Thesis progression

Thesis title: Phage genome characterization and effect of phage-antibiotic combination against *Burkholderia pseudomallei* in *ex vivo* and *in vivo*

Thesis progression title: Phage genome purification, hemolytic activity of phages and wound healing determination in mouse model

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1. Introduction

Burkholderia pseudomallei is a gram-negative bacilli found in soil and water throughout tropical regions, especially Southeast Asia and Northeast Thailand, causing melioidosis. Melioidosis is a serious infectious disease with a high mortality rate of 20-50% worldwide. Symptoms may present as a primary skin lesion, encephalitic illness with fever and seizures (Robert et al, 2020), and can spread to the pulmonary, blood or cause septicemia. *B. pseudomallei* is susceptible to many antimicrobial agents, and the recommended treatment for melioidosis includes ceftazidime or meropenem as intravenous therapies, and co-trimoxazole or doxycycline as oral therapy. In case of severe infectious disease, prolonged treatment for 2-6 months is recommended to prevent relapses (Wiersinga et al, 2018). The infections caused by *B. pseudomallei* are difficult to treat because of significant antibiotic resistance (Rhodes & Schweizer, 2016). A case report from India described a 39-year-old diabetic patient, particularly the development of ceftazidime resistance in *B. pseudomallei* infection (Behera, Prasad Babu, Kamalesh & Reddy, 2012). From previous concern of antibiotic-resistant bacteria have increased, leading to treatment failures. The alternative therapeutic use of bacteriophages has been more considered according to the raising prevalence of antibiotic-resistant bacteria.

Bacteriophages or phages are specific in killing bacteria, and there are numerous reports of their success. Interest in phage therapy has been renewed due to recent clinical successes in case studies involving personalized phage cocktails. Several clinical trials have been successful, and some trials are in progress. The recent progress in the therapeutic use of phages has led to contemplation of the key factors influencing the opportunities and challenges, with strong safety profiles (Hatfull, Dedrick & Schooley, 2022). Advantage of phages are significantly safe and tolerated, as they attach, replicate, and lyse only the target bacteria and some disadvantages of phage are virulence and antibiotic resistance gene should be transfer to the bacterial genome, that are the reason why phage genome analysis will be performed before using in animal model and clinical trials (Torres, 2018). The studies of single or cocktail phage therapy in animal models and clinical trial have been reported. For example, phage Abp1 and a four-phage cocktail against *Acinetobacter baumannii* infection in a mouse model effectively reduced bacterial load (Yin et al, 2017). Combination therapies involving antibiotics and phages may be valuable for treating multidrug-resistant bacteria. Both phages and antibiotics have the potential to treat bacterial infections (Osman et al, 2023). The combination of ceftazidime and phages have a synergistic effect to reducing bacterial population in a mouse wound model, according to a study of multidrug-resistant *Pseudomonas aeruginosa* infections (Engeman et al, 2021). In addition, topically administered bacteriophage treatment was suggested in chronic infection, especially in concurrence with wound surgery (Mendes et al, 2013).

Therefore, the aim of this study is to characterize and analyze phage genomes and the effects of the phage and ceftazidime combination against *B. pseudomallei* in pig skin and mouse wound models because no evidence of combination procedure against *B. pseudomallei* infection in various situations will be determined.

2. Objectives

1. To purify and measure the amount and purity of DNA in a phage.
2. To measure the A540 and calculate the percentage of hemolysis of phage.
3. To update the process of wound healing in mouse model.

3. Materials and methods

3.1 Phage titration by spot assay

Bacterial host strain P37 suspension was adjusted with phosphate-buffered saline (PBS, pH 7.4) containing 10^8 CFU/mL for pour onto $CaCl_2$-NA (Oxoid, UK), excess suspension was discarded, and left for surface dry. Phage lysates were diluted and spotted using 20 µL and incubated at 37°C for 18 hours. The presence of plaques (translucent spot) indicating bacterial cell lysis activity, was quantified as plaque forming unit (PFU/mL).

3.2 Phages genome extraction

Five phages were obtained from the Melioidosis Research Center (MRC) at the faculty of Medicine, Khon Kaen University (Table 1). All phages were propagated and concentrated (>10^9 PFU/mL) before DNA extraction by using polyethylene glycol 6000 (PEG6000). Briefly, the phage lysate was transferred into each a 15 mL centrifuge tube and added the filtered 20%-PEG6000 (Research Products International, USA) / NaCl (RCI Labscan, Thailand) solution to achieve a final concentration of 10% (for example, 5 mL of phage lysate solution and 5 mL of 20%-PEG6000/NaCl) and mixed by inverting 5-6 times. The lysate was cooled overnight at 4 °C. After cooling, 1 mL of the mixed solution will be transferred into a 1.5 mL microcentrifuge tube. The tubes were centrifuged at 15,000 *xg* at 4 °C for 45 minutes, and then supernatant was discarded, repeated until the mixed solution is emptied. Phage pellet was suspended in 100 µL of SM buffer and left overnight at room temperature. Then, SM buffer was covered the pellet and resuspend by carefully pipette up and down and left soak for 45 minutes. The solution was mixed and transferred into new 1.5 mL microcentrifuge tube. All phages were stored at 4 °C for phage DNA extraction.

Before extraction, contaminated bacterial DNA in 200 µL of phage lysate was eliminated by DNase I (New England Biolabs, UK) for 1 U, follow by incubation at 37 °C for 45 minutes, and stop reaction by incubated at 65°C for 10 minutes. Phage DNA was extracted

from previous lysate by the virus DNA/RNA kit (TIANamp Virus DNA/RNA Kit, Tiangen, Beijing, China) following the company's suggestions (TIANGEN Biotech, 2022). The types of nucleic acids were identified by 0.7% agarose gel in 1X TAE buffer and run at 70V for 40 minutes. Agarose gel containing DNA was stained by 0.5 ug/mL of ethidium bromide for 15 minutes. DNA sizes were visualized under UV light and DNA amount and purity were measured by NanoDrop.

Table 1 List of isolated phages of *B. pseudomallei*

No.	Phages	Family	Source	References
1	ST88	*Myoviridae*	Soil, Khon Kaen	(Yordpratum et al, 2011)
2	ST96	*Myoviridae*	Soil, Khon Kaen	(Yordpratum et al, 2011)
3	F4	*Podoviridae*	Soil, Songkhla	(Siriprayong, Wongratanacheewin & Yordpratum, 2021)
4	S4	*Myoviridae*	Soil, Khon Kaen	Unpublished
5	W12	*Myoviridae*	Water, Khon Kaen	Unpublished

3.3 Phages genome purification by Low-melting point (LMP) agarose gel

Five phage DNA samples from previous step were extracted only target band by low-melting point (LMP) agarose gel and were purified by phenol-chloroform method. Briefly, 50 ul of phage DNA were run through 1% LMP agarose gel (HiMedia®, India) in 1X TAE buffer at 70V for 40 minutes, cut the target band by sterile razor blades and weigh the gel for calculate volume of solution in the next step. The gel was contained by 1.5 µL microcentrifuge tube and add 5X volume of 1X TE buffer. The tube was incubated at 65 °C for 10-15 minutes, pending gel were melted, transfer the tube to 37°C. An equal volume of buffer saturated phenol (stored at 37°C) were added, mixed well, centrifuged at 10,000 *xg* for 5 minutes at room temperature and transfer upper aqueous layer to new tube. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) were added, mixed well, centrifuged at 10,000 *xg*

for 5 minutes at room temperature and transfer upper aqueous layer to new tube. An equal volume of chloroform:isoamyl alcohol (24:1) were added, mixed well, centrifuged at 10,000 *xg* for 5 minutes at room temperature and aliquot 400 µL of upper aqueous layer to new tube. Three molars of sodium acetate were added to final concentration of 0.3M and precipitate DNA with 2X volume of absolute ethanol at -20 °C for 18 hours. For pellet the DNA. The tube was centrifuged at 10,000 *xg* for 10 minutes at 4 °C and discard supernatant, add equal volume of 70% ethanol and centrifuged at 10,000 *xg* for 10 minutes at room temperature. Supernatant were removed and left the tube for dry the DNA pellet for 10-15 minutes. After that, DNA were resuspended with small volume of 1X TE buffer. The quality of target DNA were identified by 1% agarose gel in 1X TAE buffer and run at 70V for 40 minutes. Agarose gel containing DNA was stained by 0.5 ug/mL of ethidium bromide for 15 minutes. DNA sizes were visualized under UV light and DNA amount and purity were measured by NanoDrop.

3.4 Hemolysis assay

Hemolytic properties determination is a common method for preliminary cytotoxicity evaluation of drugs, chemicals, compounds or any blood-contacting device (Sæbø et al, 2023). If the compounds or sample causes hemolysis, hemoglobin from erythrocyte will be release to supernatant and determine hemolytic activity by spectrophotometer.

Forty milliliter of sheep blood were centrifuged at 2,000 rpm for 10 minutes at 25 °C and remove supernatant. Pellet of erythrocyte was washed with 5 mL of PBS buffer (pH 7.4) and centrifuged at 3,000 rpm for 5 minutes at 25 °C and remove supernatant for 3 times. Erythrocyte from washing step were diluted for 20%(V/V) with PBS buffer (pH 7.4).

Five-hundred microliters of 20%(V/V) erythrocyte and 500 µL of sample (5 phages including ST96, ST88, W12, S4 and F4 in high ($6x10^7$-$2.5x10^8$ PFU/mL) and low ($1x10^5$ PFU/mL) concentration, positive control; 10% Triton-X and negative control; PBS buffer (pH 7.4)) were mixed in 1.5 microcentrifuge tube and incubated at 100 rpm for 1 hours at 37 °C. After incubation, the mixed were centrifuged at 3,000 rpm for 10 minutes at 25 °C. The degree of

hemolysis was measured in supernatant by plate reader to provide absorption values and the percentage of hemolysis were calculated as below formula.

$$\text{Hemolysis (\%)} = \frac{A_{sample} - A_{neg}}{A_{pos} - A_{neg}} x100$$

3.5 Wound healing determination process in mouse model

The experiments will be conducted on BALB/C mouse that are free from specific pathogens and bred under controlled conditions. The 6-8 weeks female BALB/C mouse (Guang-Han et al, 2016) will be used in this study. BALB/C mouse preparation will be followed Rhea & Dunnwald (2020). The research involving the animals will be carried out with the necessary approval before.

To examine the antimicrobial effect of the phage-CAZ combination in *B. pseudomallei*-infected onto wound in a mouse model with the best condition from pig skin treatment experiment, this experiment followed by in the study of Rhea and Dunnwald (2020) and Piranaghl et al. (2022) with slight modifications. First, thirty of 6-week-old mice will be anaesthetized by thiopental sodium 30-40 mg/kg via IV and then the back hair in the dorsal areas will be removed. A wound will be formed, a diameter of 6 mm on the dorsal area of the mouse (n=2) using biopsy punch and sterile forceps. The mice will be separated into five groups, each comprised of 6 mouse/group, will be employed for this study. Group 1, ten microliter of SM buffer will be applied onto wound (positive control). Mid-log *B. pseudomallei* strain B110 ($1x10^5$ CFU/mL) will be inoculated onto wound of 4 groups for 2 hours before applying the treatment. The group 2-5, mice will be applied of 10 µL of SM buffer, 10 µL of CAZ (1/2MIC), 10 µL of 10^7 PFU/mL of phage S4 and 10 µL 10^7 PFU/mL of phage S4+CAZ (1/2MIC), respectively. A swab for all groups was taken to determine the bacterial count presenting the wound on the day 1 ,3, 7, 10, and 14 days post-infection. Additionally, the wound will be rated and photographed via a camera by veterinarian. The wound area will be estimated using a digital caliper, and the percentage wound healing rate will be calculated according to below formula.

$$\text{Wound healing (\%)} = [\, 1 - (\, \text{Wound area on given day} / \text{Wound area on day 0}\,)\,] \times 100$$

On day 28, the mouses will be sacrificed Thiopental sodium (overdose) 120 mg/kg via IP, followed by the removal of the tissue from the wound bed to assess the wound healing. Blood will be collected on day 2 via retro-orbital. Lung, liver and spleen will be obtained aseptically and homogenized in sterile PBS using a 100 µm cell strainer. (Conejero et al, 2011; Reimi & Kinoshita, 2008). Homogenized tissue solution or blood will be inoculated in 10-fold serial dilutions onto Ashdown's agar for 48-72 hours and $CaCl_2$-NA with *B. pseudomallei* for 18 hours, the culture plate will be incubated at 37°C. Bacterial burden and phage titer in the lung, liver and spleen of the mouse will be determined.

4. Results

4.1 Phages genome extraction and purification

Phages DNA amount, and purity was shown in Figure 1 and Table 2. The results showed that ST88 and W12 phage DNA were purified from the gel, the purity more than not purity, length approximately 23,130 bp and another phage DNA are lost after purify process (Figure 3). From previous results, ST96, F4, S4 phage DNA cannot measure phage DNA target concentration and quality.

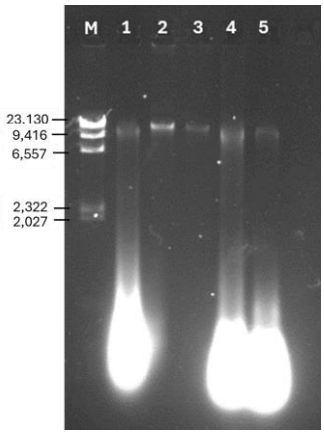

Figure 1 DNA size of phages in 0.7% agarose gel. M, DNA marker; lane 1, ST96 phage DNA; lane 2, ST88 phage DNA; lane 3, W12 phage DNA; lane 4, F4 phage DNA; lane 5, S4 phage DNA.

Table 2 Nanodrop measurement of DNA extracted from phages.

Phages DNA	Date (extract)	A260	A280	A230	A260/280	A260/230	Concentration (ng/µL)
ST96_DNA	20/03/2024	26.183	11.974	12.235	2.19	2.14	1309.1
ST88_Pure	26/04/2024	0.427	0.249	0.220	1.72	1.94	21.3
W12_Pure	30/03/2024	0.584	0.357	0.334	1.64	1.80	29.2
F4_DNA	08/06/2024	41.660	19.322	17.803	2.16	2.34	2083
S4_DNA	02/07/2024	55.699	25.959	23.4595	2.14	2.37	2779.9

4.2 Hemolytic activity of phages

Visualization of hemolytic activity in microtube, absorption measurement and percentage of hemolysis was shown in Figure 2 and 3.

The result shown that, the visualize show the most hemolysis present in S4 phage with high concentration. After incubation, the degree of hemolysis was measured in supernatant and calculated the percentage of hemolysis. The result showed that S4 phage in high concentration present high A540 values and %hemolysis for 0.2244 and 4.534%, respectively. And other condition shows a low A540 values less than 0.05.

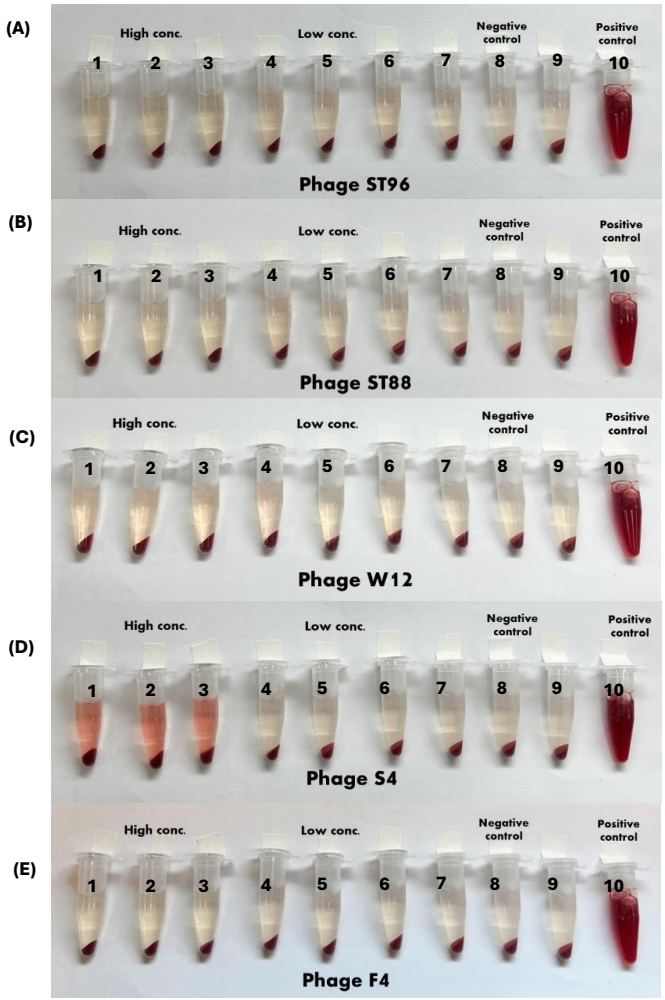

Figure 2 Visualization of hemolytic activity of ST96 phage (**A**), ST88 phage (**B**), W12 phage (**C**), S4 phage (**D**), and F4 phage (**E**). Tube 1-3=high concentration, 4-6= low concentration, 7-9= negative control (PBS buffer), 10=positive control (Triton-X)

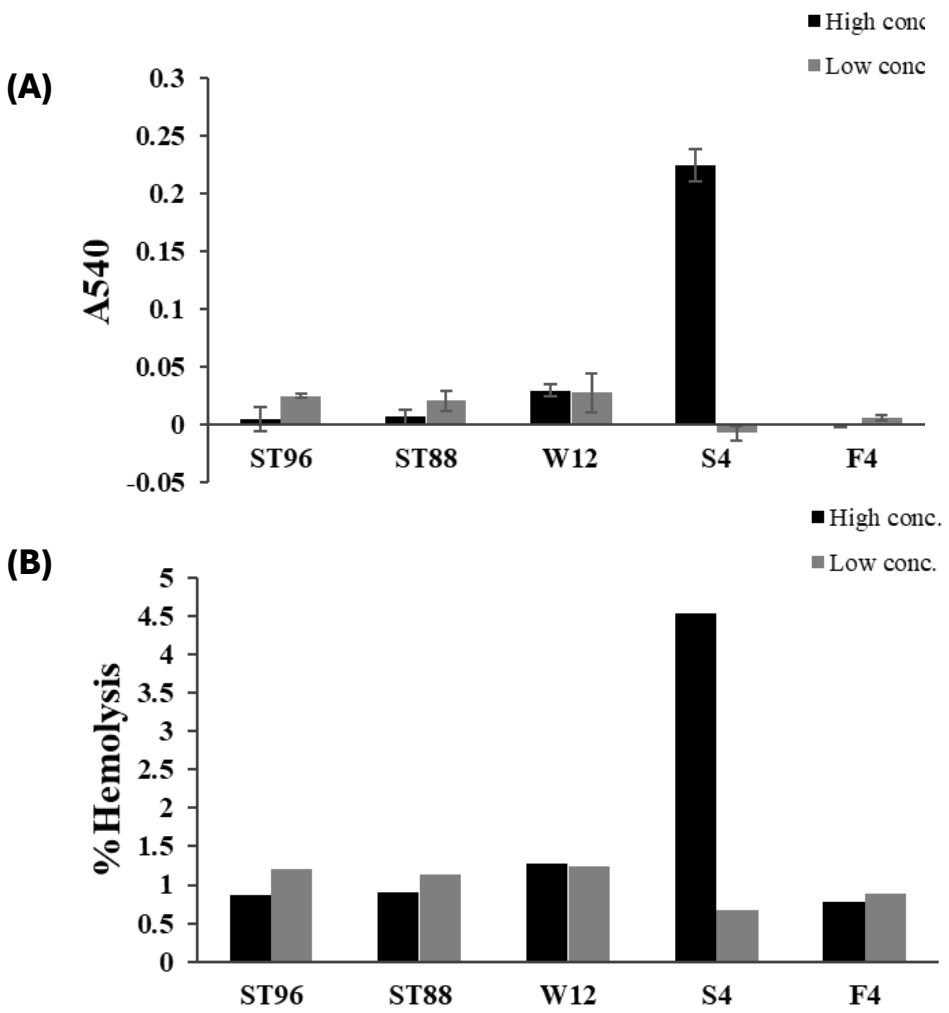

Figure 3 Absorption measurement at 540 nm (Y-axis) (**A**) and percentage (Y-axis) (**B**) of free hemoglobin in a 10% erythrocyte from high and low concentration of 5 phages**.**

5. Conclusion

From above results, the results showed that DNA of ST88 and W12 phage can measure a concentration but ST96, S4 and F4 cannot measure because target band cannot extract from the gel. For solve previous problem, phage DNA with degraded DNA will be measured by Qubit fluorometer that have a high accuracy and specificity for target DNA for reduce time and cost. The results of hemolytic activity show S4 has highest A540 values and percentage of hemolysis. And mouse model project, currently, the project is in the process of editing documents for project approval.

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