International Food Research Journal 24(2): 888-896 (April 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Distribution of bacteriophages in food and environment samples

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Article history

Received: 9 November 2016
Received in revised form: 20 December 2016
Accepted: 2 January 2017

Keywords

Foodborne pathogens
Bacteriophages
Double layer assay
Plaques
Bacteriophage titer

Abstract

Foodborne pathogens have become a constant threat to the consumer and food industry. Reduce efficacy of antibiotics with emergence of resistant bacteria has limited the opportunities for controlling pathogenic bacteria in food commodities and treating foodborne infections. Bacteriophages can be a promising alternative for alleviate the risk of transmitting pathogenic bacteria via food commodities. Therefore, this research was conducted to find distribution of bacteriophages in diverse niches in order to identify suitable sources for isolating bacteriophages to use controlling foodborne pathogens. Firstly bacterial strains were screened for lysogenic and selected suitable host bacterial strains were used for isolating and determining bacteriophage titer in fresh raw food and environmental samples. Eighteen different lytic bacteriophages effective against *Campylobacter, S. aureus, L. monocytogenes* and *E. coli* were isolated from this study. Bacteriophages titer was determined within range of 10^2 to 10^{10} PFU/mL and bacteriophages were most frequently isolated from chicken (60%) samples. The isolated bacteriophages could be potential candidates for controlling foodborne diseases.

Introduction

Campylobacter, Staphylococcus aureus, Listeria monocytogenes and *Escherichia coli* are some of the leading foodborne pathogens that cause major public health issue around the world (Behravesh *et al.*, 2012; Newell *et al.*, 2010). Food products, especially food of animal origin can be contaminated with these foodborne pathogens at any stage during the production process (Sillankorva *et al.*, 2012; Carvalho *et al.*, 2010).

High *Campylobacter* contamination level was reported in chicken and vegetables sold in Malaysia (Tang *et al.*, 2009; Chai *et al.*, 2007; Wong *et al.*, 2017). Dairy and milk products sold in Malaysian markets were contaminated with S. aureus (Sasidharan *et al.*, 2011). Neela *et al.* (2009) reported the isolation of MRSA from pigs and pig handlers in Malaysia. *L. monocytogenes* recovered from various food sources such as ready to eat foods (Marian *et al.*, 2012), ducks (Adzitey *et al.*, 2013), raw salad vegetables (Ponniah *et al.*, 2010), burger patties (Wong *et al.*, 2012), chicken (Goh *et al.*, 2013) and chicken offal (Kuan *et al.*, 2013). The *E. coli* O157: H7 occurred in beef samples (Son *et al.*, 1998); milk (Lye *et al.*, 2013); ready-to-eat food, popiah (Elexson *et al.*, 2017) and chicken (Chang *et al.*, 2013) sold in Malaysia. Also, *E. coli* was isolated from raw vegetables (Loo *et al.*, 2013), pigs (Ho *et al.*, 2013) and water samples (Alhaj *et al.*, 2007) in the country. Also high antibiotic resistance was reported among the isolated foodborne pathogens (Tang *et al.*, 2009; Chai *et al.*, 2007). Though not significant, GMO that may carry the antibiotic resistant gene as marker has also been reported in Malaysia (Lisha *et al.*, 2017).

Therefore, to combat foodborne illnesses

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and antibiotic resistance, novel, and efficient strategies have become paramount in the current global context (Gálvez *et al.*, 2010; García *et al*., 2010). Bacteriophages have been identified as a potential alternative to antibiotics (Haq *et al*., 2012; Sillankorva *et al.*, 2012). Bacteriophages also known as phages are bacterial viruses composed of nucleic acid genome enclosed within a protein or lipoprotein coat (Sillankorva *et al*., 2012). Phages are ubiquitous in the environment and all most all the bacteria possess their own bacteriophages that can be isolated from the environment where the host bacteria occur (Sillankorva *et al*., 2012; Kutter, 2005).

More than 1031 bacteriophages contain in the earth biosphere (Endersen *et al*., 2014). Further, bacteriophages that infect various host bacteria were isolated from diverse sources such as human faeces, animal faeces, food, water, soil and sewage (Atterbury, 2009). However, worldwide still an only limited number of bacteriophages were identified (Casjens, 2008). Even in Malaysia, only a few bacteriophages such as that effective against *Vibrio cholera* (Al-Fendi *et al*., 2014) and colibacillosis (Lau *et al.*, 2010) were investigated. Therefore, need to study and isolate prevailing bacteriophages in the country that could be used as a potential agent to control pathogenic and multidrug resistance bacteria. Therefore, the objectives of this study were to assess the distribution and concentration of *Campylobacter, S. aureus, L. monocytogenes,* and *E. col*i bacteriophages in food and environmental samples.

Materials and Methods

Host-bacterial cultures and growth conditions

Foodborne pathogenic bacteria including *Campylobacter jejuni, Campylobactere coli* isolated from this study and *Staphylococcus aureus,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes* and *E. coli* obtained from the Food Safety Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia were used as the target host bacteria.

Campylobacter strains used in this study were maintained in 20% (v/v) glycerol supplemented with Brain Heart Infusion (BHI) broth (Merck, Germany) at −20˚C until use. When necessary, working cultures were prepared on Blood Agar plates (PB0114, Oxoid, UK). *Campylobacter* host strains were grown according to the method by Carvalho *et al*. (2010). Briefly, *Campylobacter* was grown in New Zealand Casamino Yeast Medium (NZCYM, Sigma-Aldrich, USA) supplemented with 400 µg/mL $CaCl_2$ and 400 µg/mL $MgSO_4$ at 42˚C under microaerophilic conditions generated by Anaerocult C system (Merck, Germany) with shaking (150 rpm) (MaxQ 4000; Barnstead International, USA). The broth reached the mid-log phase were used for bacteriophage enrichment.

The bacterial strains, *S. aureus, L. monocytogenes* and *E. coli* utilized in this study, were stored in 20% (v/v) glycerol at −20˚C. A 100 µL of the frozen stock culture was inoculated into Luria Bertani (LB) broth (Merck, Germany) and incubated overnight at 37°C with shaking. Secondary cultures were prepared on Tryptone Soy agar (TSA) (Merck, Germany) slants by transferring from the activated bacterial broth culture grown in Luria-Bertani (LB) broth (Merck, Germany). Then the agar slants were sealed and stored at 4˚C until use for the preparation of working cultures. When necessary a loop full of culture from agar slants was transferred onto TSA (Merck, Germany) (incubated overnight at 37°C), then checked for purity and transferred single colony into LB broth (Merck, Germany) to prepare working cultures. The bacterial host strains (*S. aureus, L. monocytogenes,* and *E. coli*) grown in LB broth (Merck, Germany) with shaking incubation (150 rpm) at 37˚C until it reaches the mid-log phase were used for bacteriophage enrichment.

Screening bacterial strains for lysogeny

Bacterial strains were screened for lysogeny by using mitomycin C as inducing agent. A 10 mL of fresh NZCYM broth growth media was inoculated with 100 µL of an overnight culture of the *Campylobacter* strain to be tested for lysogeny. The *Campylobacter* cultures were incubated at 42°C under microaerobic conditions (Anaerocult C system (Merck, Germany). Once the bacteria reached the exponential growth phase, 5 µL of 1 mg/mL mitomycin C was added and for the negative control 5 µL of distilled water was added. Then absorbency was measured at 600 nm in each hour for 12 h. The strains that reduced turbidity were excluded from the study.

A 10 mL of fresh LB broth growth media was inoculated with 100 µL of an overnight culture of the *S. aureus, L. monocytogenes* or *E. coli* strain to be tested for lysogeny. Then cultures were incubated at 37°C, and once they reached the exponential growth phase, 5 µL of 1 mg/mL mitomycin C was added and for the negative control 5 µL of distilled water was added. Then absorbency was measured at 600 nm in each hour for 8 h. The strains that reduced turbidity were excluded from the study.

Sample collection

Fresh food samples including; dairy products,

poultry, seafood, vegetables, and beef were purchased from different wet markets in Selangor, Malaysia. Also, environmental samples such as water and sewage samples were collected from surrounding area of the wet markets in Selangor and Universiti Putra Malaysia, Malaysia. Samples were collected and stored inside an insulated cool box containing ice pack and immediately processes at the laboratory.

Enrichment of bacteriophages

Samples were mixed with 1:10 (w/v) SM buffer [50 mM Tris-HCl (pH 7.5)], 0.1 M NaCl, 8 mM $MgSO_4 \bullet 7H_2O$ and 0.01% (w/v) gelatine (Sigma-Aldrich, USA) and the mixture was pummeled. Then the homogenised sample was inoculated with exponential phase Campylobacter culture at 9:1 (v/v) ratio. Then followed by overnight shaking incubation (150 rpm) at 42°C microaerobically (Anaerocult C system; Merck, Germany) for potential bacteriophages to amplify and dissociate. The homogenized sample was inoculated with exponential phase bacterial culture of *S. aureus,* MRSA, *L. monocytogenes*, and *E. coli* at 9:1 (v/v) ratio and followed by overnight shaking incubation (150 rpm) at 37ºC for potential bacteriophages to amplify and dissociate.

Isolation of bacteriophages

After incubation, the mixture was centrifuged for 10 min at 10,000 rpm to remove bulk debris from the sample. Bacterial cells were removed by filtering the supernatant through 0.2 µM membrane filter (Sartorius, Germany). Then, the filtrate was checked for phage activity using spot plate assay (Chang *et al*., 2005; Adam, 1959). Briefly, an aliquot of 100 µL of mid-log phase bacteria *Campylobacter* cultures were inoculated into 3 mL of molten NZCYM soft agar (NZCYM broth containing 0.6% (w/v) agar). This mixture was poured evenly onto NZCYM bottom agar (NZCYM broth containing 1.5% (w/v) agar). Once the top agar solidified, 10 µL of the filtrate was spotted onto the onto NZCYM bottom agar containing *Campylobacter.* Then the plates were dried for 30 mins and incubated overnight at 42°C under microaerobic conditions (Anaerocult C system; Merck, Germany).

An aliquot of 100 µL of mid-log phase bacterial cultures of *S. aureus*, MRSA, *L. monocytogenes* and *E. coli* were separately inoculated into 3 mL of molten LB soft agar (LB broth containing 0.6% (w/v) agar). This mixture was poured evenly onto LB agar (Merck, Germany). Once the top agar solidified, 10 µL of the filtrate was spotted onto the LB agar containing host bacteria (*S. aureus,* MRSA, *L. monocytogenes*, and *E. coli*). Then the plates were dried for 30 mins and all culture plates were incubated overnight at 37°C.

Plaque morphology

After incubation, the plates were observed for the presence of visible clear zones or plaques that are indicative of bacterial lysis by phage activity. Plaques were scored on a scale from 0 to 5 depending on the plaque morphology (Al-Fendi, 2014). Plates with no interaction or minimal effect were given a score 0 and 1 respectively. A Clear and complete bacterial lysis was scored as 2 while turbid plaques that were not able to clearly distinguish as lytic activity was given a score 3 or 4. Plaque with a clear margin and turbid centre that resembles a bulls-eye was given score 5. Initially, plaques scored 2-5 were considered as phage activity.

Double layer assay

Samples that displayed phage activity were reconfirmed using double layer assay as described by Adams (1959). Briefly, a 100 µL of enriched sample filtrate and 100 µL mid-log phase *Campylobacter* bacterial culture was added to 3 mL of molten NZCYM soft agar (NZCYM broth containing 0.6% (w/v) agar). Then the mixture was mixed well and poured evenly onto NZCYM bottom agar (NZCYM broth containing 1.5% (w/v) agar). Once the top agar solidified, the plates were incubated overnight at 42°C under microaerobic conditions (Anaerocult C system; Merck, Germany).

Briefly, a 100 µL of enriched sample filtrate and 100 µL mid-log phase bacterial culture of *S. aureus*, MRSA, *L. monocytogenes,* and *E. coli* was separately added to 3 mL of molten LB soft agar (LB broth containing 0.6% (w/v) agar). The mixture was mixed well and poured evenly onto LB agar (Merck, Germany). Once the top agar solidified, the culture plates were incubated overnight at 37°C.

After incubation, plates were observed for the presence of plaques. Samples indicated presences of plaques were confirmed as phages. Detected plaques were picked using a sterile pipette tip and resuspended in 100-500 µL of SM buffer and stored at 4°C for future use.

Bacteriophages titration

Bacteriophages titration was conducted using the double layer assay as described by Adams (1959). A ten folds dilution of phage lysate was prepared by using SM buffer. Then double layer assay was conducted for each dilution in triplicates.

After incubation, the plates were observed for the presence of visible clear zones of bacterial lysis. The dilution that formed 30 and 200 plaques were selected

and counted the number of plaques. The obtained data were used to calculate the plaque forming units (PFU/ mL) using standard formula (Adams, 1959).

Results and Discussion

Screening for lysogeny

Out of the 36 tested bacterial strains for prophage activity using mitomycin C, two of the *S. aureus* strains harboured prophages in the genome. Incubating with mitomycin C, the optical density reduced in the bacterial strains that harboured prophage. At the initial phase of phage isolation, selecting bacterial hosts that do not harbour prophages in the genome is important (Garcia *et al*., 2007). The prophages in the bacterial genome can be induced by application of mitomycin C, chloroform or ultraviolet (UV) light (Weinbauer, 2004; Garcia *et al*., 2007). In this study, mitomycin C was used to induce the prophages, and two *S. aureus* strains (FQ3 and FQ5) were identified to be lysogenize by containing prophages in the genome. Therefore, those two *S. aureus* strains excluded from phage isolation procedure.

Plaque formation

First samples were screened using the spot plate assay, observed various plaque morphologies were given a score and recorded. Different scores given for plaque morphologies indicate in Figure 1. Initially, 131 experiments were able to produce clear to turbid plaques from the spot assay method. In the spot plate method, once the enriched sample applied to a lawn of host bacteria on agar medium, phages in the sample will be absorbed to the host bacterium and commence lysis. This bacterial lysis produces a visible clear zone on the bacterial lawn that named as a plaque (Adams, 1959; Kutter, 2005; Yoon *et al*., 2007). Samples demonstrated bacteriophage activities on spot plate method was then reconfirmed with double layer assay. In the food samples tested, highest phage activity was demonstrated in seafood samples however with confirmation by double layer assay identified as lysogenic phages (data not shown). However, following the double layer assay, only 18 samples were able to produce clear plaques characteristic of a lytic phage while the rest formed turbid, very small or hardly visible plaques typical for lysogenic phages (Adams, 1959; Yoon *et al*., 2007). The temperate phages lead to lysogenic infection in which transfers the phage genome into the bacterial progeny without lysis of the host bacterium. Phages that enter lysogenic growth cycle produce turbid plaques on susceptible bacterial lawns (Adams, 1959; Ai *et al*., 2008). The lytic nature of the phages to be

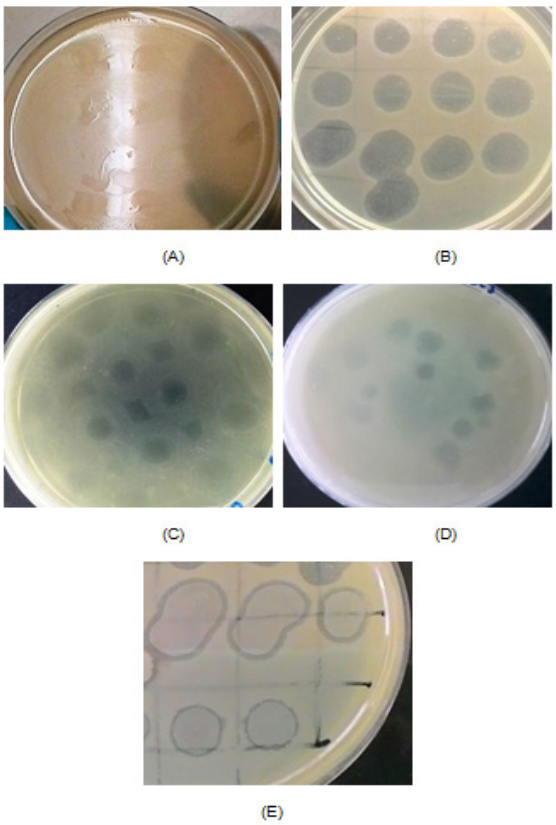

Figure 1. Observed plaque morphologies

verified before using for phage therapy because often lysogenic phages may not be likely to inhibit bacterial pathogens and might become resistant to phage lysis (Skurnik *et al*., 2007; Sulakvelidze, 2011). The isolated phages produced around 1.0 to 3.0 mm lytic plaques in the double layer assay (Figure 2). The size and appearance of a plaque can be associated with the volume and density of agar, concentration and stage of growth of the host bacterium and constancy of the top agar (Adams, 1959; Cormier and Janes, 2014).

Occurrence of phages

A total of 18 bacteriophages confirmed to demonstrate lytic activity for *L. monocytogenes, E. coli, S. aureus,* MRSA, and *Campylobacter* (Table 1). From the tested samples, the highest frequency of isolating bacteriophages was reported in chicken (60%) whilst vegetables found to have the lowest frequency (9.4%) (Table 1). The occurrence of bacteriophages was closely related to the host bacterium (Akhtar *et al*., 2014). Therefore, bacteriophages could be isolated from natural environment of the targeted host bacterium (Adams, 1959; Endersen *et al*., 2014). However, none of the phage ativity showed by environmental samples was able to produce lytic activity (Table 1). Similarly, Bigwood and Hudson, (2009) were unable to isolate *Campylobacter* phage from water samples taken from various locations can be related to a low concentration of host bacterium

Table 1. Isolation of phages from different samples

Sample Type	Number of samples tested	*L. monocytogenes* (%)	*E. coli* (%)	*S. aureus* (%)	MRSA (%)	*C. jejuni* (%)	*C. coli* (%)	*Total* (%)
Beef	15	1 (6.7)	1 (6.7)	1 (6.7)	1 (6.7)	0 (0.0)	0 (0.0)	4 (26.7)
Chicken	15	4 (26.7)	1 (6.7)	1 (6.7)	0 (0.0)	3 (20)	1 (6.7)	9 (60)
Vegetables	32	1 (3.1)	1 (3.1)	0 (0.0)	0 (0.0)	2 (6.3)	0 (0.0)	3 (9.4)
Clam	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Cockles	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Shrimp	10	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Environmental samples	20	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	112	6 (5.4)	3 (2.7)	2 (1.8)	1 (0.9)	5 (4.5)	1 (0.9)	18 (16.1)

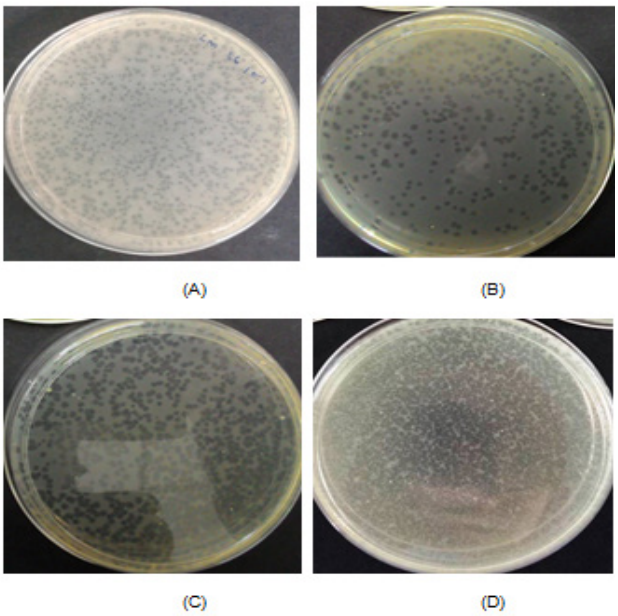

Figure 2. Morphology of lytic plaques. Phage active against A: *L. monocytogenes*; B: *S. aureus*; C: *C. jejuni*; D: *E. coli.*

present in the environmental samples (Bigwood and Hudson, 2009).

In this study, six different phages effective for *Campylobacter* were isolated from chicken and vegetable samples. Similar to these findings, previously phages effective against *Campylobacter* were isolated from variety of samples including; chicken (Atterbury *et al.,* 2003), chicken intestine (Carrillo *et al.*, 2007; Hansen *et al*., 2007; Carvalho *et al*., 2010), duck intestine (Hansen *et al*., 2007), chicken ceca (Atterbury *et al.*, 2005; El-Shibiny *et al*., 2005; Carrillo *et al*., 2007), chicken feces (Owens *et al*., 2013), and from abattoir wastewater (Hansen *et al*., 2007). However, phages for *Campylobacter* could not be isolated from water samples taken from rivers, streams, ponds and lakes (Bigwood and Hudson, 2009) and frozen chicken (Atterbury *et al.*, 2003). Interestingly, this study was able to isolate two phages effective against *C. jejuni* from vegetable samples. High level of *Campylobacter* contamination in vegetables was previously reported from Malaysia (Chai *et al*., 2007). Phages are naturally present in the environment in which the host bacterium inhabits (Brüssow, 2002). Therefore, an abundance of *Campylobacte*r host in vegetables may have associated with the isolation of phages acting against *C. jejuni.*

In the current study, the bacteriophages effective against *E. coli* were isolated each from chicken (n=1), beef (n=1) and vegetables (n=1). Previously E. coli phages were isolated from; sewage sludge (Fan et al., 2012), human stools (Tomat *et al.*, 2013), chicken meat (Shousha *et al*., 2015), poultry feces (Bhensdadia *et al*., 2014) and industrial cucumber fermentation (Lu and Breidt, 2015).

Three bacteriophages effective against *S. aureus* were isolated from chicken and beef samples and one of which was effective against MRSA. Previously, phages effective against *S. aureus* were isolated from a cow infected with mastitis (Kwiatek *et al*., 2012), sewage samples (Synnott *et al*., 2009; Alves *et al*., 2014) and endotracheal tubes used by patients (Hsieh *et al.*, 2011). The isolated phage ØMRSA1 lysed both *S. aureus* and MRSA strains.

Listeria phages (5.4%) were the most frequently isolated pahes in this study, a total of six phages from chicken (n=4), beef (n=1) and vegetables (n=1) that effective against *L. monocytogenes*. Earlier *Listeria* phages were recovered from fish waste treatment seafood (Arachchi *et al*., 2013), turkey processing plant (Kim *et al*., 2008), sewage of a dairy processing facility (Carlton *et al*., 2005) and environmental samples (Loessner and Busse, 1990). Though more than 500 different phages effective for *Listeria* were isolated; only a few were studied at the genomic level (Zimmer *et al*., 2003; Carlton *et al*., 2005; Dorscht *et al*., 2009; Schmuki *et al*., 2012).

Table 2. Bacteriophage Titers

Source	Bacterial Host	Bacteriophage	Bacteriophage titer (PFU/mL)
Chicken	*L. monocytogenes*	ØLM1	$5.3 \times 10^3 \pm 3.21$
Chicken	*L. monocytogenes*	ØLM2	$5.0 \times 10^4 \pm 2.14$
Chicken	*L. monocytogenes*	ØLM3	$1.3 \times 10^7 \pm 5.26$
Chicken	*L. monocytogenes*	ØLM6	$2.0 \times 10^7 \pm 3.44$
Beef	*L. monocytogenes*	ØLM4	$7.0 \times 10^8 \pm 2.51$
Vegetables	*L. monocytogenes*	ØLM5	$4.7 \times 10^2 \pm 1.04$
Beef	*E. coli*	ØEC1	$1.78 \times 10^{10} \pm 2.23$
Chicken	*E. coli*	ØEC2	$8.1 \times 10^{10} \pm 4.02$
Vegetables	*E. coli*	ØEC3	$4.6 \times 10^3 \pm 1.21$
Beef	*S. aureus*	ØSA1	$3.0 \times 10^3 \pm 2.11$
Vegetables	*S. aureus*	ØSA2	$1.1 \times 10^3 \pm 3.47$
Beef	MRSA	ØMRSA1	$4.4 \times 10^4 \pm 3.31$
Chicken	*C. coli*	ØCC1	$7.27 \times 10^{10} \pm 2.15$
Chicken	*C. jejuni*	ØCJ1	$9.73 \times 10^{10} \pm 3.64$
Chicken	*C. jejuni*	ØCJ2	$8.78 \times 10^{10} \pm 2.37$
Chicken	*C. jejuni*	ØCJ3	$9.90 \times 10^8 \pm 2.56$
Vegetables	*C. jejuni*	ØCJ4	$3.87 \times 10^6 \pm 2.04$
Vegetables	*C. jejuni*	ØCJ5	$8.40 \times 10^4 \pm 2.11$

Bacteriophage titers

The bacteriophage titer levels in samples ranged from $8.1 \times 10^{10} \pm 4.02$ to $4.7 \times 10^2 \pm 1.04$ (Table 2). The highest bacteriophage titer at 1010 PFU/mL was detected for *Campylobacter* and *E. coli* specific bacteriophages that isolated from the chicken and beef. While the lowest titer was recorded for phage isolated from vegetables that active against *L. monocytogenes*. Comparatively vegetable samples carried low phages titer than meat samples. Atterbury et al., (2003) was able to isolate 1 x 10^2 to 4 x 10^6 PFU/mL *Campylobacter* phages from chicken and later Atterbury *et al*., (2005) and El-Shibiny *et al*., (2005) recovered 10^2 to 10^7 PFU/g *Campylobacter* phages from the cecal content of broiler chickens. Enrichemnt incoperated in the bacteriophage isolation step was reported to facilitaed isolation of phages (Carvalho *et al*., 2010) may be associated with the favourable titers reported in this study.

Conclusion

In conclusion, phages specific for *Camphylobacter jejuni, C. coli, Staphylococcus aureus, MRSA, Listeria monocytogenes*, and *Escherichia coli* were isolated from various food commodities in Malaysia. Therefore, findings of this study indicate that phages can be readily isolated from food products, and people can be exposed to phages frequently via food. The high titers and lytic ability exhibited by these phages stipulate them as promising and potential candidates to use as effective biocontrol agents against pathogenic bacteria.

Acknowledgements

This research was supported by the Fundamental Research Grant Scheme (FRGS) of Ministry of Higher Education (MOHE), Malaysia with project code 02-01-14-1475FR and reference code FRGS/1/2014/SG05/UPM/01/2 and in part, by a fund for Research on international cooperation in medical science, Research on global health issues, Health and Labor Science Research Grants, the Ministry of Health, Labor, and Welfare of Japan, and a fund from Kyoto University Research Coordination Alliance, Japan, by a fund by Kakenhi Grant-in-Aid for Scientific Research and from the Japan Society for the Promotion of Sciences.

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