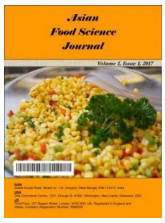

Asian Food Science Journal

10(1): 1-14, 2019; Article no.AFSJ.49654
ISSN: 2581-7752

Characterization of Bacteriophage against *Enterococcus faecium* Resistant to Vancomycin Isolated from Chicken Skin

Hazzierah Syaffieqah An Nadiah Azlan[1], Muhajir Hamid[1*] and Adelene Ai-Lian Song[1]

[1]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Malaysia.

Authors' contributions

This work was carried out in collaboration among all authors. Author HSANA designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MH and AALS managed the analyses of the study and wrote the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AFSJ/2019/v10i130026
Editor(s):
(1) Dr. Surapong Pinitglang, Assistant Professor, Department of Food Business Management, School of Science and Technology, University of the Thai Chamber of Commerce, Bangkok, Thailand.
Reviewers:
(1) Debarshi Kar Mahapatra, Dadasaheb Balpande College of Pharmacy, India.
(2) Daniel Boamah, Centre for Plant Medicine Research, Ghana.
Complete Peer review History: http://www.sdiarticle3.com/review-history/49654

Original Research Article

Received 30 March 2019
Accepted 15 June 2019
Published 22 June 2019

ABSTRACT

Aims: To characterize bacteriophages with strong *in vitro* lytic activity against vancomycin resistant *Enterococcus faecium* before testing on the chicken skin for their efficacy.
Study Design: An experimental was carried out to characterize two isolated bacteriophages against *Enterococcus faecium* and test for their efficacy on chicken skin.
Study Place: The study was carried out in Laboratory of Vaccine and Immunotherapeutics, Institue of Bioscience, Universiti Putra Malaysia in Selangor, which is the most populous state in Malaysia.
Methodology: Two host specific lytic phages against vancomycin resistant *Enterococcus faecium* strain FM8, designated as FM8-P1 and FM8-P2 were physiological characterized. This includes determination of their adsorption rate, multiplicity of infection, and single step growth kinetics. The optimum pH and temperature for both bacteriophages activity were also determined before tested

**Corresponding author: Email: muhajir@upm.edu.my;*

on chicken skin at 4°C and 25°C, which represent chiller and room temperature in poultry production line.

Results: Based on the result of single-step growth kinetics, the latent period of FM8-P1 was 35 min with a burst size of 460 particles per infected cells, while FM8-P2 has a shorter latent period (20 min) but a smaller burst size of 60 particles. The highest adsorption rate for FM8-P1 was 83% and FM8-P2 was 90% at 2 min and 4 min respectively. Both bacteriophages also exihibited a wide range of pH and temperature for their activity.

Conclusion: The specificity, lytic activity and stability of FM8-P1 and FM8-P2 emphasized their potential in effectively eliminating the vancomycin resistant *Enterococcus faecium* strain FM8. However, further works are required to validate their *in situ* reliability.

Keywords: Bacteriophage; vancomycin resistant enterococcus; enterococcus faecium; chicken skin.

1. INTRODUCTION

Chicken meat production is a major product from Malaysian poultry industry [1] Despite the efficiency of the intensive farming system, many problems remain to be resolved. One of the problem involves pathogens such *as Enterococcus* spp., namely *E. faecalis* and *E. faecium*, which is under the spotlight of the authorities in this industry [2,3].

Despite the fact that *E. faecalis* is more pathogenic than *E. faecium*, the latter exhibits more resistance towards antibiotics [2]. The *E. faecium* is also of higher concern to veterinary inspectors due to their regular presence in poultry and livestock products [3,4]. The relative importance of *E. faecium* as a pathogen has increased with the occurrence of high-level resistance to multiple antimicrobial drugs such as aminoglycosides, ampicillin, linezoid, teicoplanin and vancomycin [5,6]. Once the patients are infected by these bacteria, it becomes a health concern as vancomycin is used as the last resort of antibiotics [2]. Hence, other methods to overcome vancomycin resistant *Enterococcus* (VRE) should be taken into consideration.

One of the alternative approaches to control the bacteria load is by phage therapy. The lytic bacterial viruses are capable of lysing bacteria, and specific lytic phages can kill pathogenic bacteria in their own habitat without interrupting other organisms [7].

In this study, isolated phages with strong *in vitro* lytic activity against vancomycin resistant *E. faecium* were characterized and tested on the chicken skin for their efficacy. This study is designed to test the hypothesis that bacteriophage against *Enterococcus* spp. resistant to vancomycin could be isolated in the chicken fecal contaminated area, including the chicken carcass. The isolated bacteriophages are also able to be optimized for eradicates its host effectively.

2. MATERIALS AND METHODS

2.1 VRE Strains and Characterization

Vancomycin resistant *E. faecium* (VREfm) isolate (FM8) was obtained from the Faculty of Veterinary Medicine, Universiti Putra Malaysia. It was isolated from a human source and contained p1 *van*A gene [8]. A single colony from the Brain Heart Infusion (BHI) agar plate was selected and inoculated into 3 ml BHI broth and incubated at 37 °C for overnight. The VREfm FM8 minimum inhibitory concentration (MIC) against vancomycin was confirmed by streaking on BBL Enterococcosel™ agar with up to 48 µg/ml vancomycin incorporated. All the plates were incubated at 37°C for overnight. *Enterococcus* spp. resistance to vancomycin formed white colonies with blackening background on the agar.

2.2 Phage Isolation, Detection and High Titer Preparation

Samples were obtained from chicken skin in Selangor, Malaysia. The skin samples were brought to the laboratory to be processed separately for phage screening.

Liquid sample was filtered through 0.2 µm-pore-size membrane filter and 10 ml of the filtrate was mixed with 10 ml of double strength BHI broth containing the 1 % (v/v) log phase VREfm FM8 isolate. For solid samples, 2 g was suspended in 20 ml of the double strength BHI broth inoculated with 1% (v/v) mid-log phase VRE isolate. The mixture was incubated at 37°C with agitation at for 24 h. The culture was then centrifuged at

6500 x g for 20 min at 4°C. The supernatant was filtered through 0.2 µm-pore-size membrane filter and the filtrate was used for phage screening by spot assay as described by Wang *et al.* [9]. An individual plaque was isolated and picked out via stab sampling by stabbing and extracting the plaque out using pipette before storing in 1 ml SM buffer in a microcentrifuge tube. The tube was slightly tapped before being used for enumeration using double layer agar assay as described by Kropinski et al. [10], but with slight modification. A 100 µl VREfm FM8 in the mid-log phase was inoculated with 100 µl of SM buffer containing the plaque. The mixture was incubated at room temperature for 5 min, mixed with 3 ml of melted BHI soft top agarose [15% (w/v) BHI broth, 0.6% (w/v) agarose], vortexed at low speed and poured uniformly over the surface of hard BHI agar base plate [15% (w/v) BHI broth, 1.5% (w/v) agar]. The plate was left to stand at room temperature to allow the top agar to solidify. The plate was inverted and incubated at 37°C for 5 h. After incubation hours, the formation of plaques was being observed and enumerated. Plaques were counted in the plate containing 30 - 300 plaques and expressed as plaque forming unit per milliliter (PFU/ml).

Pure phage strain was obtained by three serial single-plaque isolations. The phage isolates were designated as FM8-P1 and FM8-P2. The purified phage was prepared by CsCl step gradient and dialysed against 1 liter of SM buffer containing 1 M NaCl, at 4ºC for overnight. The phage then was filter sterilized using 0.2 µm-pore-size membrane and stored in the dark at 4ºC.

2.3 Host Specificity of the Phage

The lytic ability of the isolated phage against several selected bacteria (Table 1) was conducted. Three hundred µl of indicator bacteria (mid-log phase; OD_{600} = 0.3; ~ 1 x 106 CFU/ml) was incubated with 100 µl of isolated phages (~ 1 x 10^7 PFU/ml) for 30 min. The mixture was then serially diluted in 900 µl maximum recovery diluent (MRD) (1 g peptone, 8.5 g NaCl in 1 L distilled water) and at appropriate dilutions, 100 µl of the sample was obtained for enumeration of the bacteria on selective agar (Table 1). After incubation at suitable condition (Table 1), the total numbers of bacterial colonies formed were counted. VREfm FM8 was used as the positive control. The experiment was carried out in triplicate and repeated three times.

2.4 Multiplicity of Infection

An optimum multiplicity of infection (MOI) is important as it conveys the activity and effective ratio for the elimination of host bacteria, as the method counts only those phages that have attached to and then infected the host, to form active interaction between both microorganisms [10]. The determination was done by mixing the respective phages lysate (FM8-P1 and FM8-P2) with 10^6 CFU/ml mid-log phase host bacteria at MOI 0.01, 0.1, 10.0, and 100.0. The mixtures were then incubated at 37°C with agitation (180 rpm). Samples were taken at 60 min intervals for the first 2 h and 30 min intervals for the next 2 h to determine the titer of phages by double layer agar method and the number of viable bacteria was determined by spread plate technique. The experiment was done in triplicate and repeated three times. Optimum MOI of the phages were identified and subjected for subsequent studies.

2.5 Growth Kinetic Study of Phage

Single step growth curve was conducted to determine the latent period and burst size of the phage as described by Hyman & Abedon [11]. In this study, phage lysate was added to mid-log phase host bacteria at MOI of 0.01. After a 10 min of incubation, free phage was removed by centrifugation at 5000 x g for 5 min at 4°C. The infected pellet was resuspended thoroughly in pre-warmed BHI broth. The suspension was then incubated again at 37°C with agitation at 180 rpm. Samples were taken at 10 min intervals and titrated immediately to enumerate the phage titer. The titer of phage was then plotted against time intervals. The experiment was done in triplicate and repeated three times.

2.6 Adsorption Rate of Phage

Determination of the adsorption rate of phage FM8-P1 and FM8-P2 was performed according to Hyman and Abedon [11]. Mid log phase host bacteria were infected with a known titer of phage at a MOI 0.01 at 37°C. After infection, 300 µl of the sample was taken out every 2 min for the first 20 min, followed by every 5 min for the next 15 min. The samples were centrifuged at 5000 x g for 3 min at 4°C to remove the bacteria and adsorbed phage. The supernatant was serially diluted immediately with SM buffer, and the concentration of free phage was determined by double layer agar method. The experiment was done in triplicate and repeated three times. The time point for the highest percentage of

adsorption to occur for both phages was used for the subsequent experiments.

2.7 Determination of Optimum pH

Determination of optimum pH for phage activity was done by adding 0.5 ml of mid-log phase of host bacteria to 0.5 ml of phage lysate (10^7 PFU/ml) at MOI 0.01 and resuspended in 4 ml BHI broth with pH 3, 4, 5, 6, 7, 8, 9, 10 and 11. Control tubes which contained either phage only or host bacteria only were set up for each of the tested pH. All the tubes were incubated at 37°C with agitation. After incubation, 1.0 ml of samples from phage control and phage-bacteria mixture tubes were taken out, centrifuged at 13,000 x g for 0.5 min and the supernatant was used for determination of the remaining free phage titer.

Incubation proceeded until 3 h. At this point, samples were removed from host bacteria control and phage-bacteria mixture tubes to determine the reduction of host bacteria by using spread plate technique and phage count was done by using double layer agar technique. The experiment was done in triplicate and repeated three times.

2.8 Determination of Optimum Temperature

In determination of optimum temperature for phage activity, 0.5 ml of phage lysate (10^7 PFU/ml) was added to 0.5 ml of mid-log phase host bacteria (MOI = 0.01) which was then resuspended in 4 ml BHI broth (pH 7.0) and incubated at different temperatures (4°C, 25°C, 37°C and 60°C). Control tubes which contained either phage only or host bacteria only were set up for each of the tested temperatures. All the tubes were incubated at designated temperatures with agitation. After incubation, samples from phage control and phage-bacteria mixture tubes were taken out, centrifuged at 13,000 x g for 0.5 min and the supernatant was used for determination of the remaining free phage titer. Incubation proceeded until 3 h. At this point, samples were removed from host bacteria control and phage-bacteria mixture tubes to determine the reduction of host bacteria by using spread plate technique and phage count was done by using double layer agar technique. The experiment was done in triplicate and repeated three times.

2.9 VREfm FM8 Antibiotic Susceptibility Test

VREfm FM8 were streaked onto Enterococcosel agar, containing streptomycin sulphate, penicillin G, methicillin-sodium salt monohydrate, ampicillin sodium, or kanamycin monosulphate. The concentration of the antibiotics assigned was 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml and 1 mg/ml. All the plates were incubated at 37°C for overnight. Then, the plates were observed for colony formation. Plates containing *Enterococcus* sp. resistant to the antibiotic tested formed blackening of Enterococcosel agar and formation of white colony bacteria.

2.10 Vancomycin Resistant Enterococcus on Chicken Skin Samples

The experiment was done as described by Hungaro et al. [12]. Fifteen samples of chicken's skin from a local market in Serdang were obtained and individually wrapped in an autoclave bag. The skin was then removed from the bag and air dried for 30 min in the laminar flow. About 10 cm^2 random area of three different parts on a piece of chicken skin was swabbed by using cotton wool swab. The sample was eluted in 1 ml MRD and vortexed for 30 sec. The eluted sample was serially diluted and was plated on BBL Enterococcosel™ agar containing 32 µg/ml vancomycin. The plates are incubated at 37°C for overnight, before being observed. The formations of white colonies with blackened agar indicate the presence of VRE. VREfm FM8 was used as a negative control. The experiment was performed in triplicates and repeated three times.

2.11 Elimination of VRE load on chicken skin at low MOI

The elimination of VRE load on chicken skin at low MOI experiment was done as described by Hungaro et al. [12]. Chicken skin pieces (8 cm^2) were inoculated in triplicate with 5.5 ml of 10^6 CFU/ml VRE FM8 and the inoculum was distributed over the surface using a glass spreader to produce host density of approximately 10^3 CFU/cm2. The samples were then incubated at 4°C, for 2 h in sterile plastic containers. After the incubation, 5.5 ml of phage (10^3 CFU/ml) was sprayed onto the surface of the chicken skin using a garden sprayer, to obtain 10 PFU/cm^2. The MOI tested in this experiment was 0.01. Prior to phage treatment, cotton wool swab samples were taken from three separate 10 cm^2 areas of each chicken skin

portion. Subsequently, swabs were collected at 6, 12, 18, 24, 36, 48, and 72 h after treatment with phage. These swab samples were taken twice at the same area and eluted in 1 ml of MRD and SM buffers separately. The target bacteria (VREfm FM8) from MRD buffer were quantified by plating appropriately diluted samples on BHI agar with 32 µg/ml vancomycin. The BHI agar was added with 32 µg/ml vancomycin, as referred to the result of Section 2.11, which VREfm FM8 known to be able to tolerate 32 µg/ml, while wild *Enterococcus* spp. did not. The number of viable VRE per cm^2 was estimated from the result of the three swab samples. Whereas for the enumeration of phage, plaque assay using the double layer agar method was performed. A set of control was prepared by applying the required concentration of phage without bacteria; the host without phage and without host and phage. The experiment was repeated by incubating the samples at 25°C and swab samples were taken at swabs were collected at 3, 6, 12, and 24 h after treatment with phage. Shorter experiment time applied for incubation at 25°C is based on the result of Section 3.8, which conveyed both phages has high lytic activity at room temperature. The experiment was done in triplicates and repeated for five times.

2.12 Elimination of VRE load on chicken skin at high MOI

The experiment was set up as described in Section 2.12, except that 5.5 ml of 10^5 PFU/ml of phage was used. The MOI tested in this experiment was 100. The target bacteria and phages were quantified as described in Section 2.12. The experiment was done at 4°C and 25°C. The experiment was done in triplicates and repeated for five times.

2.13 Statistical Analysis

The significant differences of mean PFU/ml and mean CFU/ml obtained from chicken skin with different treatments were discriminated using analysis of variance and paired t-test. The analysis of variance is used to determine significant differences between the means of three and more independent groups of samples tested. While paired t-test was used to compare a sample group's score before and after an intervention. The confidence interval used for both were 95% and all data were analyzed with SPSS for Windows version 17.

3. RESULTS AND DISCUSSION

3.1 Host Specificity of the Phage

FM8-P1 and FM8-P2 were examined for their ability to infect other selected bacteria species (Table 1). The phages could not lyse other bacteria apart from the original host in which they were isolated from, including other VRE strains tested. Host specificity is an important aspect of phage therapy [13]. Most phages have narrow host range, such as MAK 745 against *V. cholera*, P100 against *L. monocytogenes* and ECP-100 against *E. coli* O157:H7 [14]. Similarly, the phages in this study also only recognized the host in which they were isolated from.

In phage therapy, only lytic phages are used as they are able to kill the bacteria at the end of the infection cycle. In the present study, both phages produced clear plaques and led to broth lysis within 3 h. This provided an indication on the high susceptibility of the bacteria host to the isolated phages which successfully lyzed the bacteria [15].

3.2 Bacteriolytic Activity

The efficiency of bacteria reduction and propagation of the phage is affected by the multiplicity of infection (MOI) used. MOI refers to the number of phage particles added to the bacteria per cell [16]. Optimum MOI was considered as MOI value at which the phage exhibit highest phage increment rate (ratio of total phage increment to time recorded) and host reduction rate (ratio of total host reduction to time recorded). Various times of highest increment of phage titer was recorded, suggesting the phages might have different times of adsorption. FM8-P1 and FM8-P2 have optimum MOI 0.01. The phages signified one particle of phage could lyse 100 host cells. Both phages exhibited the lowest MOI value, indicated the phages has the ability to lyse the host with higher density.

3.3 Single Step Growth Curve of Isolated Phages

The single step growth curves of both phages, FM8-P1 and FM8-P2 are presented in Fig 1. In the growth kinetic study which was done by single step growth experiment, FM8-P1 was found to have a longer latent period compared to FM8-P2, with bigger burst size. Fig. 1 (a) shows the FM8-P1 burst size (460 PFU/infected cell) was bigger than the average for tailed phage, which is 50-100 PFU/ infected cell [16]. Whereas,

FM8-P2 (Fig. 1(b)) has a shorter latent period (30 min) compared to the average for tailed phage (40-60 min) [17]. According to Ackermann [18] medians of latent period and burst size for tailed phages of *Caudovirales* are typically 40–60 min and 50–100, respectively, to be regarded as having effective lytic activity. Thus, both FM8-P1 and FM8-P2 are able to be considered as effective lytic phages.

The growth kinetic study also informs us the complete replication cycle of FM-P1 is 60 min (Fig. 1 (a)), while for FM8-P2 is 40 min (Fig. 1 (b)). In biocontrol and therapeutic use, phages with long lysis cycle are not preferred because it would delay the initiation of new infections [19]. A phage with short lysis time would be able to infect and eradicate more host cells in the environment earlier [18]. Thus, new progenies of phage can be produced rapidly and abundantly, which could then repeat the infection cycle [19].

3.4 Adsoption Rate of Isolated Phages

The phage specificity is conferred by its specificity of adsorption. This is dependent on the unique structural receptors on the bacterial cell surface, its localization on the cell surface, their amount and density at various cell wall sites [19,20]. The adsorption rate of the phage to the host was determined and both phages have slightly different adsorption rate. Both isolates have rather a rapid adsorption activity, in which 83% of FM8-P1 and 90% of FM8-P2 adsorb to the host within 2 min and 4 min, respectively (Fig. 2). As compared to ØfEF24C, phage against vancomycin resistant *E. faecalis*, the adsorption rate within 5 min was about 90% [21].

3.5 Determination of Optimum pH

The adsorption of the phage may be affected by physical-chemical factors, such as pH, temperature, presence of certain substances and ions in the medium, and also depending on the host physiological state and cultural conditions [16,22]. Thus, the activities of the phage isolates at different pH and temperature conditions were determined.

External factor such as acidity can inactivate a phage through damage of its structural elements (head, tail, envelope), lipid loss, and/or DNA structural changes [23]. Thus, it is important for us to determine optimum storage pH and suitable for the phage application to acquire maximum host elimination. The adsorption of FM8-P1 occurs at all tested pH with more than 1.0 log PFU/ml, except pH 11 (Fig. 3 (a)), as the host cannot withstand the alkaline condition up to that pH. At the end of 3 h infections, total elimination of host was observed at pH 4, 5, 6, 7, 8, 9, and 10 (Fig. 3 (b)). On the other hand, although FM8-P2's titer was reduced at pH 3, adsorption to host still occurred. The adsorption of phage took place at pH 4, 5, 6, 7, 8, and 9, with highest adsorption occurring at pH 8 with 1.55 log PFU/ml (Fig. 3 (a)). There was no significant difference of phage adsorbed with the initial titer of phage at pH 10 and 11, indicating no adsorption occurred at this tested environment. A significant host reduction occurred after 3 h infection at all pH tested, except pH 11. At pH 5, 6, 7, 8, 9 and 10, more than 6 log CFU/ml host was reduced, with total elimination at pH 7 (Fig. 3(d)).

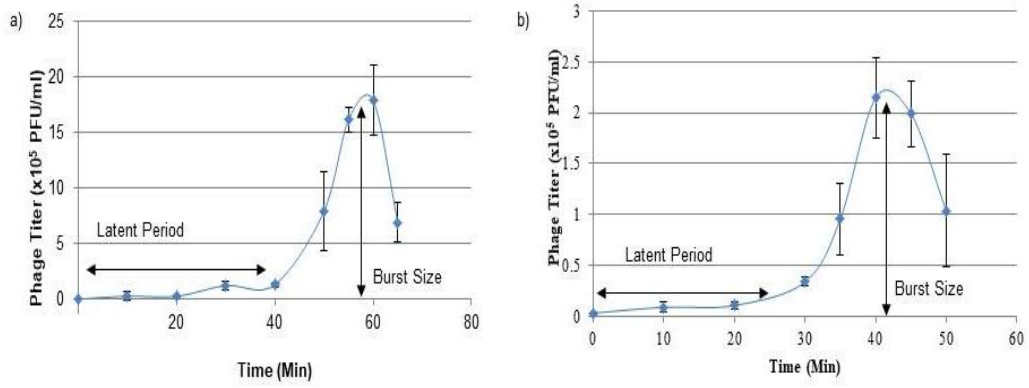

Fig. 1. Single step growth curves of (a) FM8-P1 and (b) FM8-P2
Values represent the mean ± SD of triplicate

Table 1. Growth condition of different indicator bacteria

Bacteria	Selective agar	Incubation temperature (°C)	Incubation period (h)
Enterococcus faecium FM3	Enterococcosel agar	37	24
Enterococcus faecium FM8	Enterococcosel agar	37	24
Enterococcus faecium FM9	Enterococcosel agar	37	24
Enterococcus faecium FM10	Enterococcosel agar	37	24
Enterococcus faecium FM11	Enterococcosel agar	37	24
Escherichia coli 0157:H7	MacConkey agar	37	24
Escherichia coli E113	MacConkey agar	37	24
Escherichia coli E30	MacConkey agar	37	24
Salmonella typhimurium S1000	LB agar	37	24
Salmonella typhimurium S1205	LB agar	37	24
Campylobacter jejuni	MCCDA	39	48
Staphylococcus epidermidis S168	MS agar	37	24
Klebsiella pneumonia K36	MacConkey agar	37	24
Enterobacter aerogenes ATCC 13048	EMB agar	37	48
Enterococcus feacalis E227	Enterococcosel agar	37	24
Proteus mirabilis P184	EMB agar	37	24
Proteus vulgaris P147	EMB agar	37	24
Lactobacillus reuteri C1	MRS agar	39	48
Lactobacillus reuteri C10	MRS agar	39	48
Lactobacillus reuteri C16	MRS agar	39	48
Lactobacillus gallinarum I16	MRS agar	39	48
Lactobacillus gallinarum I26	MRS agar	39	48
Lactobacillus brevis I12	MRS agar	39	48
Lactobacillus brevis I25	MRS agar	39	48
Lactobacillus brevis I218	MRS agar	39	48
Lactobacillus salivarus I24	MRS agar	39	48

LB, Luria-Bertani; MCCDA, Modified Charcoal Cefoperazone Deoxycholate Agar; MS, Manitol-Salt; EMB, Eosin Methylene Blue, MRS, Man Rogosa Sharpe

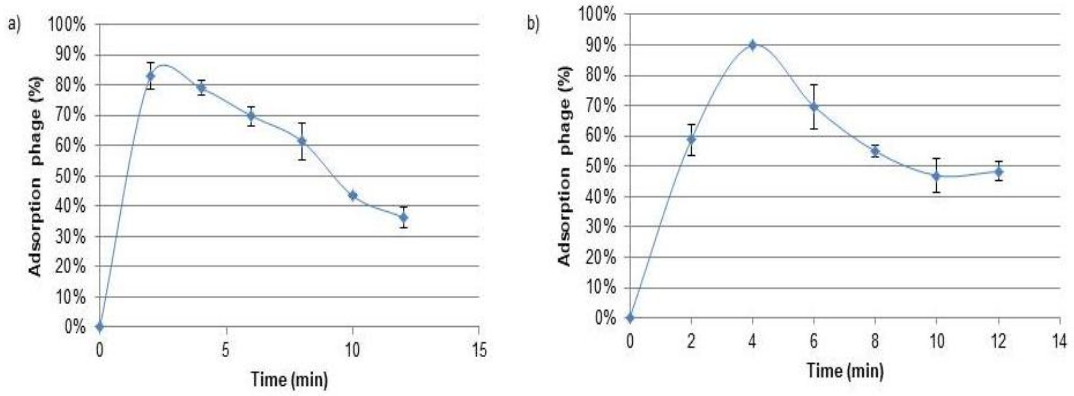

Fig. 2. Adsorption ability of phage of (a) FM8-P1 and (b) FM8-P2
Values represent the mean ± SD of triplicate

3.6 Determination of Optimum Temperature

Apart from pH, temperature plays an important role to determine the occurrence, viability, and storage of bacteriophages [24,25]. The determination of optimum temperature for phage activity was carried out to observe the effectiveness of phage adsorption to occur, survivability and lytic capability of the phage, under different temperatures. The result shows FM8-P1 was significantly adsorbed onto the host at 25°C and 37°C with 1.13 log PFU/ml and 1.18 log PFU/ml, respectively (Fig. 4 (a)). In the other

hand, the total reduction of host challenged with FM8-P2 was observed after 3 h infection at 25°C and 37°C. At 4°C, only a reduction of 1.1 log CFU/ml host was observed (Fig. 4(b)). Both phages were able to propagate and increase by approximately 3 log PFU/ml at 25°C and 37°C. FM8-P1 could lyse all hosts bacterial resulting with total elimination at both temperatures tested, while FM8-P2 reduce the host at approximately 4 log CFU/ml at 25°C and 6 log CFU/ml at 37°C. As for the phage, FM8-P2 was significantly adsorbed at 25°C and 37°C, with the highest adsorption of phage equivalent to 1.6 log PFU/ml at the later temperature (Fig. 4 (c)). The host reduction after 3 h FM8-P2 infection at 37°C was 2 log CFU/ml higher than 25 °C with a resultant reading of 6.4 log CFU/ml and 4.1 log CFU/ml respectively (Fig. 4(d)). Both phages show the ability to withstand 60°C.

Both phages also show the ability to resist 60°C temperature. The phages higher temperature tolerance than the host, suggests the viability of the phage in the environment could be prolonged [23,24]. However, at lower temperatures (4°C), both phages experienced no significant change in concentration, and the same occurrence was observed to the host. The temperature below optimum might effects the numbers of coalition among phage and host. Apart from that, low temperature also suggests the possibility of phage inactivation [25]. Thus, fewer phage genetic material penetrates into the bacterial host cell and fewer of them can be involved in the multiplication phase [25].

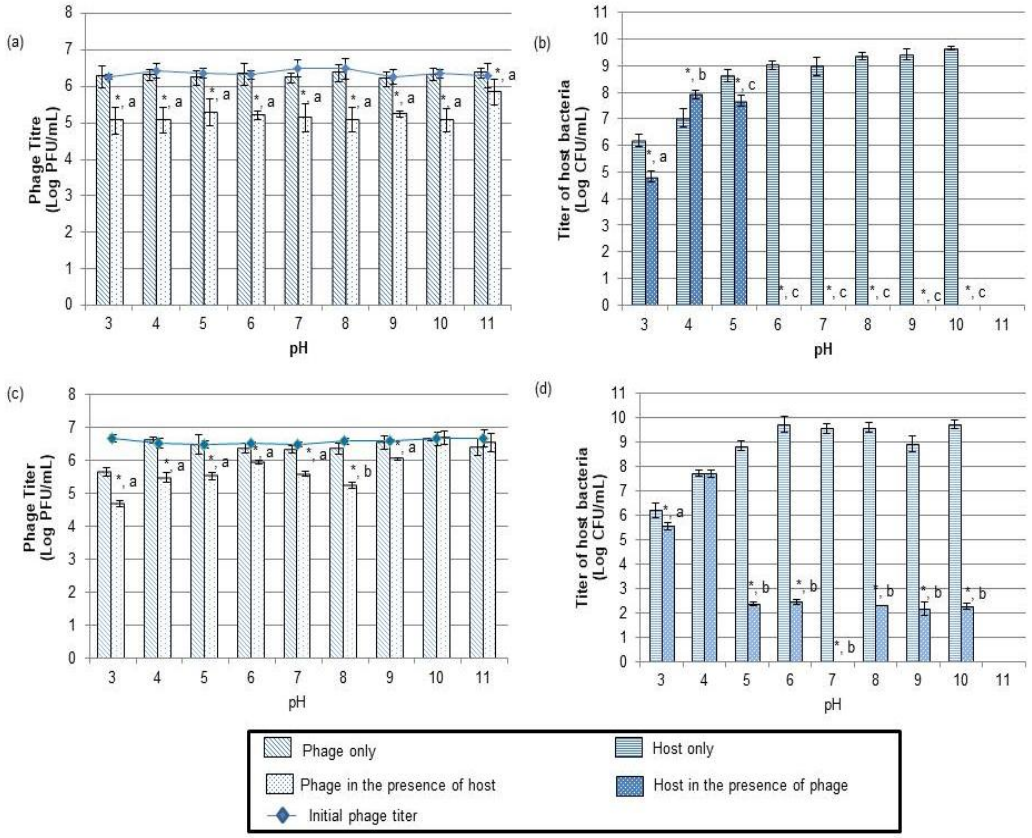

Fig. 3. Determination of optimum pH for lytic activity of the phage. (a) Titre of free FM8-P1 before and after 2 min incubation with host bacteria. (b) Titre of host bacteria after 3 h infection with FM8-P1. (c) Titre of free FM8-P2 before and after 2 min incubation with host bacteria. (d) Titre of host bacteria after 3 h infection with FM8-P2

** indicate significant difference (P = 0.05) from control group (phage or bacteria exposed to different pH). a-c Different superscripts indicated significant difference (P = 0.05) in reduction of phage titre of bacteria titre at different pH. Values represent the meant ± SD of triplicate*

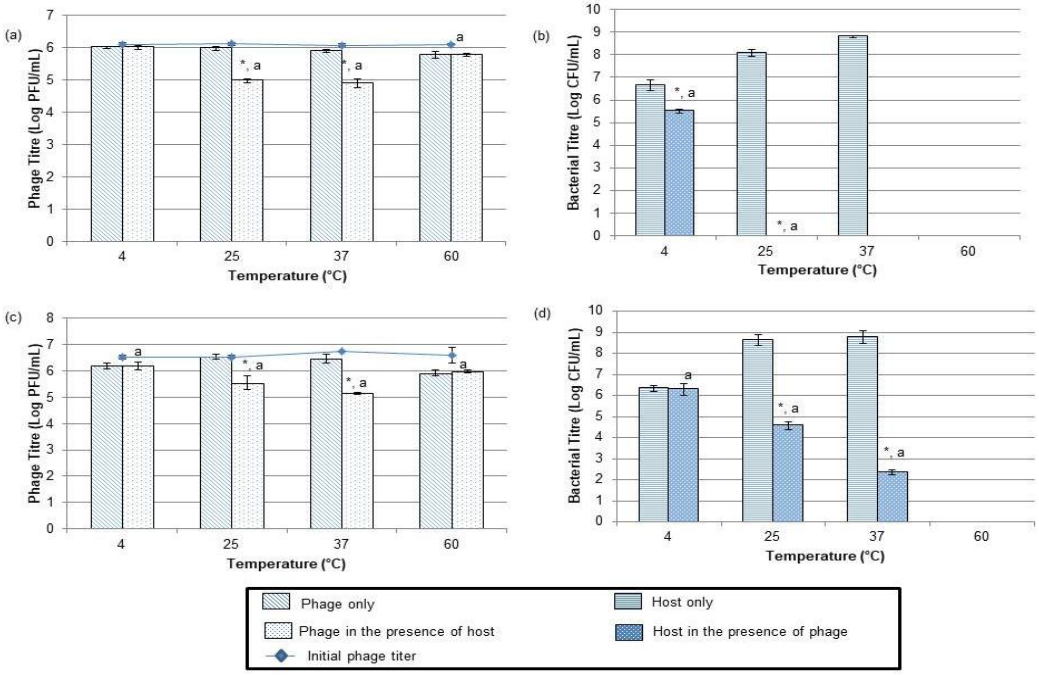

Fig. 4. Determination of optimum temperature for lytic activity of the phage. (a) Titre of free FM8-P1 before and after 4 min incubation with host bacteria. (b) Titre of host bacteria after 3 h infection with FM8-P1. (c) Titre of free FM8-P2 before and after 4 min incubation with host bacteria. (d) Titre of host bacteria after 3 h infection with FM8-P2

** indicate significant difference (P = 0.05) from control group (phage or bacteria exposed to different temperature). a-c Different superscripts indicated significant difference (P = 0.05) in reduction of phage titre of bacteria titre at different temperature.Values represent the meant ± SD of triplicate*

3.7 Efficiency of Phage Isloates to Reduce VREfm FM8 on Chicken Skin

3.7.1 Antibiotic resistance profile of VREfm FM8

VREfm FM8 was susceptible to different antibiotics at different levels. As referred to Table 1, growth of the bacteria was not observed at the concentration of 0.01 mg/ml and above for ampicillin and kanamycin. The growth of VREfm FM8 was inhibited at 0.1 mg/ml of methicillin, penicillin and streptomycin.

3.7.2 Presence of vancomycin resistant Enterococcus on chicken skin samples

From all 15 samples tested, all show no availability of the *Enterococcus* spp. that can withstand 32 µg/ml of vancomycin. Although there were *Enterococcus* spp. existences on the chicken skin, they are not resistant to vancomycin. The normal flora of the chicken skin in this study was found to be insusceptible to vancomycin above 32 µg/ml, while VREfm FM8, which contained the *van*A gene was recorded to have a MIC of 64 mg/ml [26]. This outcome assures, there is no *Enterococcus* spp. in the background that can disturb the result of subsequent tests. Thus, the effects of the phages on VREfm FM8 population can be determined by enumeration on Enterococcocel agar plate containing 32 µg/ml vancomycin. 32 µg/ml is MIC for mild vancomycin resistance in *Enterococcus* spp.

3.7.3 Elimination of phage to reduce VREfm FM8 load on chicken skin

The effectiveness of phage to eliminate target bacteria depends on several factors such as the phages over target bacteria ratio, the mode of treatment, and environmental conditions. Thus, it is essential to test the phages using a model in which the phage would be eventually applied on in order to measure its efficiency. In the present study, as the phages are intended to reduce VRE load on processed chickens, the isolated phages, FM8-P1 and FM8-P2, were tested on chicken

skin at 4°C and 25°C, using a high (MOI 100) and low (MOI 0.01) phage to bacteria ratio. Both of the temperatures were selected based on the condition of chiller storage in hypermarkets (4°C) and wet markets (25°C). At 4°C with a MOI 0.01, FM8-P1 was able to survive for 36 h, in the presence or absence of the host bacteria (Fig. 5 (a)). In the absence of host bacteria, the phage titer maintained at 3.4 – 2.9 log PFU/ml for 36 h as shown in Fig. 5 (a). In the presence of bacteria host, the phagetitre was significantly higher than the initial titer at 6-24 h, with the highest increment of phage (2 log PFU/ml) at 12 h. At a higher MOI (MOI 100), the phage titre of FM8-P1 at the end of the experiment was generally lower (3.6 log PFU/ml) than the initial titre (4.2 log PFU/ml) for all incubation period until 72 h (Fig. 5 (c)). In the presence of bacteria, the phage titer was generally lower than the initial titer in the absence of bacteria. However, the FM8-P1 experienced increments of 0.2 log PFU/ml (4.9 - 5.1 log PFU/ml) at 18 - 24 h, before it gradually decreased until 72 h (2.8 log PFU/ml).

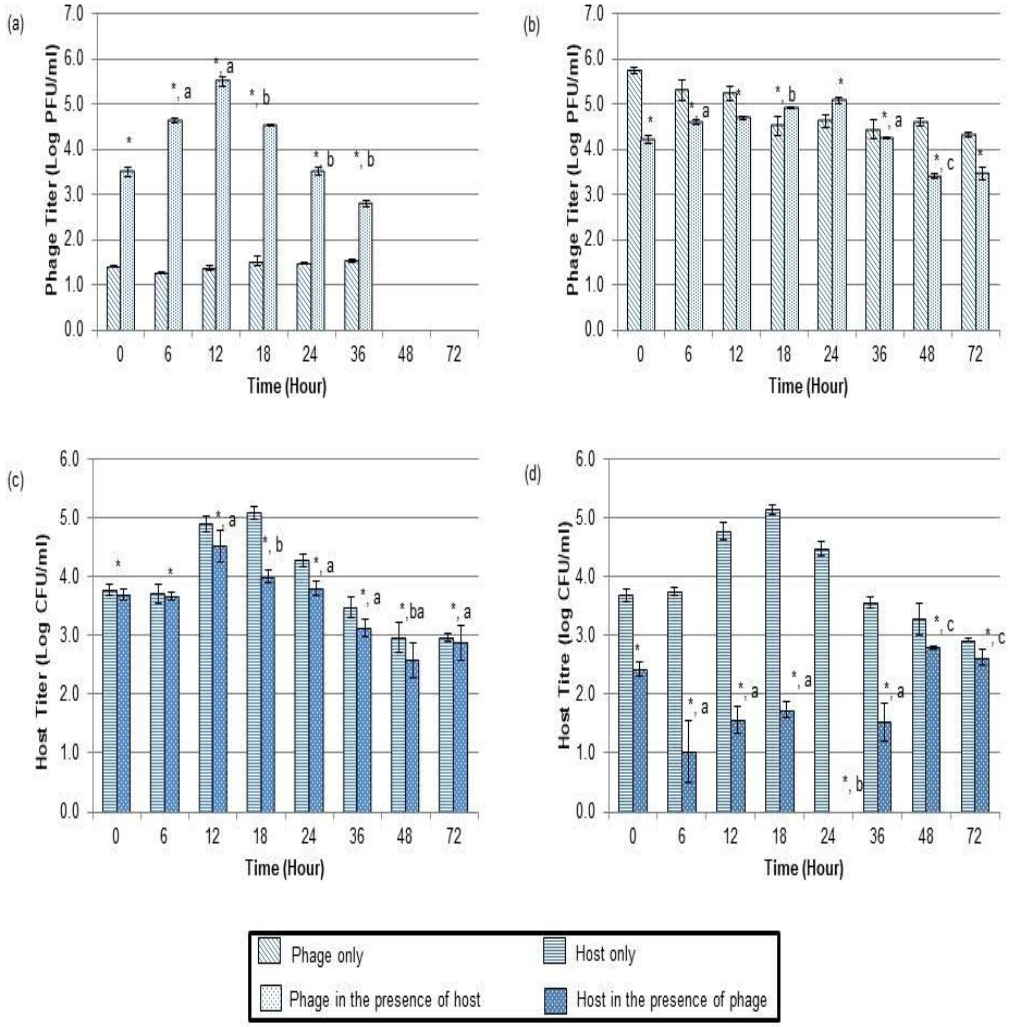

Fig. 5. FM8-P1 and host availability on chicken skin incubated at 4°C. Titer of the phage and host were taken at specific time intervals for four days. The experiment was done in triplicate. (a) Titer of unabsorbed FM8-P1 treated with low MOI (0.01). (b) Titer of unabsorbed FM8-P1 treated with high MOI (100). (c) Titer of free host when treated with FM8-P1 at MOI (0.01). (d) Titer of free host when treated with FM8-P1 at high MOI (0.01)

** Indicate significant difference (P = 0.05) from control group (phage or bacteria exposed along the time). a-b Different superscripts indicated significant difference (P = 0.05) in reduction (phage or bacteria titre at different time).Values represent the meant ± SD of triplicate*

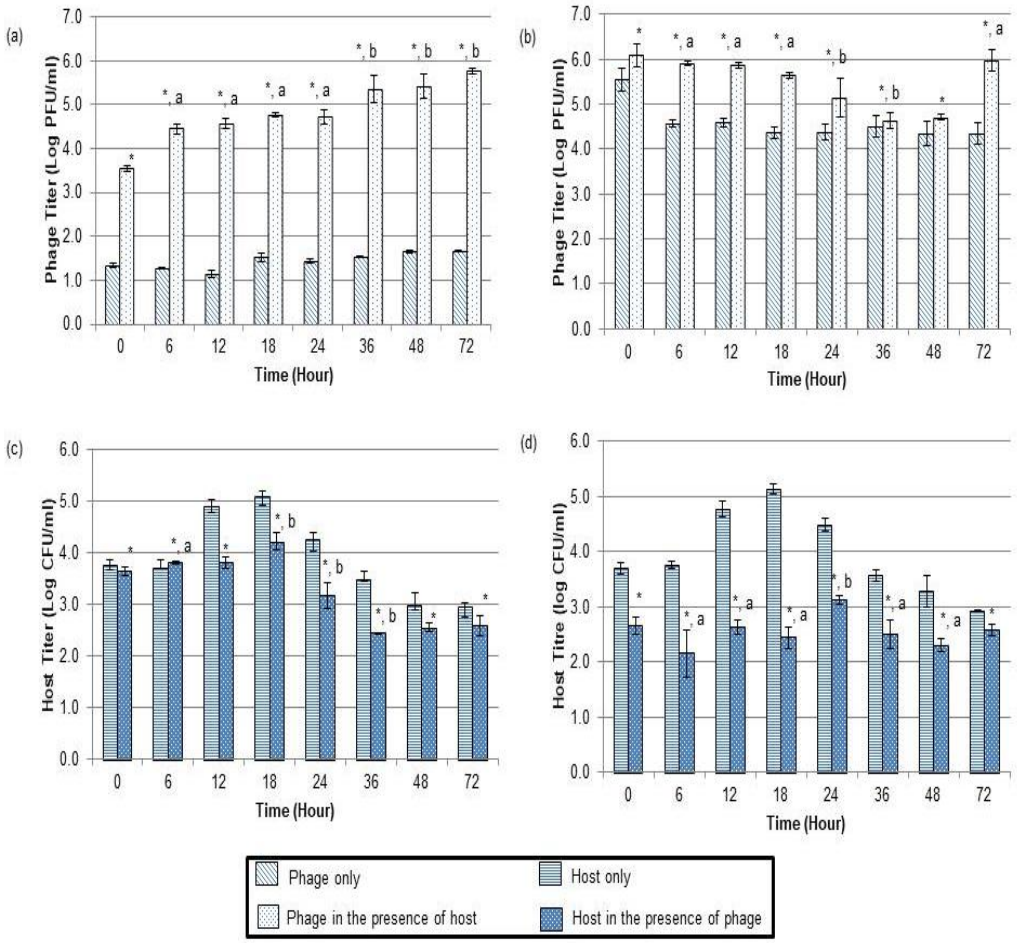

Fig. 6. FM8-P2 and host availability on chicken skin incubated at 4°C. Titer of the phage and host were taken at specific time intervals for four days. The experiment was done in triplicate. (a) Titer of unabsorbed FM8-P2 at low MOI (0.01). (b) Titer of unabsorbed FM8-P2 at high MOI (100). (c) Titer of free host when treated with FM8-P2 at MOI (0.01). (d) Titer of free host when treated with FM8-P2 at high MOI (0.01)

** Indicate significant difference (P = 0.05) from control group (phage or bacteria exposed along the time). a-b Different superscripts indicated significant difference (P = 0.05) in reduction (phage or bacteria titre at different time).Values represent the meant ± SD of triplicate*

The population of bacteria decreases throughout time in the absence of phage at 4°C (Fig. 5 (b)). The population of VREfm FM8 at this temperature when challenged with FM8-P1 at low MOI, increased from 3.6 log CFU/ ml at 0 h to 5.2 log CFU/ml at 18 h. Subsequently, the population was reduced to 2.9 log CFU/ml at 72 h. The same trend of bacteria growth was observed in the presence of phage except that the highest population of bacteria achieved was lower than in the absence of phage, which is 4.6 log CFU/ml at 12 h. FM8-P1 reduced the host by 2.9 log CFU/ml during the first 48 h, with total elimination at 24 h (Fig. 5 (d)). However, a regrowth of bacteria was observed subsequently.

On the other hand, FM8-P2 showed a different trend. The phage was found to be stable for at least 72 h in the absence of host (Fig. 6 (a)). In the presence of host, an increment of phage (5.8 log PFU/ml) was observed at the end of the experiment. As for bacteria host challenged with FM8-P2, a significant reduction (4.2 – 2.6 log CFU/ml) of VRE FM8 was observed at 12 - 48 h (Fig. 6 (c)). For FM8-P2, the phage titer maintained at 4.5 log PFU/ml for 6-72 h in the absence of host (Fig. 6 (c)). In the

presence of the host, the phage titer was significantly higher for all incubation period. The reduction of bacteria host was higher for both phages at higher MOI compared to the lower MOI. In the presence of FM8-P2, the VREf FM8 load was maintained at 2.6 – 3.1 log CFU/ml during the experimental period (Fig. 6 (d)). Highest reduction (2 log CFU/ml) of bacteria occurred at the 12th and 18th hour.

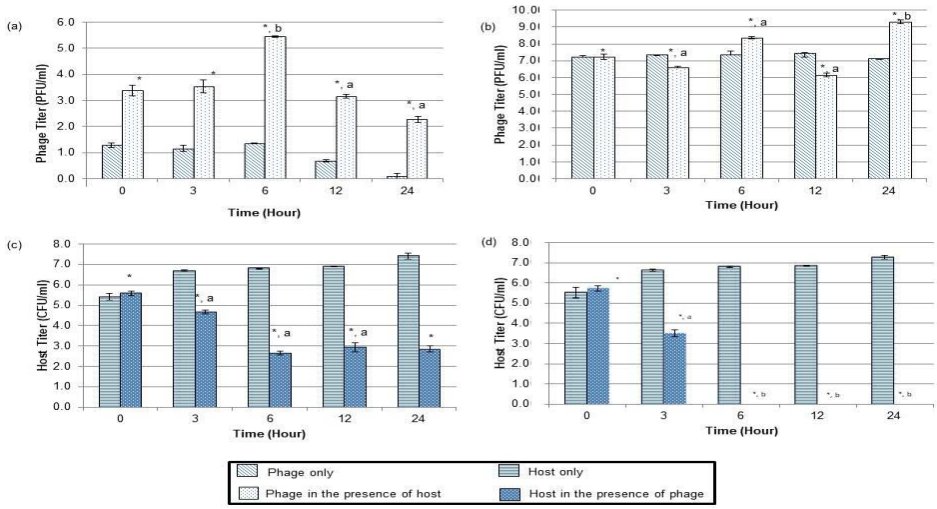

Fig. 7. FM8-P1 and host availability on chicken skin incubated at 25 °C. Titer of the phage and host were taken at specific time intervals for one day. The experiment was done in triplicate. (a) Titer of unabsorbed FM8-P1 at low MOI (0.01). (b) Titer of unabsorbed FM8-P1 at high MOI (100). (c) Titer of free host when treated with FM8-P1 at MOI (0.01). (d) Titer of free host when treated with FM8-P1 at high MOI (0.01)

** Indicate significant difference (P = 0.05) from control group (phage or bacteria exposed along the time).*
a-b Different superscripts indicated significant difference (P = 0.05) in reduction (phage or bacteria titre at different time).Values represent the meant ± SD of triplicate

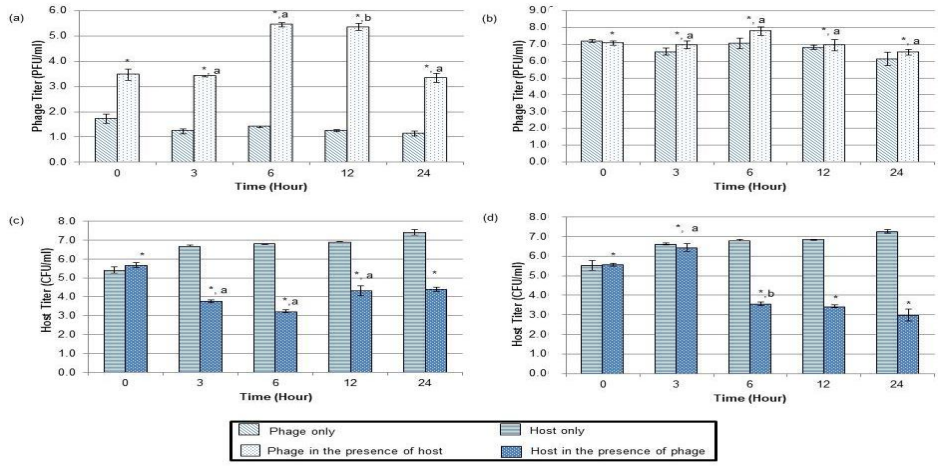

Fig. 8. FM8-P2 and host availability on chicken skin incubated at 25 °C. Titer of the phage and host were taken at specific time intervals for one day. The experiment was done in triplicate. (a) Titer of unabsorbed FM8-P2 at low MOI (0.01). (b) Titer of unabsorbed FM8-P2 at high MOI (100). (c) Titer of free host when treated with FM8-P2 at MOI (0.01). (d) Titer of free host when treated with FM8-P2 at high MOI (0.01)

** Indicate significant difference (P = 0.05) from control group (phage or bacteria exposed along the time).*
a-b Different superscripts indicated significant difference (P = 0.05) in reduction (phage or bacteria titre at different time).Values represent the meant ± SD of triplicate

At 25°C, with MOI 0.01, the phage FM8-P1 reduced gradually from 5.6 log PFU/ml to 2.8 log PFU/ml after 24 h incubation without bacteria host (Fig. 7 (a)). In the presence of bacteria host, the phage titer increased from 3.5 log PFU/ml to 5.5 log PFU/ml at 6 h but subsequently decreased to 2.2 log PFU/ml until 24 h (Fig. 7 (c)). During this period of time (6 - 24 h) the bacteria population was maintained at 2.8 log CFU/ml, in comparison to 7.4 log CFU/ml in the absence of phage. At MOI 100, phage titer of FM8-P1 was 2 log PFU/ml higher than the titer of phage in the absence of host at the end of 24 h (Fig. 7 (c)). Total elimination of the host bacteria occurred at 6-24 h (Fig. 7 (d)). No regrowth of the host is observed towards the end of the experiment.

For FM8-P2 challenged in 25°C, the phage maintained at 2 log PFU/ml throughout the experimental period in the absence of host (Fig. 8 (a). In the presence of the host, the phage titer increased 5.2 - 5.4 log PFU/ml at 6 - 12 h. At 6 h the bacteria reduced to 3.2 log CFU/ml in comparison to 6.8 log CFU/ml in the absence of phage (Fig. 8 (c)). As for FM8-P2, the phage titer was higher by 0.6 log PFU/ml at 6 h in comparison to the titer in the absence of host (Fig. 8 (c)). Furthermore, the bacteria host reduction was significant (2.0 log CFU/ml) at 6-24 h (Fig. 8 (d)).

4. CONCLUSION

Two isolated phages, FM8-P1 and FM8-P2, against vancomycin resistant *Enterococcus. faecium* strain FM8 (VREfm FM8) were physiologically characterized. The specificity and lytic activity of the phages for VREfm FM8 emphasized the potential used of FM8-P1 and FM8-P2 for inhibition of VRE in the environment. FM8-P1 and FM8-P2 had optimum lytic activity against VREfm FM8 at MOI of 0.01 in a bacteriolytic activity test. Both phages had stability in wide range pH and temperature, indicating that it can be stored and positively applied at various conditions. Thus, FM8-P1 and FM8-P2 are expected to be effectively used as biocontrol of VRE-contaminated chicken skin surface and to prevent threat caused by VRE infection. However, further studies about their genomic characteristic and antibacterial activity of these phages in situ will be required to expand its application.

ACKNOWLEDGEMENTS

To the memory of the late Assoc. Prof. Dr. Sieo Chin Chin. We would like to thank Ministry od Science and Technology, Malaysia for their National Science Fellowship that had funded study. We are also thankful to Dr. Zunita Zakaria from the Department of Veterinary Pathology & Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia for providing VRE bacterial strains to carry out our phage host range experiments.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Anonymous. Faderation of Livestock Farmers' Malaysia.
Available:http://www.flfam.org.my/images/Statistics/statistics_6_1ei.com
Accessed 11 November 2017
2. Driscoll TO, Crank CW. Vancomycin-resistant enterococcal infections : Epidemiology , clinical manifestations , and optimal management. Infection and Drug Resistance. 2015;8:217-230.
3. Ullah SR, Andleeb S, Raza T, Jamal M, Mehmood K. Effectiveness of a lytic phage SRG1 against vancomycin-resistant enterococcus faecalis in compost and soil. BioMed Research International. 2017; 2017:1-8.
4. Cattoir V, Leclercq R. Twenty-five years of shared life with vancomycin-resistant enterococci: Is it time to divorce? The Journal of Antimicrobial Chemotherapy. 2013;68(4):731–42.
5. Baldır G, Engin DÖ, Küçükercan M, İnan A. High-level resistance to aminoglycoside, vancomycin and linezolid in enterococci strains. Journal of Microbiology and Infectious Diseases. 2013;(3):100–103.
6. Willems RJL, Top J, Santen M. Van, Robinson DA, Coque TM, Baquero F, Bonten MJM. Global spread of vancomycin- resistant enterococcus faecium from distinct nosocomial genetic complex. Emerging Infectious Diseases. 2005;11(6):821–828.
7. Akhtar M, Viazis S, Diez-Gonzalez F. Isolation, identification and characterization of lytic, wide host range bacteriophages from waste effluents against *Salmonella enterica* serovars. Food Control. 2014;38:67–74.
8. Getachew Y, Hassan L, Zakaria Z, Abdul Aziz S. Genetic variability of vancomycin-

resistant enterococcus faecium and enterococcus faecalis isolates from humans, chickens, and pigs in Malaysia. Applied and Environmental Microbiology. 2013;79(15):4528–33.

9. Wang J, Woo M, Yan C. Spot plating assay for the determination of survival and plating efficiency of *Escherichia coli* in sub-MIC Levels of Antibiotics. 2017;1(June), 26–29.
10. Kropinski AM, Mazzocco,A, Waddell TE, Johnson RP. Chapter 7 Enumeration of Bacteriophages by Double Agar Overlay Plaque Assay. 2009;501:69–76.
11. Hyman P, Abedon ST. Bacteriophage host range and bacterial resistance. Advances in Applied Microbiology. 2010;70:217–48.
12. Hungaro MH, Célia R, Mendonça S, Meireles D, Cristina M, Vanetti D, Pinto DO. Use of bacteriophages to reduce Salmonella in chicken skin in comparison with chemical agents, Food Research International. 2013;52:75–81.
13. Cooper I. A review of current methods using bacteriophages in live animals, food and animal products intended for human consumption. Journal of Microbiological Methods. 2016;130:38-47.
14. Ly-Chatain MH. The factors affecting effectiveness of treatment in phages therapy. Frontiers in Microbiology. 2014;5: 51.
15. Cornelissen A, Ceyssens PJ, T'Syen J, Van Praet H, Noben JP, Shaburova OV, Lavigne R. The T7-related *Pseudomonas putida* phage φ15 displays virion-associated biofilm degradation properties. PloS One. 2011;6(4):e18597.
16. Parasion S, Kwiatek M, Mizak L, Gryko R, Bartoszcze M, Kocik J. Isolation and characterization of a novel bacteriophage φ4D lytic against *Enterococcus faecalis* strains. Current Microbiology. 2012;65(3): 284–9.
17. Abedon ST. Phage therapy dosing: The problem(s) with multiplicity of infection (MOI). Bacteriophage. 2016;6(3):1-5.
18. Ackermann, H.-W. 5500 Phages examined in the electron microscope. Archives of Virology. 2007;152(2):227–43.
19. Shao Y, Wang IN. Bacteriophage adsorption rate and optimal lysis time. Genetics. 2008;180(1):471–82.
20. Rakhuba DV, Kolomiets EI, Dey ES, Novik GI. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. Polish Journal of Microbiology. 2010;59(3):145–55.
21. Abedon ST, García P, Mullany P, Aminov R. Editorial: Phage therapy: Past, present and future. Frontiers in Microbiology. 2017; 8:1–7.
22. Uchiyama J, Takemura I, Hayashi I, Matsuzaki S, Satoh M, Ujihara T, Daibata M. Characterization of lytic enzyme open reading frame 9 (ORF9) derived from *Enterococcus faecalis* bacteriophage φEF24C. Applied and Environmental Microbiology. 2011;77(2):580–585.
    Available:https://doi.org/10.1128/AEM.01540-10
23. Zaburlin D, Quiberoni A, Mercanti D. Changes in environmental conditions modify infection kinetics of dairy phages. Food Environental Virology. 2017;9:270–276.
24. Jończyk E, Kłak M, Międzybrodzki R, Górski A. The influence of external factors on bacteriophages--review. Folia Microbiologica. 2011;56(3):191–200.
25. Schijven JF, Sadeghi G, Hassanizadeh S. M. Long-term inactivation of bacteriophage PRD1 as a function of temperature, pH, sodium and calcium concentration. Water Research. 2016;103:66–73.
26. Hassan L, Getachew YM, Zunita Z, Kamaruddin MI. Distribution of van genes of vancomycin-resistant enterococcus Isolated from Broilers in Peninsular Malaysia, Proceedings, The 15th Congress of FAVA -OIE Joint Symposium on Emerging Diseases. 2008;2–23.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/49654