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 precipitation for genome extraction and recovery of phage from phage and ceftazidime combination

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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. Objective

1. To increase phage concentration for high yield extracted (>10^9 PFU/mL).
2. To extract and measure the amount and purity of DNA in a phage.
3. To determine stability of phage in phage and ceftazidime combination.

3. Materials and method

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 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.

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 Recovery of phage from phage/CAZ combination

One-hundred microliters of phage lysate solution (final concentration 10^6 PFU/mL) and 100 µL of CAZ (Sigma-Aldrich, Germany) (1/2MIC) or 100 µL of CAZ (1/2MIC) were mixed in a 1.5 mL microcentrifuge tube. This mixture was incubated at 37 ℃, room temperature and 4 ℃ for 4 hours. After incubation, phage stability number was determined by a spot assay.

4. Results

4.1 Phages genome extraction

(1) Phage precipitation and concentration by PEG6000

Phages titer after precipitation by PEG6000 was shown in Table 2. The result showed that only F4 phage was concentrated with PEG6000, while others were not increased concentration and still all gone.

Table 2 Phages titer after precipitation

Phages	Phage titer (PFU/mL)	
	Before precipitation	After precipitation
ST96	7.25×10^6	-
ST88	2×10^7	-
W12	2×10^6	-
S4	7.25×10^7	-
F4	3×10^8	7.5×10^9

(2) Length, amount, and purity of DNA extracted from phages

Phages DNA length, amount, and purity was shown in Figure 1 and Table 3. The results showed that F4 phage DNA length approximately 23,130 bp and another phage DNA not being noticeable (Figure 1). F4 phage DNA present highest yield for 426.4 ng/ µL but all of phage DNA was shown A260/280 ratio of >1.8, that is not pure for DNA.

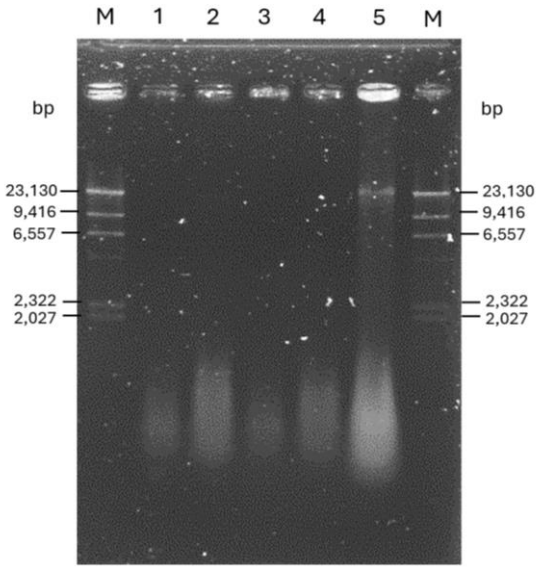

Figure 1 DNA size of phages in 1% agarose gel. M, DNA marker; lane 1, ST96 phage DNA; lane 2, ST88 phage DNA; lane 3, W12 phage DNA; lane 4, S4 phage DNA; lane 5, F4 phage DNA.

Table 3 Nanodrop measurement of DNA extracted from phages.

Phages	Phage titer (PFU/mL)	A260	A280	A260/280	Concentration (ng/µL)
ST96	3×10^8	1.913	0.667	2.87	95.7
ST88	1.1×10^9	2.473	0.935	2.65	123.7
W12	2×10^8	1.917	0.667	2.87	95.8
S4	1.2×10^9	2.606	0.920	2.83	130.3
F4	7.5×10^9	8.529	3.748	2.28	426.4

4.2 Recovery of phage from phage/CAZ combination

Phage S4 and CT (cocktail) was tested for antibiotic stability with CAZ concentration of 1/2MIC (64 µg/mL) and 1/4MIC (32 ug/mL) in 37 ℃, room temperature, and 4 ℃ and was found to not be different between mixture in each temperature but if that was used high concentration of CAZ, phage will be slightly lost when compared with low concentration (Figure 2).

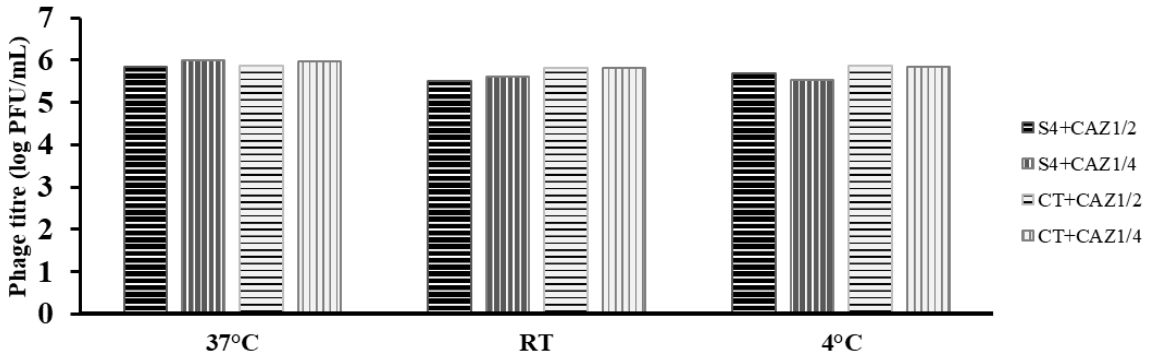

Figure 2 S4 and CT phage titer from phage combine with CAZ in different temperatures.

5. Conclusion

From previous results, the results showed that precipitation and concentration by PEG6000 can use for F4 phage only, high concentration of phage that effective to present band of DNA in agarose gel electrophoresis, and S4 and CT phage are stable with CAZ which could be suitable for treatment. In future work, phage will be propagated via liquid lysate method and phage DNA will be extract via phenol-chloroform method.

6. References

Behera, B., Prasad Babu, T.L., Kamalesh, A., & Reddy, G. (2012). Ceftazidime resistance in *Burkholderia pseudomallei*: first report from India. **Asian Pacific journal of tropical medicine**, 5(4), 329-330.

Engeman, E., Freyberger, H.R., Corey, B.W., Ward, A.M., He, Y., Nikolich, M.P., et al. (2021). Synergistic Killing and Re-Sensitization of *Pseudomonas aeruginosa* to Antibiotics by Phage-Antibiotic Combination Treatment. **Pharmaceutical**, 14, 184.

Hatfull, G.F., Dedrick, R.M., & Schooley, R.T. (2022). Phage Therapy for Antibiotic-Resistant Bacterial Infections. **Annual review of medicine**, 73, 197-211.

Mendes, J. J., Leandro, C., Corte-Real, S., Barbosa, R., Cavaco-Silva, P., Melo-Cristino, J., Górski, A., & Garcia, M. (2013). Wound healing potential of topical bacteriophage therapy on diabetic cutaneous wounds. **Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society**, 21(4), 595-603.

Osman, A.H., Fleischer, C.N., Kotey, A.O., Samuel, D., Raphael, K.Y., Nicholas, T.K.D.D., & Eric S.D. (2023). The Potential of Bacteriophage-Antibiotic Combination Therapy in Treating Infections with Multidrug-Resistant Bacteria. **Antibiotics**, 12(8), 1329.

Rhodes, K.A., & Schweizer, H.P. (2016). Antibiotic resistance in *Burkholderia* species. **Drug resistance updates: reviews and commentaries in antimicrobial and anticancer chemotherapy**, 28, 82-90.

Robert, M., Joseph, W., Nathan, J., Samir, S., Robert, C., & Karen, M. (2020). *Pseudomonas*, *Burkholderia*, and *Stenotrophomonas*. **Nelson Textbook of Pediatrics**, Chapter 232, 1529-1533.e2.

Siriprayong, A., Wongratanacheewin, R., & Yordpratum, U. (2021). Characterization of novel phage F4 with lytic activity against *Burkholderia pseudomallei* in vitro. Paper presented at the 3rd National and International Virtual Conference on Multidisciplinary Research, Manisa Celal Bayar University, Turkey.

TIANGEN Biotech. (2022). **TIANamp Virus DNA/RNA Kit manual**. Retrieved Sep 28, 2023, from https://en.tiangen.com/upload/file/20220509/20220509170045_59351.

Torres, B.C. (2018). Phage Therapy Faces Evolutionary Challenges. **Viruses**, 10(6), 323.

Wiersinga, W.J., Virk, H.S., Torres, A.G., Currie, B.J., Peacock, S.J., Dance, D.A.B., et al. (2018). Melioidosis. Nature reviews. **Disease primers**, 4, 17107.

Yin, S., Huang, G., Zhang, Y., Jiang, B., Yang, Z., Dong, Z., et al. (2017). Phage Abp1 Rescues Human Cells and Mice from Infection by Pan-Drug Resistant *Acinetobacter Baumannii*. **Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology**, 44(6), 2337-2345.

Yordpratum, U., Tattawasart, U., Wongratanacheewin, S., & Sermswan, R.W. (2011). Novel lytic bacteriophages from soil that lyse *Burkholderia pseudomallei*. **FEMS Microbiology Letters**, 314(1), 81-88.