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Isolation of food-borne pathogen bacteriophages from retail food and environmental sewage

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Abstract

Bacteriophages are the viruses of bacteria and are widely distributed in the biosphere, exhibiting dramatic manifestations both in liquid cultures and on solid media. In this study, bacteriophages were isolated from different types of food (beef, chicken meats, cucumber, lettuce, clam, cockles and shrimp) and sewage samples using 6 reference pathogen strains (*Salmonella* Enteritidis, *Salmonella* Typhimurium, *Campylobacter jejuni, Vibrio parahaemolyticus, Listeria monocytogenes* and *Escherichia coli*). A total of 29 bacteriophage isolates were obtained and further examined for titer via agar overlay assay. The titers were determined within the range of 10^8 to 10^{11} PFU/mL. Our results showed that diverse of bacteriophages are naturally present in a variety of foods.

Introduction

Food-borne illnesses of microbial origin and safety of genetically modified organism are among food safety issues worldwide. In Malaysia, genetically modified organism in various food products and animal feeds that may contain the antibiotic resistant gene marker has been reported (Lisha *et al*., 2017). In addition, the epidemiology of food-borne diseases due to antibiotic resistant bacteria pathogens (e.g., *Salmonella, Campylobacter, Listeria monocytogenes* and *Vibrio parahaemolyticus*) varies among the pathogens, as do the routes by which various bacteria contaminate food products. Hence, the use of bacteriophages to remove pathogens from food has recently become an option for the food industry as a novel method for biocontrol of bacteria without interfering with the natural microflora, as well as enhancing the safety of food products (Hagens and Loessner, 2010).

Bacteriophages, also known as phages, are bacterial viruses that infect bacteria. Since their host specific nature of infection, bacteriophages have been used as powerful agents for controlling the composition of prokaryotic communities. In this context, bacteriophages play an important role in ecology (e.g., bacteriophage impact on the cycling of organic matter in the biosphere at a global level) (Fuhrman, 1999) and evolution of bacterial genomes (most obviously in the development of bacterial pathogenicity) (Boyd and Brussow, 2002). Thus, the impact of bacteriophages in the environment has resulted in significant interest in the issues of both bacteriophage abundance and bacteriophage diversity. Accordingly, bacteriophages are widely distributed in the environment including sewage (Akhtar *et al*., 2014) and can also be isolated from a wide range of foods such as ground beef, chicken and other meat products (Kennedy *et al.*, 1986; Hsu *et al*., 2002), chilled and frozen crabmeat (DiGirolamo *et al.*, 1972), fermented dairy products like cheese and yoghurt (Kilic *et al.*, 1996), and fermented foods such as red pepper paste and soybean paste (Shin *et al*., 2011).

The most common and useful method for enumerating bacteriophages is the plaque assay. Also, it is well established as the gold standard for bacteriophages quantification (Cormier and Janes, 2014). This method is based on the ability of bacteriophages to infect and lyse the host bacteria

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and allow bacteriophages to propagate in a confluent lawn of bacterial host cells immobilized in a layer of soft agar, which overlay on the surface of a base layer of nutrient-containing agar supporting bacterial growth. After incubation, a circular transparent area of lysed cells will form and known as plaques; each plaque is considered to be derived from an infectious bacteriophage particle. The plaques are counted, and the bacteriophage titer or concentration is expressed as plaque forming units per milliliter (PFU/mL) of the assayed preparation.

In Malaysia, very few reports are available in literature concerning the isolation of bacteriophages from retail food sources. Moreover, the isolation of obligately lytic bacteriophages from different sources has always been the subject of interest to researchers for biocontrol of pathogens. Therefore, the objectives of this study were to isolate bacteriophage from different food samples and environmental sewage samples, and to determine the bacteriophage titer using the agar overlay assay.

Materials and Methods

Bacterial strains

For the present study, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Campylobacter jejuni, Vibrio parahaemolyticus, Listeria monocytogenes* and *Escherichia coli* were used to serve as host strains for bacteriophage isolation. All bacterial strains were stored in 1.0 mL aliquots 20% (v/v) sterilized glycerol stock at -80˚C. Bacterial cultures were prepared in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) and inoculated onto tryptic soy agar (TSA) (Merck, Darmstadt, Germany) slants and incubated at proper growth condition. The agar slants were kept at 4˚C and used as working cultures.

Sample collection

Different types of food samples: beef and chicken meats, vegetables (cucumber and lettuce), and seafood (clam, cockles and shrimp) were purchased from retail wet markets and hypermarkets in Selangor, Malaysia. Whereas, several sewage samples were collected from the surrounding area of the Universiti Putra Malaysia, and wastewater treatment plant in Seri Kembangan. Samples were transported to laboratory under aseptic conditions and processed for bacteriophage isolation within 2 h.

Bacteriophage isolation

Prior to bacteriophage isolation, each bacterial host strain was grown to mid-log phase ($OD_{600\ nm}$ = 0.5) by incubation at 37˚C with shaking at 150 rpm. Initially, the sample was suspended 1:10 (w/v) in salt of magnesium (SM) buffer (50 mM Tris-hydrochloride (Tris-HCl) [pH 7.5], 0.1 M sodium chloride (NaCl), 8 mM magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) and 0.01% (w/v) gelatine [Sigma-Aldrich, Saint Louis, USA]) and stomached for 60 s followed by addition of a mid-log phase bacterial culture to the ratio of 9:1 (v/v). The enrichment sample was incubated at 37˚C for 24 h on an orbital shaker at 150 rpm. After incubation, the suspension was centrifuged at 10,000 × g for 10 min to separate the host cell debris and the bacteriophage. The resultant supernatant was filtered with a 0.2 µm pore-size disposable syringe filter (Sartorius, Gottingen, Germany) to remove any remaining bacterial cells. The filtrate was then examined for the presence of bacteriophages using agar overlay assay as described by Adams (1959). Filtrates that showed bacteriophage plaques forming were stored at 4˚C and used as bacteriophage lysate solution for further analysis.

Bacteriophage titer determination

Bacteriophage titer was determined using agar overlay assay (Adams, 1959). Briefly, a series of 10-fold dilutions in SM buffer were made of bacteriophage lysate solution. Aliquots of 100 µL of diluted bacteriophage solution were mixed with 100 µL of mid-log phase bacterial culture in 3 mL of molten Luria Bertani (LB) (Merck, Darmstadt, Germany) soft agar (LB broth containing 0.6% (w/v) agar). Immediately, the soft agar mixture was poured onto solid LB agar plates (1.5% (w/v) agar) and incubated at 37˚C for 24 h. After overnight incubation, the number of visible plaques was counted on the appropriate dilutions giving between 30 and 200 plaques, and expressed as PFU/mL. Each assay was performed in triplicates.

Results and Discussion

Bacteriophage isolation

A total of 29 bacteriophages were isolated from food and raw sewage samples (Table 1). Among the six bacterial host strains used for bacteriophage isolation, *S.* Enteritidis yielded the highest number of bacteriophage isolates (n=14). Meanwhile, four bacteriophages were isolated using *S.* Typhimurium, four bacteriophages with *C.* jejuni, two with *V. parahaemolyticus*, and five with *E. coli*. Of the 81 samples, it is somewhat surprising that no bacteriophage infecting *L. monocytogenes* was isolated, but this may simply reflect the limited number of strains of this species available in this

Table 1. Isolation of bacteriophage from food and sewage samples using different pathogen host strains

Sample types	n[a]	Number of positive bacteriophage					
		S. Enteritidis	*S.* Typhimurium	*C. jejuni*	*V. parahaemolyticus*	*L. monocytogenes*	*E. coli*
Beef	9	3 (33.3%)[b]	1 (11.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (11.1%)
Chicken meat	11	5 (45.5%)	2 (18.2%)	1 (9.1%)	0 (0.0%)	0 (0.0%)	1 (9.1%)
Cucumber	11	2 (18.2%)	0 (0.0%)	1 (9.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Lettuce	9	1 (11.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Clam	9	1 (11.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Cockles	9	1 (11.1%)	0 (0.0%)	1 (11.1%)	1 (11.1%)	0 (0.0%)	0 (0.0%)
Shrimp	11	1 (9.1%)	1 (9.1%)	1 (9.1%)	1 (9.1%)	0 (0.0%)	1 (9.1%)
Sewage	12	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (16.7%)
Total	81	14 (17.3%)	4 (4.9%)	4 (4.9%)	2 (2.5%)	0 (0.0%)	5 (6.2%)

[a]Number of samples.
[b]Percentage of positive samples.

study rather than an absence of bacteriophages which affect this species. According to Akhtar *et al*. (2014), the presence of bacteriophages was tightly associated with their natural hosts. The host bacteria appeared to modify the bacteriophage in some way so as to affect its subsequent ability to infect other bacterial strains (Denes and Wiedmann, 2014).

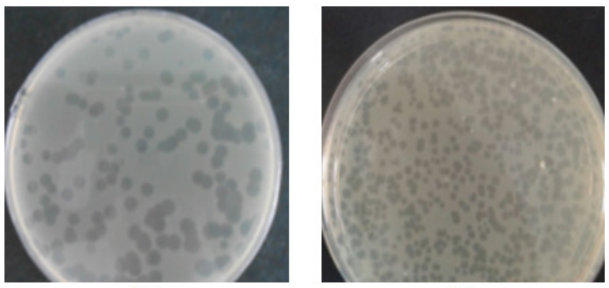
Of the food samples tested, seafood exhibited the highest frequency of bacteriophage isolation (41.4%), whereas the lowest frequency (13.8%) was found in vegetables (Table 1). Bacteriophages have often been isolated in various seafoods. For instance, Bacteroides fragilis bacteriophages often were isolated from black mussels during examining the value of bacteriophages as surrogate markers for detecting pollution in shellfish (Lucena *et al.*, 1994). In our study, shrimp sample was the most diverse source with the isolation of *S.* Enteritidis, *S.* Typhimurium, *C. jejuni, V. parahaemolyticus* and *E. coli* bacteriophages. Previously, a novel bacteriophage was isolated with *Vibrio harveyi* from a black tiger shrimp culture ponds (Pasharawipas *et al.*, 2005). They found that osmolarity might decrease the viability of any bacteriophages present. As in the vegetable samples, lettuce was nearly free of bacteriophages. Concurrent with this observation, Tsuei *et al.* (2007) reported that vegetable samples such as asparagus, mung bean sprouts, spinach, watercress and silverbeet were free of *E. coli* bacteriophages.

On the other hand, *Salmonella* bacteriophages were predominantly isolated from beef and chicken meat samples, indicating that lytic bacteriophages were found to be widely distributed in this particular region of wet markets and hypermarkets. Several studies have suggested that 100% of the ground beef and chicken meat sold at retail contain various levels of various bacteriophages (Kennedy *et al*., 1986; Hsu *et al*., 2002). To our knowledge, this study represents the first bacteriophage isolation from beef and chicken meat in Malaysia. In addition, high frequency of *Salmonella* bacteriophages might provide circumstantial evidence for *Salmonella* food contamination in Malaysia, awakening the food preparation, processing, and production.

For sewage samples, only *E. coli* bacteriophages were isolated. Similarly, bacteriophage FAHEc1, which is almost entirely specific for *E. coli* serogroup O157, was isolated from raw sewage (Hudson *et al*., 2013). Muniesa *et al*. (2004) also reported the isolation of shiga-toxin producing *E. coli* bacteriophages from sewage samples, showed a wide range of host infectivity. It has been suggested that sewage samples were found to have the most diverse bacteriophages, in which a wide host range of bacteriophages against *S.* Typhimurium, *S.* Enteritidis, *S.* Saintpaul and *S.* Newport serovars were isolated (Akhtar *et al*., 2014). However, in our study, no *S.* Enteritidis or *S.* Typhimurium bacteriophage was found in sewage samples, which could be due to the adaptation to different environment and exploitation of different vectors. It is important to note that bacteriophages can significantly modify bacterial communities, which is dependent on the potential for bacteriophage propagation within those communities. Thus, bacteriophage potential for propagation, in turn, is a function of the interaction of the biotic and abiotic components of an environment (Boyd and Brussow, 2002).

Bacteriophage titer determination

Agar overlay assays are the most commonly used approach for determining bacteriophage titers. The lytic appearance of the plaque size was ranging from 1.0 mm to 3.0 mm (Figure 1). This phenomenon might be due to several factors such as the procedure for sterilization of the agar medium, the volume and softness of the top and bottom layers, and the number and growth stage of the bacterial cells added to the lawn. Additionally, an uneven over layer could affect



(A) (B)

Figure 1. Morphology of bacteriophage plaques. (A) Plaques formed in larger size. (B) Plaques formed in smaller size.

the plaque size and efficiency of plating. Different plaque sizes have been reported where the optimized plaque assay had a medium plaque size of 2.0 mm (Cormier and Janes, 2014), while the pouring method had the largest plaque size of 3.3 mm (Lillehaug, 1997). Indeed, the spreading of plaque formation consists of expansion of the bacteriophage population as mediated by multiple rounds of bacteriophage adsorption, infection, and lysis of individual bacteria, including bacteriophage diffusion. Hence, bacterial infection, which in turn feed the plaque formation by increasing bacteriophage numbers.

In all assays performed, bacteriophage titers were observed within the range of 10^8 to 10^{11} PFU/mL (Table 2). Among the bacteriophages, SE07, isolated from chicken meat, exhibited the highest titer of 1.37×10^{11} PFU/mL. A study conducted by Shin *et al.* (2011) showed that high titer bacteriophage (>10^8 PFU/mL) was obtained by fourteen *Bacillus cereus* bacteriophages which were isolated from fermented foods. In another case, Gupta *et al.* (2013) have found that *Klebsiella pneumoniae* bacteriophage PKp6 showed a much higher titer of 1015 PFU/mL. On the contrary, the concentration of bacteriophages SE09, SE10 and CJ02, which were isolated from cucumber, were found to be fairly low of 9.10×10^8 PFU/mL, 9.53×10^8 PFU/mL and 9.90×10^8 PFU/mL, respectively. Thus, compared with other isolation sources, cucumber sample was less favorable for enumeration of bacteriophages. The viability of bacteriophages in food samples could be affected by some characteristics including the structure and composition of the food, water activity, and pH (Hagens and Loessner, 2010). Furthermore, the optimum plating condition and other parameters such as the buffer in which the bacteriophages were suspended and the incubation media, may have an impact on the assay (Cormier and Janes, 2014).

Table 2. Bacteriophage titer determination using agar overlay assay

Bacteriophage	Source	Bacteriophage titer (PFU/mL)
SE01	Beef	$9.97 \times 10^9 \pm 2.12^a$
SE02	Beef	$1.12 \times 10^{10} \pm 2.03$
SE03	Beef	$7.40 \times 10^9 \pm 2.41$
SE04	Chicken	$8.17 \times 10^{10} \pm 2.30$
SE05	Chicken	$6.73 \times 10^{10} \pm 2.06$
SE06	Chicken	$7.13 \times 10^{10} \pm 2.11$
SE07	Chicken	$1.37 \times 10^{11} \pm 2.21$
SE08	Chicken	$8.20 \times 10^{10} \pm 2.23$
SE09	Cucumber	$9.10 \times 10^8 \pm 2.23$
SE10	Cucumber	$9.53 \times 10^8 \pm 2.29$
SE11	Lettuce	$1.08 \times 10^9 \pm 2.40$
SE12	Clam	$8.93 \times 10^{10} \pm 2.31$
SE13	Cockles	$8.33 \times 10^{10} \pm 2.31$
SE14	Shrimp	$7.77 \times 10^{10} \pm 2.08$
ST01	Beef	$8.93 \times 10^9 \pm 2.22$
ST02	Chicken	$9.77 \times 10^9 \pm 1.96$
ST03	Chicken	$9.53 \times 10^9 \pm 2.03$
ST04	Shrimp	$7.03 \times 10^{10} \pm 2.10$
CJ01	Chicken	$7.27 \times 10^{10} \pm 2.15$
CJ02	Cucumber	$9.90 \times 10^8 \pm 2.56$
CJ03	Cockles	$6.87 \times 10^{10} \pm 2.04$
CJ04	Shrimp	$8.40 \times 10^{10} \pm 2.11$
VP01	Cockles	$7.83 \times 10^{10} \pm 2.21$
VP02	Shrimp	$8.17 \times 10^{10} \pm 1.95$
EC01	Beef	$8.10 \times 10^{10} \pm 2.45$
EC02	Chicken	$7.53 \times 10^{10} \pm 2.00$
EC03	Shrimp	$8.90 \times 10^{10} \pm 2.61$
EC04	Sewage	$1.14 \times 10^{11} \pm 2.15$
EC05	Sewage	$1.04 \times 10^{11} \pm 2.28$

[a]Results expressed as mean s ± standard deviations from triplicates per assay.

Conclusion

Overall, our study was successful in isolating diverse of bacteriophages from several of food samples. From a biological control point of view it is important to obtain bacteriophages that have significant lytic ability. Therefore, the bacteriophages isolated in this work may potentially be used as a tool for biocontrol of food-borne pathogens. However, further knowledge of the characteristic and other properties as well as lysis efficacy of those bacteriophages will be necessary in order to know their application to improve food safety.

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