bacterial infection through *in vitro* and *in vivo* test. Preliminary results presented here, show a total of four phages were successfully isolated from human waste. Two of the phages were successfully isolated infecting *Proteus mirabilis* that caused urinary tract infection in human whereas another two phages infecting *Escherichia coli* 0157:H7 (caused food-borne diseases) and *Escherichia coli* ATCC 13706, respectively.
Keywords: Bacteriophage, drug, *Proteus mirabilis, Escherichia coli*

Introduction

Bacterial viruses or bacteriophages (phages for short) are viruses that could infect and destroy bacteria. They have been referred to as bacterial parasites and occupy all habitats of the world where bacteria thrive. It has been estimated that there are ten phage particles for each bacterial cell (Skurnik and Strauch, 2006). Phages are among the most common biological entities on the earth and widely distributed entity in the biosphere with an estimated viral population of greater than 10^{31} particles (McAuliffe *et. al.,* 2007). Some phages are highly specific to their host while others are extremely broad in their host range (Skurnik and Strauch, 2006).

Phages, of which there are two orders with ten families infecting eubacteria and four families infecting archaebacteria as registered in the International Committee on Taxonomy of Viruses (ICTV). About 5360 phages are tailed phages and 1790 phages are reported belonged to cubic or filamentous or pleomorphic phages with at least 100 novel phages are described every year (Ackermann, 2011). This phage biology still does not include five pending families of phages that could infect archaebacteria. Phage predation kills estimated one-half of the bacterial population worldwide every 48 hours. Phage played a critical role for the evolution of the bacterial biosphere, in part by accelerating the mutation rates of bacteria. Phages which mutating at a more rapid rate than their prey, of course, finds means to overcome this resistant bacteria. The billions of years of this evolution originating from predator-prey relationship has made phages a potentially rich resource for the discovery of new antibacterial agents as a complement for the existing treatments (Deresinski, 2009).

Phage therapy serves as a potential candidate to fight pathogenic bacteria along side with the current treatments available. Phage therapy involves using phages or their products as bioagents for the treatment or as prophylaxis of infectious diseases caused by the bacteria (Matsuzaki *et. al.,* 2005). Phages have been proposed as active natural antimicrobial agents against bacterial infections in human, veterinary science, environment and agricultural importance. The using of phages has also been discussed as hygiene measures in food production facilities as well as hospitals.

Materials and Methods

Bacterial strains and culture media

A total of three bacterial strains, namely *Proteus mirabilis*, *Escherichia coli* 0157:H7 and *Escherichia coli* ATCC 13706 were used as primary host for the isolation, propagation, characterization and development of the bacteriophage. All the bacterial stock cultures were

stored at -80°C in 40% (v/v) glycerol. When needed, frozen cultures were cultivated in Luria Bertani (LB) broth and plated onto LB agar. The bacteria and isolated bacteriophages were propagated in LB broth and agar. For plaque assays, the top agar was prepared with LB broth supplemented with 0.5% bacteriological agar.

Isolation and screening of bacteriophages

Bacteriophages were isolated from human waste. The sample was taken freshly using sterile container and used immediately for isolation. A single colony of the host was cultured in 5 ml of LB broth and incubated in shaker with agitation speed of 180 rpm at 37°C. After 2-5 hours, about 5 ml of the sample was added into the cultured host with the addition of 10 ml of double-strength LB broth and the mixture was incubated again at the same condition for overnight. On the next day, the culture was harvested at 10 000 rpm for 15 minutes using cold centrifugation (4°C) before the supernatant was filter sterilized using 0.2 µm membrane filter.

The filtrate was then further used for phage screening by performing plaque assay using overlay agar technique (0.5% LB top agar). Ten-fold serial dilutions of filtrate were prepared from 10^{-2} until 10^{-10} in LB broth. For the original filtrate stock, 100 µl of fresh (2-5 hours incubation) cultured host and 100 µl of filtrate were added on ½ LB agar and the mixture was overlaid with 3 ml of top agar. The plate was moved clockwise and anticlockwise for several times to ensure complete and proper mixing. Similarly, all the prepared dilutions were performed the same steps. For control, the filtrate was replaced with 100 µl of LB broth. All the plates were inverted and incubated overnight at 37°C. The morphology of phage plaques were observed and recorded.

Purification and enrichment of bacteriophages

100 µl of fresh bacterial culture (2-5 hours incubation at 37°C, 180 rpm) that acts as a host for the isolated bacteriophage was added on ½ LB agar and overlaid with 3 ml of top agar (0.5% of melted LB agar). The top agar was mixed properly with the bacterial culture and let to be hardened. After the top agar was hardened, a well isolated plaque obtained from previous screening process plate was purified using sterile toothpick by poking the centre of the plaque and pricked onto the hardened agar (new bacterial lawn). This purification step was repeated for three times. As final step, the plaques-containing top agar was scrapped and eluted in SM buffer (50mM of Tris-HCl, 100 mM of NaCl, 10 mM of $MgCl_2.6H_2O$) for overnight at 4°C. Then, the isolated phage-containing SM buffer was centrifuged at 10 000 rpm for 15 minutes. The supernatant was filter sterilized using 0.2 µm membrane filter and the phage stock was stored at 4°C.

The enrichment of the phage stock was further performed exactly as isolation step with the addition of 5% of chloroform as the final step. Plaque assay was repeated once again for the purified phage for the determination of phage titre before the characterization steps were carried out. The plaques were observed and recorded.

Bacteriophages visualization

The enrichment of bacteriophages was carried out to obtain the titre approximately 10^{10} pfu/ml. The sample containing the high titre was spotted onto a film-coated carbon grid and negatively stained with 2% methylamine tungstate, pH 6.5 before the bacteriophage morphology could be observed using transmission electron microscope (Philips CM12 equipped with analysis system, Philips Electron Optics).

Results and Discussion

A total of four phages were successfully isolated from human waste. All of the isolated phages produced different plaques in term of size, shape and turbidity when plaque assays were performed. As shown in the Figure 1 (a), the plaque was round-shaped, clear and large with mean diameter of the plaque 29.5 mm. Both Figure 1 (b) and (c) also produced round-shaped and clear plaque with very small size of the plaques (mean diameter of 1.0 mm and 1.5 mm, respectively). Instead of clear, Figure 1 (d) showed a clear ring surrounding the turbid plaques and had similar shape like others with mean diameter of 2.0 mm.

Morphological characterization of phages using TEM, one of the most used methods for phages classification. All the isolated phages were tailed phages that constitute the order *Caudovirales* and fell into three families, characterized by contractile, long and noncontractile, or short tails and named respectively *Myoviridae*, *Siphoviridae* and *Podoviridae* (Ackermann, 2011).

Based on the electron micrograph shown in the Figure 2 (a) and (b), both the isolated phages could be assigned to the family *Siphoviridae*. This morphology was similar to PW2 phage that possessed icosahedral head and a long, non contractile tail (Phumkhachorn and Rattanachaikunsopon, 2010). As can be seen in the Figure 2 (c), the isolated phage could be assigned to the family *Podoviridae*. Similarly, phage ϕIBB-PF7A was reported morphologically belongs to the family *Podoviridae* since it has a very short non-contractile tail (Sillankorva *et. al.,* 2008). The isolated phage in the Figure 2 (d) could be assigned to family *Myoviridae* as reported for phage ϕKZ which also had very long contractile tail that fell into the same family (Krylov *et. al.,* 2003).

The isolated phages as shown in the Figure 2 (a) and (b) had a mean diameter of 66 nm and 61 nm, a tail length of 208 nm and 177 nm as well as a tail diameter of 15 nm and 11 nm, respectively. Instead of long tail, as can be seen in the Figure (c), the isolated phage had mean diameter of 109 nm, tail length of 89 nm and tail diameter of 28 nm. Meanwhile, the isolated phage as shown in the Figure 2 (d) had mean diameter of 57 nm, tail length of 120 nm and tail diameter of 22 nm. Similarly, all the isolated phages had isometric heads.

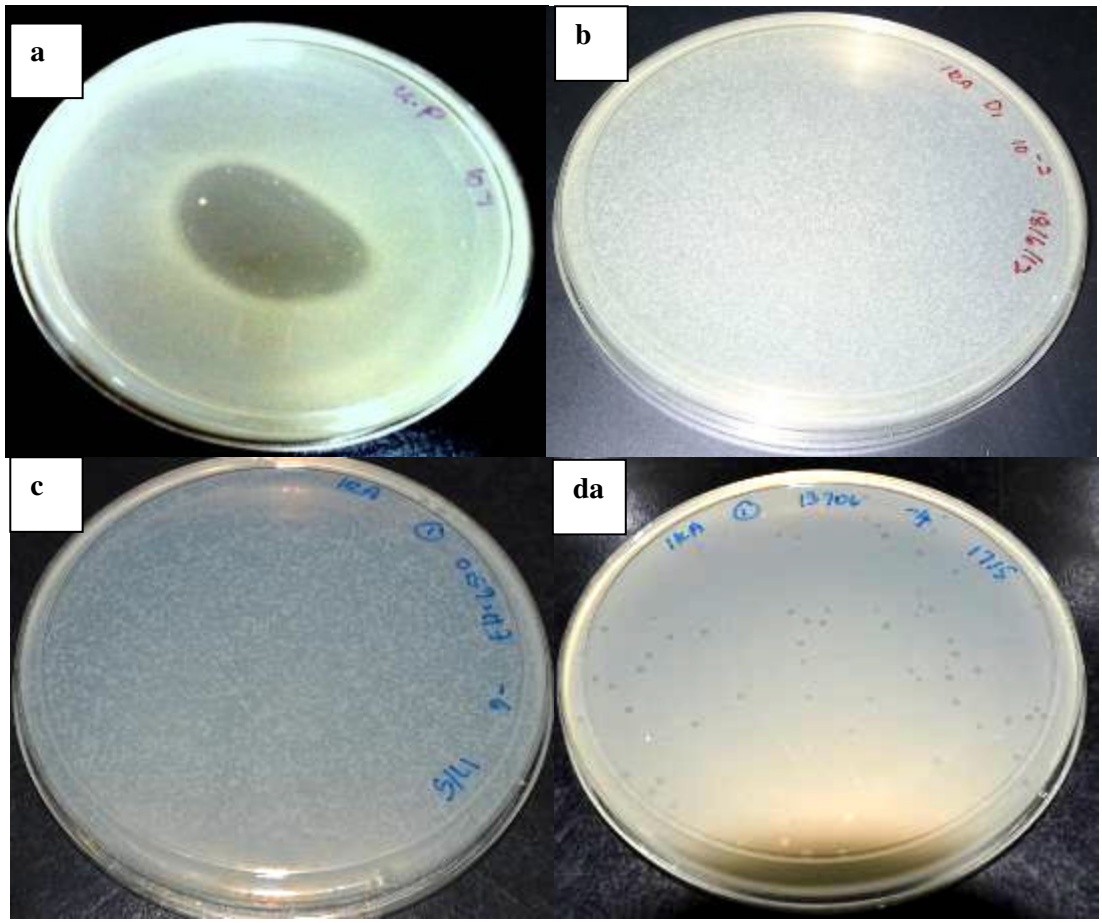

Figure 1. (a) and (b) Phages infecting *Proteus mirabilis* (c) Phage infecting *E.coli* 0157:H7; (d) Phage infecting *E.coli* ATCC 13706. Morphology of the plaques on the plates using overlay agar Technique (0.5% top agar).

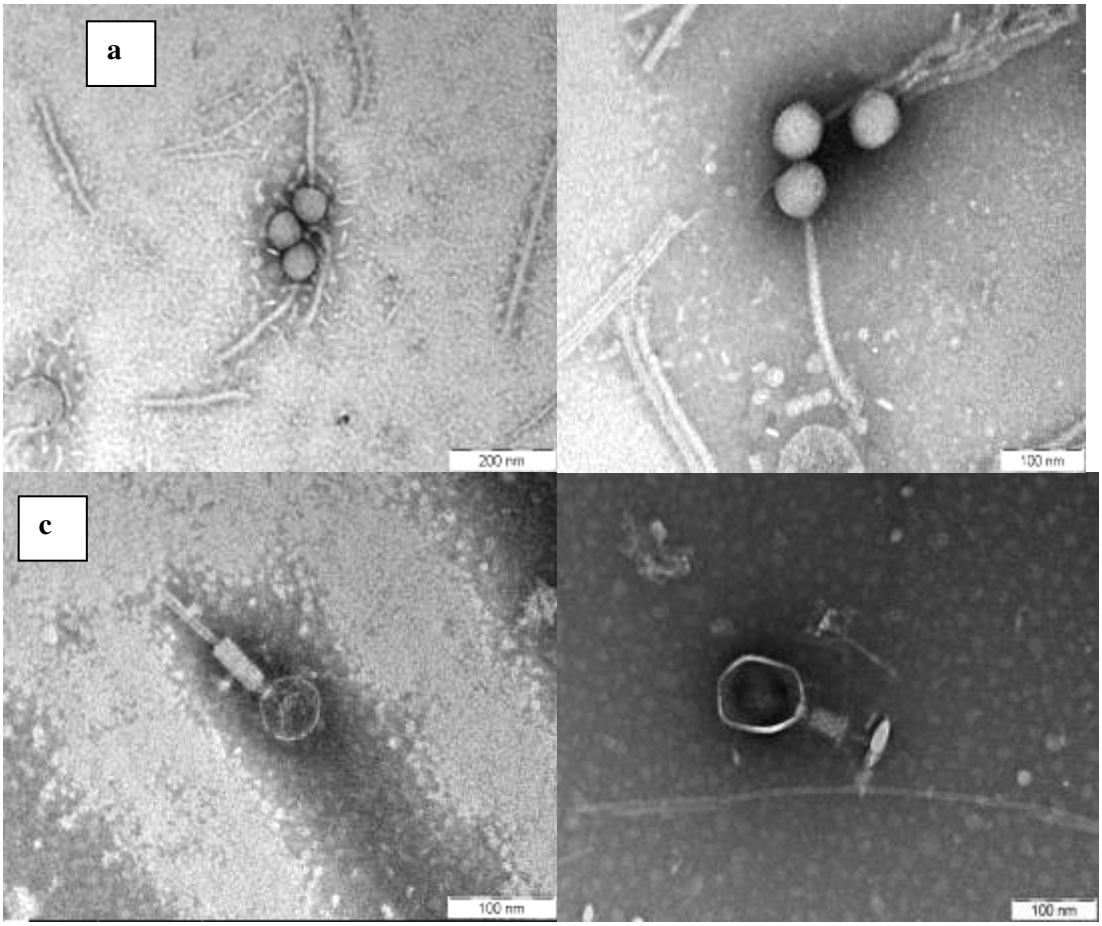

Figure 2. (a) and (b) Phages infecting *Proteus mirabilis*; (c) Phage infecting *E.coli* 0157:H7; (d) Phage infecting *E.coli* ATCC 13706. Negative stained electron micrograph of phages with 2% methylamine tungstate.

Conclusions

For the preliminary results, about four phages were successfully isolated from human waste. All the isolated phages were distinct and fell into the order of *Caudovirales* (tailed phages) with possible three families of *Siphoviridae*, *Podoviridae* and *Myoviridae*. Further characterization work are in progress before the isolated phage could be developed as phage therapy against infectious diseases that caused by pathogenic bacteria.

References

Ackermann, H.W. (2011). Bacteriophage taxonomy. Microbiology Australia.

Deresinski, S. (2009). Bacteriophage Therapy: Exploiting Smaller Fleas. Clinical Infectious Diseases 48: 1096-1101.

Krylov, V., Pleteneva, E., Bourkaltseva, M., Shaburova, O., Volckaert, G., Sykilinda, N., Kurochkina, L. and Mesyanzhinov, V. (2003). *Myoviridae* bacteriophages of *Pseudomonas aeruginosa*: a long and complex evolutionary pathway. Research in Microbiology 154: 269-275.

Matsuzaki, S. *et. al.,* (2005). Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. Japanese Society of Chemotherapy and The Japanese Association for Infectious Diseases 11: 211-219.

McAuliffe *et al*. (2007). The New Phage Biology: From Genomics To Applications in Mc Grath, S. and Van Sindren, D. (eds) Bacteriophage: Genetics and Molecular Biology Caister Academic Press.

Phumkhachorn, P. and Rattanachaikunsopon, P. (2010). Isolation and partial characterization of a bacteriophage infecting the shrimp pathogen *Vibrio harveyi*. African Journal of Microbiology 4(16).

Sillankorva, S., Neubauer, P. and Azeredo, J. (2008). Isolation and characterization of a T7-like lytic phage for *Pseudomonas fluorescens*. BMC Biotechnology 8:80.

Skurnik, M. and Strauch, E. (2006). Phage therapy: Facts and fiction. International Journal of Medical Microbiology 296: 5-14.