ISOLATION IDENTIFICATION AND CHARACTERIZATION OF BACTERIOPHAGE SPECIFIC TO ANTIBIOTICS RESISTANT BACTERIA

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TITLE ISOLATION IDENTIFICATION AND CHARACTERIZATION OF BACTERIOPHAGE SPECIFIC TO ANTIBIOTICS RESISTANT BACTERIA

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บทคัดย่อ

เรื่อง : การคัดแยกจำแนกและศึกษาคุณลักษณะของแบคเทอริโอเฟจที่จำเพาะต่อเชื้อแบคทีเรียดื้อยา

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คำสำคัญ : *Pseudomonas aeruginosa*, bacteriophage, multidrug resistant bacteria, Bacteriophage therapy

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อคัดแยกและศึกษาคุณสมบัติของแบคเทอริโอเฟจที่จำเพาะต่อเชื้อ *Pseudomonas aeruginosa* สายพันธุ์ดื้อยาหลายชนิด ผลการศึกษาพบว่าสามารถคัดแยกแบคเทอริโอเฟจ WJ9 (phageWJ9) ได้จากโรงบำบัดน้ำเสียโรงพยาบาลสรรพสิทธิประสงค์ จังหวัดอุบลราชธานีโดย phageWJ9 มีความจำเพาะต่อเชื้อ *P. aeruginosa* สายพันธุ์ดื้อยาหลายชนิดที่คัดแยกจากผู้ป่วยในโรงพยาบาลสรรพสิทธิประสงค์ การศึกษาคุณสมบัติของ phageWJ9 ได้แก่ การศึกษาเซลล์แบคทีเรียเจ้าบ้าน การทนต่อสารละลายเกลือและแอลกอฮอล์ คุณสมบัติของสารพันธุกรรม และการศึกษาสัณฐานวิทยา การศึกษาพบว่า phageWJ9 มีความจำเพาะต่อแบคทีเรีย *P. aeruginosa* สูง โดยมีค่าร้อยละเท่ากับ 70% (7/10) ของจำนวนเชื้อ *P. aeruginosa* ทดสอบทั้งหมด นอกจากนี้ phageWJ9 ไม่สามารถทำลายแบคทีเรีย *Escherichia coli* ATCC 25922 *Enterobacter* sp. *Klebsiella pneumonia Salmonella typhimurium* DMSc 5784 *Shigella dysenteriae* DMSc 2137 *Staphylococcus aureus* ATCC 25923 *Staphylococcus epidermidis* และ *Bacillus subtilis* การทดสอบการทนต่อสารละลายเกลือและแอลกอฮอล์ พบว่า phageWJ9 สามารถอยู่รอดได้ในสารละลายน้ำเกลือเป็นเวลา 30 นาที การศึกษาคุณสมบัติสารพันธุกรรมพบว่า phageWJ9 มีสารพันธุกรรมเป็น double-stranded DNA ทั้งนี้สารพันธุกรรมถูกย่อยได้ด้วยเอนไซม์ตัดจำเพาะ *Hin*dIII และ *Nco*I และเอนไซม์ DNase แต่ไม่ถูกย่อยด้วย RNase A การศึกษารูปร่างของแบคเทอริโอเฟจด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่าน พบว่า phageWJ9

มีลักษณะส่วนหัวเป็น icosahedral ขนาดเส้นผ่าศูนย์กลางประมาณ 83 นาโนเมตร ส่วนหางยาวประมาณ 200 นาโนเมตร เมื่อพิจารณารูปร่างและชนิดสารพันธุกรรมของ phageWJ9 พบว่าจัดอยู่ในวงศ์ *Myoviridae* ออร์เดอร์ *Caudovirales* ดังนั้นแบคเทอริโอเฟจที่คัดแยกได้จากการศึกษาครั้งนี้มีคุณสมบัติที่ดีและน่าสนใจนำไปศึกษาในขั้นสูงต่อไป เช่น การทดสอบการยับยั้งเชื้อ *P. aeruginosa* ในเซลล์เพาะเลี้ยง สัตว์ทดลอง และรักษาแผลติดเชื้อ *P. aeruginosa* ในผู้ป่วยต่อไป

ABSTRACT

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KEYWORDS : *PSEUDOMONAS AERUGINOSA*, BACTERIOPHAGE, MULTIDRUG RESISTANCE BACTERIA, BACTERIOPHAGE THERAPY

This study aimed to isolate and characterize bacteriophage specific to the multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains. The result found that the bacteriophage named WJ9 (phageWJ9) could be isolated from the wastewater treatment plant of Sanprasitthiprasong Hospital, Ubon Ratchathani Province. The phageWJ9 was highly specific to bacteria MDR *Pseudomonas aeruginosa*. The strains were isolated from patients who were hospitalized at Sanpasitiprasong Hospital. Bacteriophage properties that were thoroughly investigated in this study include determination of bacteriophage's host range, stability in ethanol and normal saline solution, bacteriophage genome analysis and bacteriophage morphology. The results showed that phageWJ9 was highly specific to *P. aeruginosa* at percentage of 70 (7/10) of all strains tested. However, other tested bacteria which include *Escherichia coli* ATCC 25922, *Enterobacter* sp, *Klebsiella pneumonia*, *Salmonella typhimurium* DMSc5784, *Shigella dysenteriae* DMSc2137, *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* and *Bacillus subtilis* were not killed by phageWJ9. Stability in ethanol and normal saline solution demonstrated that phageWJ9 was able to survive after incubating in 0.85 % normal saline for 30 minutes but was not

tolerated in the ethanol solution. Genome analysis revealed that phageWJ9's genome consisted of a double-stranded alcohol DNA because it was digested by restriction enzyme *Hind*III and *Nco*I and DNase enzyme, but it was not digested by RNase A. Study of phage morphology by transmission electron microscopy revealed that the phage had an icosahedral head (83nm in diameter) and a long contractile tail (200nm in length). Thus, based on phage genome and morphology, phageWJ9 can be categorized in the family *Myoviridae* of the order *Caudovirales*. In conclusion, the highly specific bacteriophage to MDR *Pseudomonas aeruginosa* had good properties and should be considered for further advanced study such as determination of the *P. aeruginosa* growth inhibition in cell line culture, animal models, and application for wound healing of *P. aeruginosa* in patients with infectious diseases.

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LIST OF ABBREVIATIONS

CHARACTER	MEANING
CFU	Colony forming unit
°C	Degree Celsius
DNA	Deoxyribonucleic acid
g	Gram
h	Hours
Kb	Kilobase pairs
µg	Microgram
µL	Microliter
µm	Micrometer
mL	Milliliter
mm	Millimeter
min	Minute
PFU	Plaque forming unit
/	Per
%	Percent
rpm	Revolutions per minute
RNA	Ribonucleic acid
TEM	Transmission electron microscope
×*g*	Times gravi
SDS	Sodium dodecyl sulfate
EDTA	Ethylenediaminetetraacetic acid
TAE	Tris-acetate

CHAPTER 1
INTRODUCTION

1.1 Background and rational of the study

Currently, the problem of antibiotic resistance bacteria has an affect worldwide. It was found that treatments of bacterial infectious diseases by antibiotics are less effective, especially tuberculosis, pneumonia and gonorrhea (Hanlon, 2007). The mechanisms of antibiotic resistance in these bacteria include, enzyme formation degrades the drug structure in β-lactams and aminoglycoside group (Ramirez and Tolmasky, 2010), transmission of the drug resistance gene between bacteria by a process known as horizontal gene transfer (Strateva and Yordanov, 2009), and by efflux pump (Lister and et al., 2009; Strateva and Yordanov, 2009). *P. aeruginosa* have emerged as important nosocomial pathogens that display not only intrinsic resistance to many antibiotics, but also a remarkable ability to develop novel mechanisms of resistance during treatment (Falagas and Kopterides 2006; Jarvis and Martone 1992; Bennett and et al., 2014).

Antibiotic resistance of *P. aeruginosa* is a current major public health problem, as this bacterium has a high mutation rate to cause antibiotic resistance and thus often resistant to many types of antibiotics, called multidrug resistant (MDR) *P. aeruginosa* (Carmeli and et al., 1999). This resistance leads to ineffective treatment and high mortality in infected patient (Mahar and et al., 2010; Lambert and et al., 2011). Therefore, treatment of MDR *P. aeruginosa* infection requires alternative effective strategies. Currently, one of the promising approaches used to treat bacterial infection is bacteriophage therapy by using bacteriophage to lyse bacterial cell (Sulakvelidze and et al., 2001; Khakhum and et al., 2010). Bacteriophage, a virus has bacterium as host cell, is being increased in the phage therapy such as using bacteriophage to treat *P. aeruginosa* chronic infections in the middle ear (Harper and Enright, 2011).

For comparison of specificity, bacteriophage has an a higher specific than antibiotic, due to antibiotics also destroy the commensal microflora especially in the intestine, which may lead to intestinal disorders (Inal, 2003). In addition, activation of bacteriophage occurs only in the presence of bacterial infection different from the action of antibiotics that act in a wide-board area. Therefore, the use of bacteriophage in the treatment gives few side effects than the use of antibiotics (Parichat, 2009).

For these reasons, it is interesting to isolate and characterize the novel bacteriophage specific for MDR *P. aeruginosa* that can be further developed for using as treatment tool for bacterial infections in the near future.

1.2 Objectives

1.2.1 To isolate bacteriophage specific to multidrug resistant *P. aeruginosa*.

1.2.2 To study the properties of bacteriophage.

1.3 Scope and limitation of research

This study aims to isolate and characterize bacteriophage specific to MDR *P. aeruginosa*. The water sample from wastewater treatment plant of Sanprasitthiprasong hospital, wastewater from Warin Chamrap wet market, and natural water from the Mekong river were used for bacteriophage isolation. Bacteriophage properties which included bacteriophage's host range, stability in ethanol and normal saline solution, analysis of bacteriophage genome and morphology, were investigated in this study.

1.4 Anticipated outcomes

To understand the properties of bacteriophage that was isolated from wastewater treatment plant of Sanprasitthiprasong hospital, wastewater from Warin Chamrap wet market, and natural water from the Mekong river.

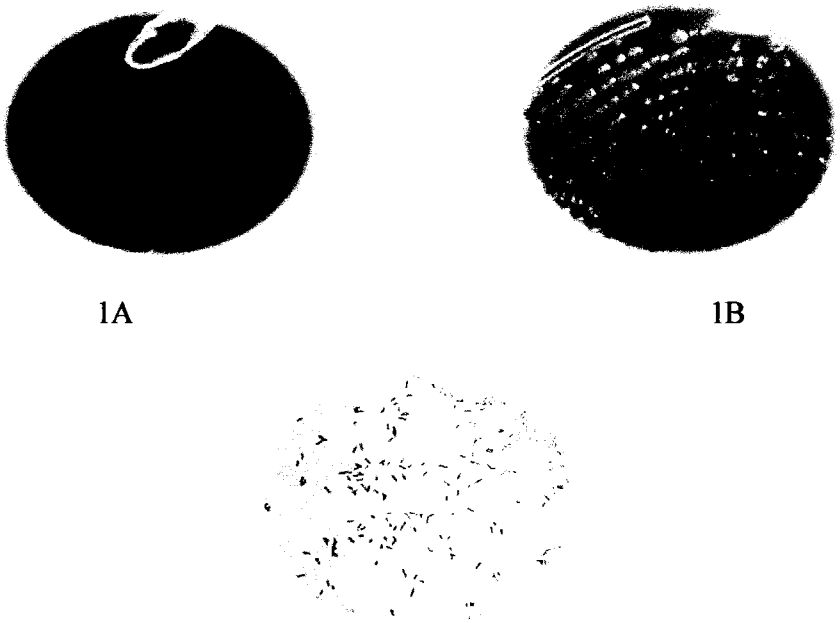
CHAPTER 2
LITERATURE REVIEWS

2.1 *Pseudomonas aeruginosa*

P. aeruginosa is in the family Pseudomonadaceae genus of *Pseudomonas*. Bacteria is a facultative anaerobe can be move freely in wet areas. *P. aeruginosa* is considered opportunistic which often cause disease in patients with immune disorders (Bhawsar and Singh, 2014) and often severe symptoms with patients in the urinary tract infection and patients with inflammation of the wound (Mousa, 1997). The disease is caused by bacteria can be treated with antibiotics and can be prevented by vaccination.

2.1.1 Cell morphology of *Pseudomonas aeruginosa*

P. aeruginosa is a common Gram-negative, rod-shaped bacterium size 0.5-1.0 × 1.5-5.0 micrometer (Figure 2.1C) distinguishing feature of *P. aeruginosa* is specific odor smells like grapes grow well at 42°C and movable by flagella (Figure 2 . 1 A) *P. aeruginosa* can produce pyocyanin, which is classified as secondary metabolite, such substances can damage the cells of other bacteria. Cell walls of *P. aeruginosa* contains lipopolysaccharide (LPS) which has a structure similar to bacteria in the family. *Enterobacteriaceae* but it is different to some chemicals. For polysaccharide side chain which extends from LPS is believed to be associated with susceptible and specificity to bacteriocin. This is a peptide or protein synthesized from ribosome and inhibits the bacteria. Bacteriocin synthesizer from lactic acid bacteria (Barefoot and Klaenhammer 1983). When *P. aeruginosa* was cultured in liquid media this bacteria are usually found on the surface of the media (Pellicle) shows that the bacteria prefer oxygen. The colonies of *P. aeruginosa* grown on solid media are mucus-like and metallic-like (Metallic sheen) (Figure 2 . 1 B). Colony can produce pyocyanin pigment is blue color and pyoverdine is yellow when illuminated by ultraviolet light both the color pigments are combined green.



1A 1B

1C

Figure 2.1 General characteristics of *P. aeruginosa*

1A = Characteristics of *P. aeruginosa* is rod shape is movable by flagellum.

1 B = Colonial characteristics of *P. aeruginosa* is small round shape and smooth white.

1 C = Characteristics under the microscope of *P. aeruginosa* is gram-negative of safranin

Source: Prince of Songkla University (2017: http://medinfo.psu.ac.th/pr/MedBoard/readboard.php?id=113)

2.1.2 Ecology of *Pseudomonas aeruginosa*

P. aeruginosa living well using oxygen than anaerobic It can grow and get energy from the sources of carbon and nitrogen from ordinary nutrients such as carbon dioxide and ammonia. *P. aeruginosa* can increase the number and live in temperatures ranging from 20-42℃ . It is generally found in nature, such as in water in soil and is well tolerated in various environments. *P. aeruginosa* is a major cause of nosocomial

infection. Especially patients with immunodeficiency, end stage cancer or ulcers such as burns (Mousa, 1997).

2.1.3 Severe factors in pathogenesis of of *Pseudomonas aeruginosa*

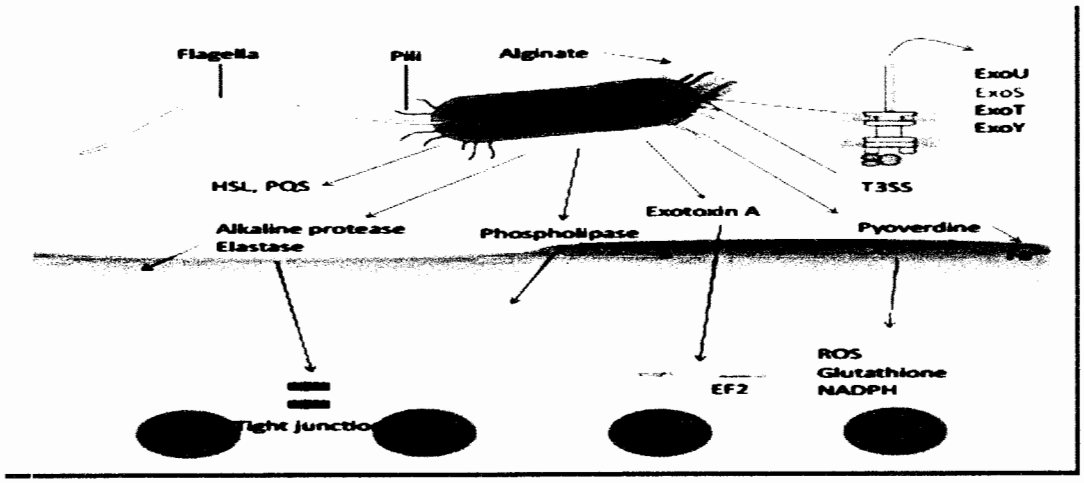


Figure 2.2 Factors virulences in the pathogenesis of *P. aeruginosa* shows the structure of *P. aeruginosa* associated with pathogenesis

Source: Gellatly and Hancock (2013)

2.1.3.1 Capsules or slime allows bacteria to stick together and stick to the surface of target cells, especially the cells of patients with chronic respiratory disease. In addition, the capsule protects the bacteria from being destroyed by the process phagocytosis (Gilligan 1991 and et al., 2014).

2.1.3.2 Pili or Fimbriae is a small hairline protruding from the skin cells to catch whit epithelium.

2.1.3.3 Endotoxin or Lipid A is a liposaccharide located in the cell wall of gram-negative bacteria. In addition, endotoxin serves as an important antigen. For Lipid A is an element of endotoxin about toxicity (Kanthakumar and et al., 1993).

2.1.3.4 Elastase is enzyme that destroys an elastic fiber at the vascular wall of an infected person. Which causes bleeding and the spread of the infection into the bloodstream and the various systems, destructive complement and antibody and inhibit the movement of neutrophil (Galloway, 1991; Read and et al., 1992).

2.1.3.5 Exotoxin A is a serious infection. It has hindered the process of protein synthesis of elongation factor-2. causing the infected cells area to die (Wick and et al., 1990).

2.1.3.6 Proteases is enzyme excreted outside the cell can be degrade the tissue of infected patients causing the infection to spread even further and can also be destroyed Immunoglobulin, complement of infected patients (Galloway, 1991).

2.1.3.7 Exotoxin S is a substance that inhibits protein synthesis difference with exotoxin A is heat resistant and found in some species of *P. aeruginosa.*

2. 1. 3. 8 Phospholipase C can decompose fat and lecithin to release the phosphatidylcholine to destroy tissue resulting bacteria spread even further.

Because *P. aeruginosa* is an opportunistic infection rarely cause disease in healthy people. But, it can cause disease in people with low immunity. For example, elderly person and people with the disease that causes the immune system is depressed leukemia, such as people who get immunized. The infection usually enters the body in the area of the wound or mucosal area. Patients in the hospital have the potential for *P. aeruginosa* infection is easy and severe. The bacteria often live on medical instruments, breathing apparatus and urinary catheter.

2.1.4 Infectious diseases from *P. aeruginosa*

2.1.4.1 Infection burns (Mousa, 1997) burns area is wet and no Neutrophil to prevents the invasion of bacteria. It causes infections easily. *P. aeruginosa* will gather at burns area and it will destroy blood vessels as a result, the bacteria can spread into the bloodstream at burns area.

2.1.4.2 Pulmonary infection *P. aeruginosa* infection of the respiratory tract from the neck to the lungs it is cause of pneumonia. This infection is usually found in patients with chronic lung disease and neutrophil less than normal (Bennett and et al., 2014). *P. aeruginosa* infection can occur for many reasons. For example;

1) Intubation and Tracheostomy usually found in water used for moisture with tools (Brewer and et al., 1996; Rello and et al., 1996).

2) The spread in the bloodstream In patients with an infection somewhere. The infection usually spreads into the bloodstream and come to the lungs as the last organ of infection in the bloodstream.

3) Choking in the mouth or from the stomach into the lungs.

4) Breathing bacteria in the air as a result, the infection can spread to the lungs directly.

2.1.4.3 Ear infection usually occurs with people who like playing water and the infection may spread into the inner ear (Rubin and Victor, 1988).

2.1.4.4 Urinary tract infections often caused by the use of a urinary catheter (Nickel and et al., 1992).

2.1.4.5 Skin infections (Maniatis and et al., 1995) *P. aeruginosa* infections of the skin, there are 4 types.

1) Vesicles and Bullae the infected area with a blister.

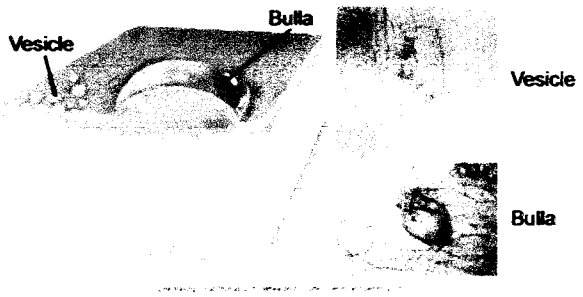


Figure 2.3 The lesions of Vesicles and Bullae

2) Ecthyma gangrenosum the infected area has a rather swollen red and quite solid. Later it becomes gangrene. Usually no pain due to scar in black robes. This infection may occur after vesicles and bullae.

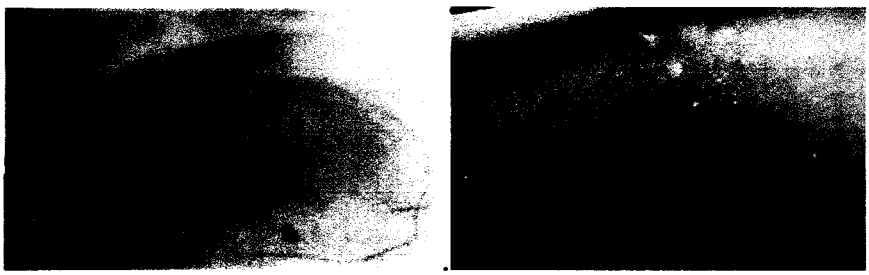


Figure 2.4 The lesions of ecthyma gangrenosum

Source: Chan and et al (2006)

3) Macular or maculopapular the infected area is characterized by spots (Macula), often found in the arms and legs.

4) Gangrenous cellulitis is a severe infection of the skin. and may spread to the tissues under the skin.

6) In addition, *P. aeruginosa* infection may also occur with other organs such as the central nervous system, digestive tract, eye lining, heart and musculoskeletal system (Gupta and et al., 1990).

2.1.5 Diagnosis

Pseudomonas can be easily cultured in conventional foods such as MacConkey agar or Blood agar (Winn, 2006) It is bacteria that needs oxygen to grow. To identify bacteria by study the following. First, morphological features of the colonies such as ability to decompose red blood cells, color and size including biological testing. For example, colonies are flat and grow faster, oxidation reaction is positive, green pigment and the smells like grapes. Second, classification of specific bacterial by these methods. Biochemical tests, Pyocyanin formation, Susceptibility to antibiotics and gene sequencing of the 16S ribosomal DNA (16S rDNA). Laboratory diagnostics can be performed by taking specimens that are contaminated isolation on selective media. For example, take contaminated specimens to isolate on cetrimimide agar, take samples of burns, blood and others to isolate on Muller-Hinton agar or take samples from the sputum of patients with chronic lung disease (Cyctic fibrosis) to isolate on MacConkey agar. Take the isolate to study by gram staining, Movement reaction oxidation test and study the formation of pigment. For diagnosis by PCR (Polymerase Chain Reaction) can analyze the level of genes of microorganisms. For example, Metallo-beta-lactamase gene (blaIMP) is a gene that makes antibiotic resistant (Senda and et al., 1996) Therefore, the use of PCR technique can detect specifically to *Pseudomonas* species.

2.1.6 Treatment

P. aeruginosa is usually resistant to several antibiotics. Therefore, there are antibiotic sensitivity test of *P. aeruginosa* to antibiotics before treatment. Treatment of *P. aeruginosa* infection often uses several antibiotics together. For example, drug group aminoglycoside such as gentamicin, tobramycin, amikacin in combination with drug group penicillin such as mezlocilln, piperacillin, ticarcillin. The other drugs used

to treat it effectively such as ciprofloxacin, cefoperaxone and ceftazidime. (Bell and et al., 1985; Zhanel and et al., 1995).

2.1.7 Antibiotic Resistance of *P. aeruginosa*

Currently, found that many *P. aeruginosa* resistant to antibiotics and it is a major problem in the treatment of infection. In some cases, resistance to several antibiotics. In this case, we would call that multidrug resistant bacteria. *P. aeruginosa* has the antibiotic resistance mechanism in Table 2.1. Table 2.2 show the data of resistance bacteria from Sunpasitthiprasong Hospital, Ubon Ratchathani. Data shows the problem of resistance to antibiotics of *P. aeruginosa* (MDR) from the total number of *P. aeruginosa*. The information is interesting that it could be a major issue in the treatment of *P. aeruginosa* infection. Although it is likely to decrease, if the average is seen every year, *P. aeruginosa* (MDR) there are still plenty of *P. aeruginosa* (MDR) that can be problematic.

Table 2.1 Resistance mechanism of *P. aeruginosa*

Mechanism	Resistance class	Example
Efflux pumps	Intrinsic	MexAB–OprM, MexCD–OprJ, MexEF–OprN, MexXY–OprM (cephalosporins, carbapenems, aminoglycosides, quinolones, ureidopenicillins)
Outer membrane impermeability	Intrinsic	OprF, OprD, OprB (carbapenems, aminoglycosides, quinolones)
β -lactamases	Intrinsic	AmpC (penicillins)
Targeted mutation	Acquired	DNA gyrase, DNA topoisomerase (quinolones) MexZ (quinolones, cefapimes, aminoglycosides)
Horizontal transfer	Acquired	Metallo- β -lactamases, ESBLs (penicillins, cephalosporins, carbapenems)
Membrane changes	Adaptive	Lipid A modification (aminoglycosides, polymyxins) AmpC upregulation (penicillins)

Source: Gellatly and Hancock (2013)

(1) Intrinsic is natural antibiotic resistance of the bacteria itself. All bacterial strains or nearly every species in the species that have the ability to resist antibiotics. For example, the resistance to vancomycin of all Gram-negative bacteria because the drug can't pass through the cell membrane into the cells of the bacteria. There are *also Klebsiella* spp resistant to ampicillin because bacteria can produce enzymes that degrade the drug.

(2) Acquired is a drug resistant in some strains or some population of bacteria in that species. As a result, bacteria that have been destroyed by antibiotics become resistant to antibiotics this is caused by genetic changes or modification of genetic structure in bacterial populations from the following mechanisms. First, mutation is a change of genetic material in bacteria cells. For example, sequence change or number of sequence in DNA.As a result, the genetic code changed from the original. This may change the sequence and type of amino acids, resulting protein synthesis is different from the original. Second, horizontal gene transfer is bacteria get antibiotic resistance gene from other bacterial cells, which may be bacteria in the same species or different species and genus.

(3) Efflux pump is a mechanism by which bacteria can drive multiple antibiotic out of cells such as tetracyclines, chloramphenical, aminoglycosides, quinolones, and β-lactams.

(4) Outer membrane impermeability is a mechanism to reduce the penetration of antibiotics into the cell. It might have modification outer membrane to reduce the Porins as a result, antibiotic into the cell less.

(5) β-lactamases are enzymes that digest drug group β – lactams.

(6) Horizontal transfer is transmission of drug resistance genes between different bacteria species by process called horizontal gene transfer.

Table 2.2 Number of antibiotics resistant bacteria from Sanpasitiprasong Hospital, Ubon Ratchathani

Bacteria	2013		2014		2015	
	Total	MDR	Total	MDR	Total	MDR
Pseudomonas aeruginosa	2,577	574	2,787	536	2,909	500
Coagulase positive *Staphylococcus*	1,443	403	1,765	417	1,709	300

From the problem of antibiotic resistance of *P. aeruginosa* as a result, the rate of antibiotic resistance is increasing lead to antibiotic previously used to treat effectively later found that antibiotics used in the original treatment less effective. Therefore, the need for new antibiotics but often more expensive and sometimes the treatment outcome is not as good as the original. In addition, it can cause more side effects. The use of antibiotic in large quantities as a result, infectious disease patients are at risk or potentially dangerous by antibiotic treatment even more. Therefore, the treatment of bacterial infectious disease by bacteriophage (Bacteriophage therapy) is interesting. In theory, bacteriophage has several advantages over antibiotics, as shown in Table 2.3.

Table 2.3 Comparison of bacteriophage and antibiotic properties for treatment

Bacteriophage	Antibiotic
Bacteriophage highly specific to target bacteria.	Antibiotic not specific to bacteria can destroy both pathogenic bacteria and normal flora.
Bacteriophage can increase the number of positions are infected.	Antibiotic is eliminated by the metabolic process of the body. As a result, the concentration of antibiotic remaining in the body is not enough to disinfect at the infected area.
No side effects.	Antibiotic has many side effects or sometimes causes allergic reactions.
Bacteriophage resistant bacteria were limited to only target bacteria.	The antibiotic resistance of bacteria is not limited to the target bacteria because effect of antibiotics is broad spectrum activity.
A short time for bacteriophage isolation to use in the treatment.	Take a long time for development or create new antibiotic for used in the treatment of bacterial infections from antibiotic resistant bacteria.

Source: Parichat (2009)

2.2 Bacteriophage or phage

Bacteriophage or phage is virus of bacteria that rely on bacterial cells to multiply. Bacteriophage can be found in environments and bacteriophage was first discovered in 1915 by Twort (Salmond and Fineran, 2015). He found that there are certain substances that can destroy bacteria cells, the *Micrococcus* group by observe clear zone in the culture medium. At that time not yet know what caused the phenomenon was a virus. Later, a scientist name Felix D'Herelle discover what makes the cells death of *Shigella* sp. while culture in broth culture media is virus of bacteria and called bacteriophage (Chanishvili, 2012). Basic features of bacteriophage found is high specificity to host since the bacteriophage specific to bacteria only one species. Currently, found that the source of bacteriophage can be isolated as well is water sources such as lakes, rivers, streams (Chan and et al., 2016). Especially the source of wastewater because it is a region with a variety of bacteria (Brüssow, 2005).

Table 2.4 The morphology and genomes of bacteriophage

Family name	Morphology	Genome
Corticoviridae	Icosahedral capsid with lipid layer	dsDNA
Cystoviridae	Enveloped, icosahedral capsid, lipids	dsRNA
Fuselloviridae	Pleomorphic, envelope, lipids, no capsid	dsDNA
Inoviridae	Rod-shaped with helical symmetry	ssDNA
Leviviridae	Quasi-icosahedral capsid	ssRNA
Lipothrixviridae	Enveloped filaments, lipids	dsDNA
Microviridae	Icosahedral capsid	ssDNA
Myoviridae	Contractile tail	dsDNA
Plasmaviridae	Pleomorphic, envelope, lipids, no capsid	dsDNA
Podoviridae	Short, non-contractile tail	dsDNA
Rudiviridae	Helical rods	dsDNA
Siphoviridae	Long, non-contractile tail	dsDNA
Tectiviridae	Icosahedral capsid with inner lipoprotein vesicle	dsDNA

2.2.1 Bacteriophage classification

All types of bacteriophages contain genome surrounded by a protein called capsid. Types of genomes include double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA). More than 90% of the bacteriophages are dsDNA. Head shape is icosahedral and in order *Caudovirales*, which consists of Families the *Myoviridae* (long, contractile tail), the *Siphoviridae* (long, noncontractile tail) and the *Podoviridae* (short tail), (Figure 2.5) (Harper and Enright, 2011). Currently, the International Committee for Taxonomy of Viruses (ICTV) identification of bacteriophages based on viral genome features, shape characteristics of virus particles, bacteriophages are divided into 1order 13 families and 30 genera based on the nature of nucleic acid and the shape of the virus (Hanlon, 2007). Each type of bacteriophage has an average size of about 20-200 nanometers. Bacteriophages known in the tailed phage group are the oldest bacteriophages, have origins before the separation of euacteria from euryarchaeota. This type of bacteriophage has a structure consisting of a head and a tail portion. Figure 6 shows the shape of bacteriophage ST70, which is tailed phage with genomes as dsDNA and specificity with *Burkholderia pseudomallei*, a Gram - negative bacteria that cause melioidosis (Khakhum and et al., 2010).

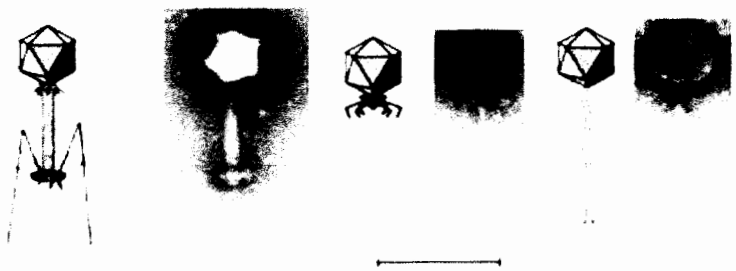


Figure 2.5 Shape of bacteriophage in order *Caudovirales*

Source: Harper and Enright (2011)

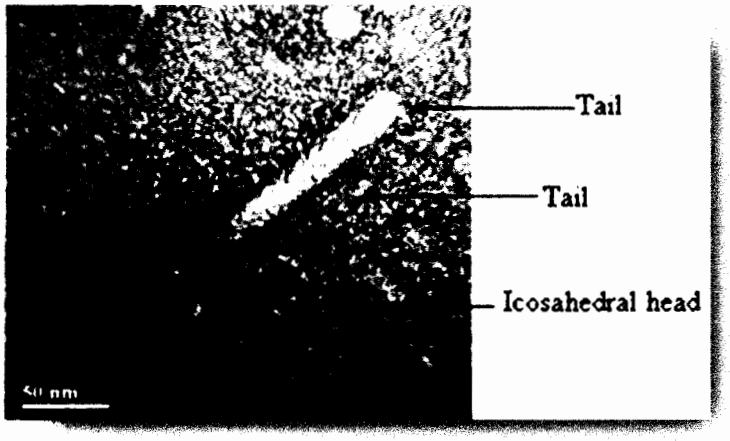


Figure 2.6 Phage ST70s photos specific to *Burkholderia pseudomallei* by transmission electron microscopy, show icosahedral head and tail
Source: Khakhum and et al (2010)

2.2.2 Phage life cycle

Bacteriophages are divided into 2 types according to the life cycle in bacteria.

2.2.2.1 Lytic bacteriophage is the type of bacteriophage normally use in bacteriophage therapy because it can infect specific to bacterial host. Lytic phages instruct the machinery in the host cell by binding to the receptor on the bacterial cell surface to make more phages. Fully viable progeny phages burst out and kill the bacteria and released phages attack new bacteria. This process continues until. When bacteriophages are mixed with bacteria, then mixed with agar and placed on the surface of the culture medium. It is observed that bacteria are destroyed is clear zone called plaque (Figure 2.7) (Khakhum and et al., 2010).

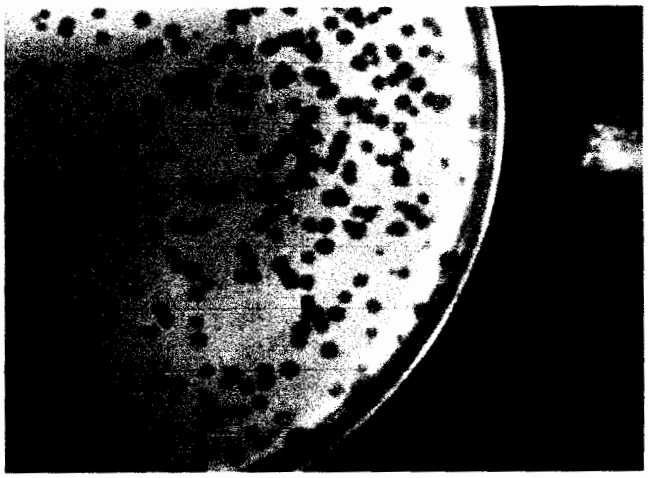


Figure 2.7 Plaque characteristics appearing on the culture medium containing the bacterium *Burkholderia pseudomallei*

2.2.2.2 Lysogeny is characterized by integration of the bacteriophage chromosomes or formations of a circular replicon in the bacterial cytoplasm (Genetic recombination). In this condition the bacterium continues to live and reproduce normally. The genetic material of the bacteriophage, called a prophage, when the chromosome of the bacterium divide, the prophage is divided equally as part of the chromosome, new cell bacteria have latent prophages, This process is called lysogenization, bacteria with latent prophages are called lysogen or lysogenic bacteria (Figure 2.8B). The genome of the bacteriophage inserted into the host cell's chromosomes often changes the host cell's properties. For example, *Corynebacterium diphtheriae* that can produce diphtheria toxin which causes diphtheria, it is a strain that inserts the bacteriophage genome into the chromosome of *C. diphtheriae* (Uchida, 1982). In addition, the insertion of genomic bacteriophages into the bacterial chromosome also makes many bacteria have the ability to cause disease. For example, *Clostridium botulinum*, which causes food poisoning and *Streptococcus* group A, which causes rash in scarlet fever. However, the life cycle of lysogenic phage can change from lysogenic cycle into lytic cycle. (Figure 2.8A), depending on factors such as the nutrient, environment, and the host cell.

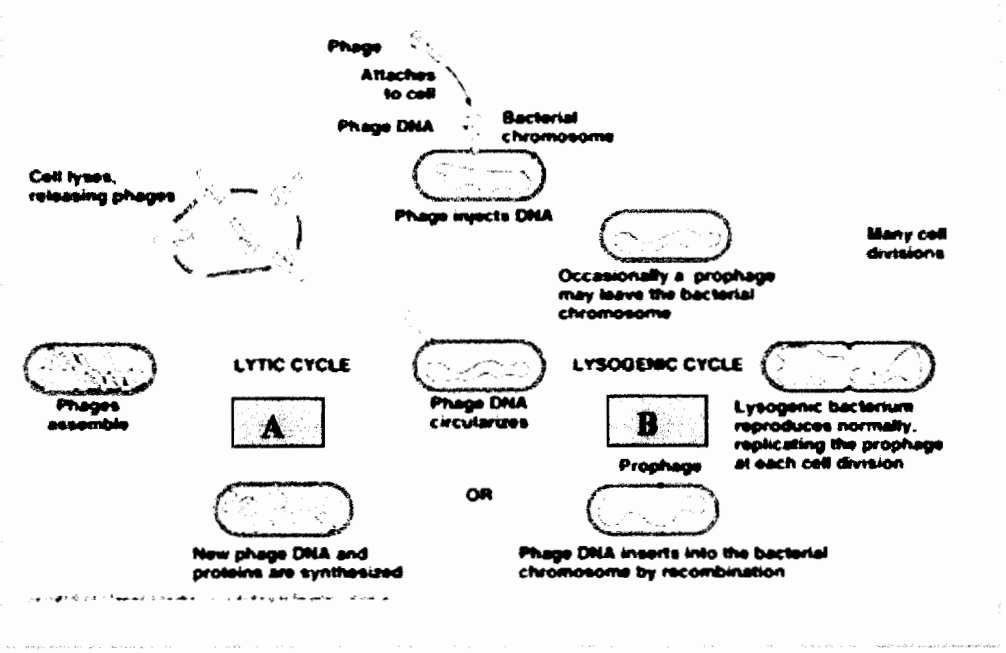


Figure 2.8 Life cycle of bacteriophage lytic (A) and lysogenic (B)

Source: Khakhum and et al (2010)

2.2.3 Lactic phage mechanism for bacterial destruction

Mechanism of lytic phage to destroy the cell wall of bacteria until the bacterial cell lysis. There are two types of protein that work together is holin and endolysin, holin is a small hydrophobic protein, which holin monomer is inserted into the cell wall of the bacteria from the inside (Cytoplasmic side) of the cells and then assembled into holin oligomer make holes in cell walls area. After endolysin, which is an enzyme, can pass into peptidoglycan for destroy and kill the host to release the newly produced bacteriophages that can in turn generate infect the host (Young and et al., 2000). Since Gram-positive bacteria and Gram-negative bacteria have different cell walls, making enzyme activity different, Gram-positive bacterial cell walls consist of layers peptidoglycan Thick and on the outside. Therefor, endolysin can work as exolysin to destroy bacteria cell from the outside. Cell walls of Gram-negative bacteria have outer membrane that do not allow enzymes to work from the outside of cell but when lypopolysaccharide is destroyed by ethylene diamine tetraacetic acid (EDTA) or detergents. As a result, it can be exterminated by the enzyme endolysin.

2.2.4 Bacteriophage applications

Currently, bacteriophages are used in many fields such as genetics, agriculture, biotechnology including medical (Khakhum and et al., 2010).

2.2.4.1 Genetic engineering

Bringing bacteriophages are used as a vector for gene of interest is introduced into the cells of the bacteria by cutting off some genetic material that is not needed for bacteriophage and then use genes that are interesting in the study to replace (Recombinant). Then introduced into bacterial cells by bacteriophages insert genetic material into the bacterial genome. The host cell mechanism was used to generate the bacteriophage protein including proteins from the genes we are interested.

2.2.4.2 Bacteria typing by bacteriophage

Bacteriophage is used for the classification of bacterial strains of different species in the genus and the same species as the group, based on susceptibility to bacteriophage of bacteria by the specificity between bacteriophage and receptor on the cell wall of bacteria. When the lysis of bacteria will notice plaque or clear zone on the surface of the culture medium (Agar surface). Bacteria typing by bacteriophage are useful for epidemiological studies in the detection of the bacteria causal agent of the outbreak.

2.2.4.3 Bio-control tool

Endolysin enzyme properties of bacteriophage that specifically destroys bacteria was applied in the food industry to destroy the unwanted bacteria, pathogenic bacteria that may be contaminated in food and the product is not cooked. In addition, bacteriophage was used to create transgenic plants, which has bacteriophage endolysin gene to resist pathogenic bacteria in plants. For example, using T4 lysozyme in potatoes to prevent the destruction of *Erwinia carotovora* is plant pathogens (de Vries and et al., 1999) and using lysozyme from *E. amylovora* phage, which was created in *E. coli* to spray and to prevent pathogens in plants on the skin of pears.

2.2.4.4 Bacteriophage therapy

Lytic bacteriophage is the type of bacteriophage normally use in bacteriophage therapy because it can infect specific to bacterial host. Therefor, if bacteriophages are used in the treatment of bacterial infections. Bacteriophage will

destroy bacteria target only, it will not affect the normal flora residents of the bodies of people and animals (Parichat, 2009). In addition, livelihoods rely on host cells of bacteriophage is another factor contributing to the use of bacteriophages in the treatment of the disease. Since, after the destruction of pathogenic bacteria by bacteriophage infected area completely. As a result, bacteriophages lack host cells for replication and can' t live up to it. As a result, there is no problem with the bacteriophage remain in the body after using bacteriophage in the treatment. In the past, bacteriophages have been used to destroy bacteria for use in the treatment of bacterial infections. But when antibiotics are found to kill more pathogens Therefore, bacteriophage treatment has been reduced interest, but in Russia has been developed continuously. At present, the medical field has introduced endolysin as a therapeutic agent. It may be used alone or in combination with antibiotics to destroy antibiotic-resistant bacteria or bacteria that make biofilm. At present, there are many studies on bacteriophage therapy. For example, phage therapy against *S. aureus* in mice, describes a bacteriophage active against *S. aureus*, including methicillin-resistant staphylococcal strains. When inoculated into mice simultaneously with *S. aureus*, phage rescued 97% of the mice (Capparelli and et al., 2007). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *P. aeruginosa*; a preliminary report of efficacy describes a bacteriophage active against antibiotic-resistant *P. aeruginosa*, clinical trial of a therapeutic bacteriophage preparation showed efficacy and safety in chronic otitis because of chemo-resistant *P. aeruginosa* (Wright and et al., 2009). There are also many other studies, such as bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *E. faecium* (Biswas and et al. , 2002), quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials (Merabishvili and et al., 2009) and bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial (Rhoads and et al., 2009).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Bacterial strains and culture conditions

Bacterial strains used in this are listed in Table 3.1. All strains of *Pseudomonas aeruginosa* were isolated from patients hospitalized at the Sanpasitiprasong hospital, Ubon Ratchathani, Thailand, during on August to September 2016. *Pseudomonas aeruginosa, Klebsiella pneumonia*e, *Salmonella typhimurium*, *Shigella dysenteriae* and *Enterobacter* sp. strains were grown in brain heart infusion (BHI) medium at 37 °C with aerobic conditions. *Staphylococcus aureus Staphylococcus epidermidis* and *Bacillus subtilis* were cultured under aerobic conditions at 37°C in nutrient broth (NB) medium. The 15g/L of biological agar was added into corresponding media to prepare BHI agar.

Table 3.1 List of bacterial strains used in this study

Strains	Antibiotic resistant properties	Source
Pseudomonas aeruginosa 268	Resistant to CAZ, TGC, IPM, ETP	Sanpasitipr asong hospital
Pseudomonas aeruginosa 288	Resistant to CAZ, CN, CIP, AK, NET, SCF, SCF, IPM	
Pseudomonas aeruginosa 392	Resistant to AK, NET, SCF, IPM, CN, CIP, NOR, CAZ	
Pseudomonas aeruginosa 689	Resistant to AMP, FOX, SXM, CN, SCF, CXM, SXT, AK	
Pseudomonas aeruginosa 698	Resistant to AK, NET, CIP, CN, IPN, SCF	
Pseudomonas aeruginosa 837	Resistant to CN, IPM, SCF, NET, AK, CIP	
Pseudomonas aeruginosa 838	Resistant to SXT, CN, AMP, SCF, FOX, CXM	
Pseudomonas aeruginosa 956-2	Resistant to NET, AK, CIP, CN, IPM, SCF	
Pseudomonas aeruginosa 3472	Resistant to SXT, SCF, FOX, AMP, CN, CXM	
Pseudomonas aeruginosa 3472	Resistant to SXT, SCF, FOX, AMP, CN, CXM	
Pseudomonas aeruginosa ATCC 27853	Antibiotic susceptible strain	American type culture collection
Escherichia coli ATCC 25922	N.D.	
Staphylococcus aureus ATCC 25923	N.D.	
Enterobacter sp.	N.D.	Collage of Medicine and Public Health
Klebsiella pneumoniae	N.D.	
Staphylococcus epidermidis	N.D.	
Bacillus subtilis	N.D.	
Salmonella typhimurium DMSc 5784	N.D.	Department of Medical Sciences
Shigella dysenteriae DMSc 2137	N.D.	

NET, Netilmicin; AK, Amikacin; CAZ, Ceftazidime; SCF, CefoperazoneCXM, Cefuroxime; FOX, cefoxitin; CN, Cefalexin; AMP, Ampicillin; CIP, Ciprofloxacin SXT, Cotrimoxazole; MEN, Meropenem; IPM, Imipenem; TGC, Tigecycline ETP, Ertapenem; NOR, Norfloxacin; N.D, not determine.

3.2 Molecular identification of *P. aeruginosa*

The accurate species of *P. aeruginosa* strains was confirmed by 16S ribosomal RNA (16S rRNA) amplification and sequencing. Polymerase chain reaction (PCR) was used to amplify the target 16S rRNA with the universal primer pairs, 27F (5'-agagtttgatcctggctcag-3') 1492R (5'-ggttaccttgttacgactt-3'). The PCR components for the amplification were done in 50 µL of PCR mixture and consisted of 2.0 µL chromosomal DNA (5-20 ng/µL), 1.0 µL forward primer (10 µM), 1.0 µL reverse primer (10 µM), 5.0 µL 10x PCR buffer, 1.5 µL MgCl2 (1.5 mM), 2.0 µL dNTP solution (10 mM dNTP), 1.0 µL *Taq* polymerase (5 units/µL) and 36.5 µL PCR-grade water. PCR conditions were performed as follows: pre-denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec. annealing at 56°C for 30 sec. and extension at 72°C for 1 min; followed by a final extension step of 72°C for 7 min.

The PCR products derived from PCR were purified by using Gel/PCR DNA Fragments Extraction Kit (RBC, Bioscience Taiwan) according to the manufacturer's instruction. In briefly, one volume (one-hundred microliters) of PCR product was added with 5 volumes of DF buffer and mixed by vortexing. The sample mixture was applied into the DF column and centrifuged at 10,000 rpm for 30 sec. The flow-through was discarded. The DF column was added with 600 µL wash buffer and centrifuged at 10,000 rpm for 30 sec. After centrifugation, the column matrix was dried by centrifugation for 2 minutes at 10,000 rpm. The column matrix was added with 20-50 µL elution buffer at the center of the column and stand for 2 min until elution buffer or water was absorbed by the matrix. The purified DNA was eluted after centrifugation for 2 min at 10,000 rpm. The eluted DNA was stored at -20 °C until used.

3.3 Antibiotic susceptibility test

Antibiotic susceptibility test was done as previously described by Kirby-Bauer's method (Hudzicki, 2009). Ten *Pseudomonas* sp. strains, *P. aeruginosa* 268, *P. aeruginosa* 288, *P. aeruginosa* 392, *P. aeruginosa* 689, *P. aeruginosa* 698, *P. aeruginosa* 837, *P. aeruginosa* 838, *P. aeruginosa* 910, *P. aeruginosa* 956-2 and *P. aeruginosa* 3472 were tested in this study. A single colony of bacteria was grown in 2 ml of BHI medium at 37 °C with aerobic conditions. After 16-18 h of incubation, the

bacteria cells were collected, suspended in PBS and adjusted the bacterial density to MacFarland no. 1.0 which is equivalent to 1.5x 10^8 CFU/ mL. One ml of 1.0 McFarland bacteria suspension was supplemented in pre-warm BHI agar 0.5% (w/v) of bacteriology agar and poured onto petri dish plate. When the BHI became solid, the paper disk containing of the different types of antimicrobial agents were put on the BHI agar, and the plate was further incubated for 24 h at 37 °C. To check the result, if the bacteria are susceptible to a particular antibiotic, a clearing area surrounds the disk where bacteria are not capable of growing (called a zone of inhibition). If the organism is resistant to an antibiotic, there will be grown around the disc, thus, zone of inhibition can be not observed. For interpretation, the diameter of zone of inhibition was measured as millimeter (mm). According to the criteria of the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing standards M02-A12, M07-A10, and M11-A8 (Table 3.2), the result could be classified as Resistant (R), Intermediate (I) or Susceptible (S). *P. aeruginosa* ATCC 27853 were used as control strains. The antibiotics used in this study were amikacin (AK), ceftazidime (CAZ), ampicillin (AMP), ciprofloxacin (CIP), cotrimoxazole (SXT), imipenem (IMP), tigecycline (TGC).

Table 3.2 Antimicrobial disk diffusion zone interpretation guide for *P. aeruginosa*

Antimicrobial Agent	Disk potency (µg)	Zone Diameter Interpretive Criteria (mm)		
		R	I	S
Amikacin	30	<14	15–16	>17
Ceftazidime	30	<14	15–17	>18
Ciprofloxacin	5	<15	16–20	>21
Piperacillin	10	<14	15–20	>21
Cotrimoxazole	25	<14	15-17	>18
Imipenem	10	<15	16-18	>19
Tigecycline	15	<11	12-14	>15

3.4 Bacteriophage isolation

Sewage water samples used for phage isolation were collected from wastewater treatment plant of Sanprasitthiprasong hospital, wastewater from Warin Chamrap wet market, and natural water from the Mekong river. Ten mL of water samples were centrifuged at 5,000 rpm for 10 min to remove bacterial cells and artificial debris. The clear supernatant was filtered through a 0.45 μM pore size of membrane filter (Corning Incorporated Corning, NY 14831, Germany), resulting in phage filtrate 1. The filtrate 1 was added with equal volume of double strength BHI broth and inoculated with an early log phase host culture *P. aeruginosa* 268 at 37 °C for 18-24 h. After incubation, the culture was centrifuged at 5,000 rpm for 10 min. The supernatant was passed through a 0.45 μM pore size of membrane filter, resulting in phage filtrate 2. The filtrate 2 was stored at 4 °C until use.

3.5 Bacteriophage detection

Phage detection was performed by using a spot test method (Parichat and Pongsak 2015). The test was used as an initial step for phage detection by observing lytic activity of phages on the host in agar plate. Three ml of soft agar, BHI broth with 0.3% agar), was seeded with 0.1 mL of a log-phase of *P. aeruginosa* 268 culture, mixed thoroughly, and poured onto petri dish plate and then the phage filtrate 2 was spotted onto the top agar layer. The plate was incubated at 37 °C for 18-24 h. After incubation, a clear zone in the plate, indicating the lysis of bacterial cells and, thus, indicating the presence of phage was observed. Spot tests were also used for host range studies.

3.6 Bacteriophage purification

A clear zone in the plate of spot test method was used for determining the phage titer. The filtrate 2 was subjected to ten-fold serial dilution using BHI broth as a diluent. 100 μL of the diluted phage solution and 100 μL of log phase *P. aeruginosa* 268 culture were added into 3 mL soft agar (0.3 % agar), mixed and poured onto a BHI agar plate. The plate was incubated at 37°C for 24 h. to allow plaques, the formation of plaques indicates the presence of a bacteriophage in the filtrate 2, a single plaque was picked up and added into phosphate buffered saline (pH 7.0). The phage-contained buffer was further used to repeat plaque assay for two rounds. The number

of plaques was used to calculate the bacteriophage titer which was expressed in plaque forming unit (PFU)/mL.

Bacteriophage titer: Plaque-forming unit/mL (pfu/mL) = Plaque × 10 × dilution

3.7 Determination of bacteriophage host range

The host range of phage was determined by spot test method as previously described in bacteriophage detection method. The bacterial cells used as host cell were *P. aeruginosa* ATCC 27853, *P. aeruginosa* 268, *P. aeruginosa* 288, *P. aeruginosa* 392, *P. aeruginosa* 689, *P. aeruginosa* 698, *P. aeruginosa* 837, *P. aeruginosa* 838, *P. aeruginosa* 910, *P. aeruginosa* 956-2, *P. aeruginosa* 3472, *Escherichia coli* ATCC 25922, *Enterobacter* sp., *Klebsiella pneumonia*, *Salmonella typhimurium* DMSc 5784, *Shigella dysenteriae* DMSc 2137, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* and *Bacillus subtilis*.

3.8 Bacteriophage Stability

The stability of phage in ethanol was performed in this study. The different concentration of ethanol included 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 % (v/v). Phage was incubated in different concentrations of ethanol solution at 37°C for 30 min. After incubation, the stability of bacteriophage was calculated by following formula

% bacteriophage survival = [bacteriophage in chemical treatment / bacteriophage control] ×100

3.9 Bacterial growth inhibition by Bacteriophage

P. aeruginosa 268 (10^8cfu/ml) and phage (10^8pfu/ml) was mixed in ratio 1:1 (mixture) and then incubated at 37°C. Every 3 h (0 h, 3 h, and 6 h) of incubation, the mixture was used to detect live bacteria cell by using serial dilution and spread plate methods.

% bacteria survival = [Mixture / bacteria control] ×100

3.10 Bacteriophage genome extraction

A 100 µL of bacteriophage (10^8-10^9pfu/mL) was mixed by 10% sodium dodecyl sulfate and incubated at 65 °C for 15 min. Equal volume of Phenol: Chloroform: Isoamyl Alcohol (1:1:24) was added and mixed by inversion. The upper phage of supernatant of the mixture was collected by centrifugation at 13,000 rpm for 10 min at 4°C. The suspension was added with 300 µL of 3 M sodium acetate (pH 4.8) and mixed by inversion. Equal volume of isopropanol was added to the suspension and incubated at -20 °C for 1 h. The genomic DNA was collected by centrifugation at 13,000 rpm for 10 min at 4°C. Finally, the DNA pellet was rinsed with 1 mL of 70 % ethanol followed by air-dried and suspended in 50 µL of sterile distilled water or TE (pH 8.0). The genomic DNA was kept at -20°C for further analysis.

3.11 Genome analysis by restriction enzyme digestion

The purified phage genome was digested by restriction enzyme *Eco*RI, *Hin*dIII, N*col* RNase A, and DNase. The lambda DNA digested with *Hin*dIII marker (Thermo Fisher Scientific) was used as standard DNA ladder. The results were analyzed by 0.7% agarose gel electrophoresis.

3.12 Agarose gel electrophoresis

The molecular size of DNA was determined by gel electrophoresis as previously described by Sambrook (Sambrook and Russell, 2001). Briefly, the DNA was mixed with the 6X gel-loading buffer in a ratio (1:2). The mixture was then loaded into the wells covered by electrophoresis buffers. The electrophoresis was carried through appropriate voltage (V) until the bromophenol blue have migrated the appropriate distance through the gel. The agarose gel was stained with ethidium bromide and destained with water. Finally, the gel was visualized under UVP's ChemiDoc-ItTS2 Imagers (Analytik Jena). The molecular size of DNA was determined by comparing its bands with standard size DNA.

3.13 Bacteriophage morphology

A 10 µL of phage suspension was spotted on top of a copper grid and allowed to adsorb for 5 min at room temperature. After 5 min, excess stain was removed and bacteriophage was stained by adding of 2% phosphotingstate (pH 7.0). After 5 min, excess stain 2% phosphotingstate was removed and the grid was allowed to air dry for 2 h. The stained bacteriophage was examined under electron microscope (JEOL Ltd.) operated at 80 kV at Collage of Medicine and Public Health, Ubon Ratchatani University.

CHAPTER 4
RESULTS

4.1 Molecular identification of *Pseudomonas aeruginosa* species

Ten *P. aeruginosa* strains originally isolated from the patients, who were admitted at Sanprasitthiprasong hospital, had been identified by phenotypic profiling using biochemical testing. However, this method was reported to be non-reliable, thus, the molecular approach, 16S rRNA amplification and sequencing, was used for species confirmation in this study. The partial 16S rRNA PCR product with the length of 1,500 bp (Figure 4.1) was amplified from the genomic DNA of *Pseudomonas aeruginosa* strains (strain 268, 288, 392, 689, 698, 837, 838, 910, 956-2 and 3472) with the universal primers 27F and 1492R and then was sequenced with the same primers. Analysis of the sequence of 16S rRNA PCR product revealed that all strains showed percentage of identity of 97 to 98 % with 16S rDNA genes of several *P. aeruginosa* strains in database as shown in Table 4.1. Therefore, this result confirmed that all the *P. aeruginosa* strains are *P. aeruginosa*.

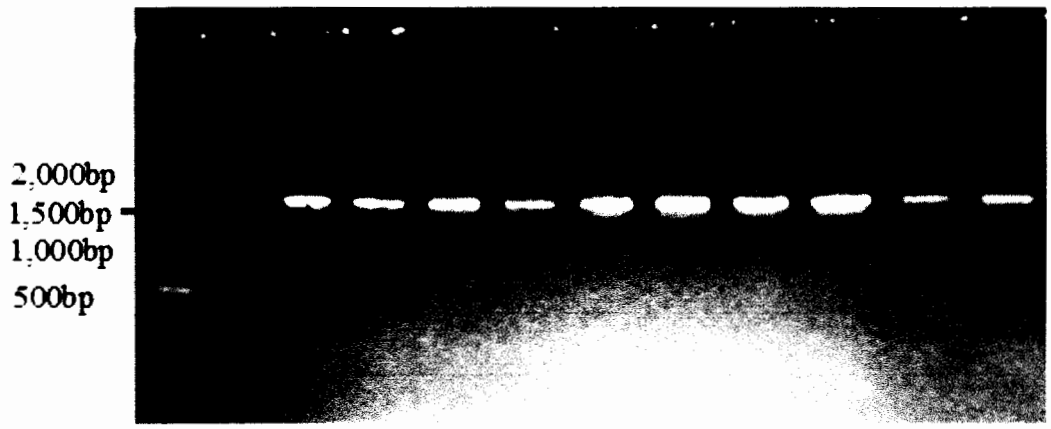


Figure 4.1 Agarose gel electrophoresis shown nucleic acid of *P. aeruginosa*

Ethidium bromide staining gel of the amplified product of 16S rDNA of *P. aeruginosa* strains. Lane 1 = DNA ladder with size in bp (left side), Lane 2 = negative control, Lane 3 to 12 = amplified product of 16S rDNA gene from *P. aeruginosa* strains (arrow indicates the size of the amplified product)

Table 4.1 The comparison of 16S rDNA sequence of *P. aeruginosa* strains with those of *P. aeruginosa* hold in GenBank database

NO.	Strain	Identified species	GenBank accession no.	% of identity
1	*P. aeruginosa* 268	N.D.	-	N.D.
2	*P. aeruginosa* 288	*P. aeruginosa* strain R14	DQ095879.1	98%
3	*P. aeruginosa* 392	*P. aeruginosa* strain S25	DQ095913.1	98%
4	*P. aeruginosa* 689	*Pseudomonas* sp. Pseudo-EJB5	GU966670.1	97%
5	*P. aeruginosa* 698	*Pseudomonas* sp. Hex35	JX436400.1	97%
6	*P. aeruginosa* 837	N.D.	-	N.D.
7	*P. aeruginosa* 838	*Pseudomonas* sp. SK4	LT545682.1	97%
8	*P. aeruginosa* 910	*Pseudomonas* sp. SK4	LT545682.1	97%
9	*P. aeruginosa* 956-2	N.D.	-	N.D.
10	*P. aeruginosa* 3472	*P. aeruginosa* strain HK1-2	JN661695.1	97%

N.D., not determine

4.2 Antibiotic susceptibility test of *Pseudomonas aeruginosa*

Table 4.2 shows that all strains of *P. aeruginosa* isolated from Sanpasitiprasonghospital, Ubon Ratchathani, Thailand, resisted to ceftazidime, ampicillin, cotrimoxazole, imipenem and tigecycline except for *P. aeruginosa* ATCC 27853, the antibiotic susceptible strains, showed susceptible to all antibiotic tested which include amikacin, ceftazidime, ampicillin and ciprofloxacin. In addition, *P. aeruginosa* 838 and *P. aeruginosa* 910 showed resistant to all antibiotic tested.

Table 4.2 Antibiotic susceptibility testing of *P. aeruginosa*

NO	Bacteria	Inhibition zone (mm)						
		AK	CAZ	AMP	CIP	SXT	IMP	TGC
1	*P. aeruginosa* 268	30 (S)	0 (R)	0 (R)	28 (S)	0 (R)	0 (R)	10 (R)
2	*P. aeruginosa* 288	30 (S)	0 (R)	0 (R)	30 (S)	0 (R)	0 (R)	10 (R)
3	*P. aeruginosa* 392	35 (S)	0 (R)	0 (R)	25 (S)	0 (R)	0 (R)	10 (R)
4	*P. aeruginosa* 689	35 (S)	0 (R)	0 (R)	25 (S)	10 (R)	0 (R)	10 (R)
5	*P. aeruginosa* 698	32 (S)	0 (R)	0 (R)	25 (S)	10 (R)	0 (R)	10 (R)
6	*P. aeruginosa* 837	30 (S)	0 (R)	0 (R)	23 (S)	15 (I)	0 (R)	14 (I)
7	*P. aeruginosa* 838	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	10 (R)
8	*P. aeruginosa* 910	0 (R)	0 (R)	0 (R)	0 (R)	10 (R)	0 (R)	10 (R)
9	*P. aeruginosa* 956-2	25 (S)	0 (R)	0 (R)	35 (S)	10 (R)	0 (R)	10 (R)
10	*P. aeruginosa* 3472	0 (R)	0 (R)	0 (R)	15 (I)	15 (I)	0 (R)	10 (R)
11	*P. aeruginosa* ATCC 27853	35 (S)	25(S)	20(S)	23(S)	20 (S)	25 (S)	25(S)

Amikacin (AK), Ceftazidime (CAZ), Ampicillin (AMP), Ciprofloxacin (CIP), cotrimoxazole (SXT), imipenem (IMP), tigecycline (TGC)

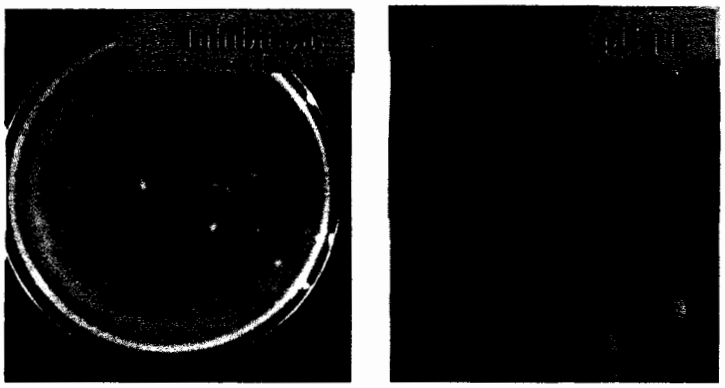
4.3 Bacteriophage isolation and purification

As shown in Table 4.3 by using spot test bacteriophage specific to *P. aeruginosa* 2 6 8 was only isolated from the water sample from wastewater treatment plant of Sanprasitthiprasong hospital (Fig 4.2 A), while the other samples from Warin Chamrap Market and Mekong river were not. The presence of bacteriophage was confirmed by plaque assay. It was found that bacteriophage produced many small plaques with size of 1.0 mm in diameter on BHI agar (Fig 4.2 B). In addition, the bacteriophage titer was 10^8 pfu/mL. The name of this bacteriophage is phageWJ9.

Table 4.3 Bacteriophage isolation and detection

Sample	Spot test	Plaque assay	Bacteriophage titer (PFU/mL)
Wastewater treatment plant of Sanprasitthiprasong hospital	+	+	10^8
Wastewater from Warin Chamrap Market	-	N.D.	N.D.
Natural water from the Mekong river	-	N.D.	N.D.

N.D., not determine



A B

Figure 4.2 Bacteriophage detection by spot test and plaque assay

Detection of bacteriophage from wastewater treatment plant of Sanprasitthiprasong hospital on the lawn of BHI agar supplemented with *P. aeruginosa* 268. Figure 4.2 shows an inhibition zone of spot test (A) plaques of plaque assay (B).

4.4 Determination of bacterial host range

Host range of phageWJ9 in a different bacteria genera and species was determined by using the spot test method. It was found that phageWJ9 was able to infect only *P. aeruginosa* as the formation of clear zones after overnight incubation on lawns of only *P. aeruginosa* with phageWJ9 was occurred, indicating that bacteriophage lysed bacterial cells. This clear zones were not obtained from the culture of bacteriophage with bacteria *Escherichia coli* ATCC 25922, *Enterobacter* sp, *Klebsiella pneumoniae*, *Salmonella typhimurium* DMSc 5784, *Shigella dysenteriae* DMSc 2137, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* and *Bacillus subtilis* (Table 4.4). The results demonstrated that the phageWJ9 had a highly specific host range for *P. aeruginosa*.

Table 4.4 Bacteriophage host range

Bacterial strains	Spot test
Pseudomonas aeruginosa ATCC 27853	+
Pseudomonas aeruginosa 268	+
Pseudomonas aeruginosa 288	+
Pseudomonas aeruginosa 392	+
Pseudomonas aeruginosa 689	+
Pseudomonas aeruginosa 698	+
Pseudomonas aeruginosa 837	-
Pseudomonas aeruginosa 838	-
Pseudomonas aeruginosa 910	+
Pseudomonas aeruginosa 956-2	-
Pseudomonas aeruginosa 3472	+
Escherichia coli ATCC 25922	-
Enterobacter sp	-
Klebsiella pneumoniae	-
Salmonella typhimurium DMSc 5784	-
Shigella dysenteriae DMSc 2137	-
Staphylococcus aureus ATCC 25923	-
Staphylococcus epidermidis	-
Bacillus subtilis	-

4.5 Stability of bacteriophage

Stability of bacteriophage in different concentration of ethanol and normal saline solution was examined. It was found that phageWJ9 could be survived at the percentage of 98.79% in 0.85 % normal saline for 30 min after expose to the solution. However, all bacteriophage could not survive in all concentration of alcohol solution as shown in Table 4.5.

Table 4.5 Stability of bacteriophage to chemical

Chemical	% bacteriophage survival
10% Ethanol	-
20% Ethanol	-
30 % Ethanol	-
40 % Ethanol	-
50 % Ethanol	-
60 % Ethanol	-
70 % Ethanol	-
80 % Ethanol	-
90 % Ethanol	-
100 % Ethanol	-
0.85 % Normal saline	98.79 %

4.6 Bacterial growth inhibition assay

To determine the efficiency of bacteriophage to inhibit bacterial growth in different time points, bacterial growth inhibition assay was performed. The method used in this assay is serial dilution and spread plate method. The survival of bacteria was counted as colony forming unit (CFU). The *P. aeruginosa* 268 strain was used as host cell for bacteriophage infection for 6 h. The survival of bacteria was counted every 3 h of incubation (0 h, 3 h and 6 h). It was found that phageWJ9 showed growth inhibition of *P. aeruginosa* 268. The percentage of bacterial survival expressed by CFU was reduced to 83.89 % after 6 h of incubation, compared to that of the control which showed more than 100% of survival, due to bacterial cell division in BHI medium. This result indicated that phageWJ9 could inhibit the growth of *P. aeruginosa* 268. The result show in Figure 4.3 and Table 4.6.

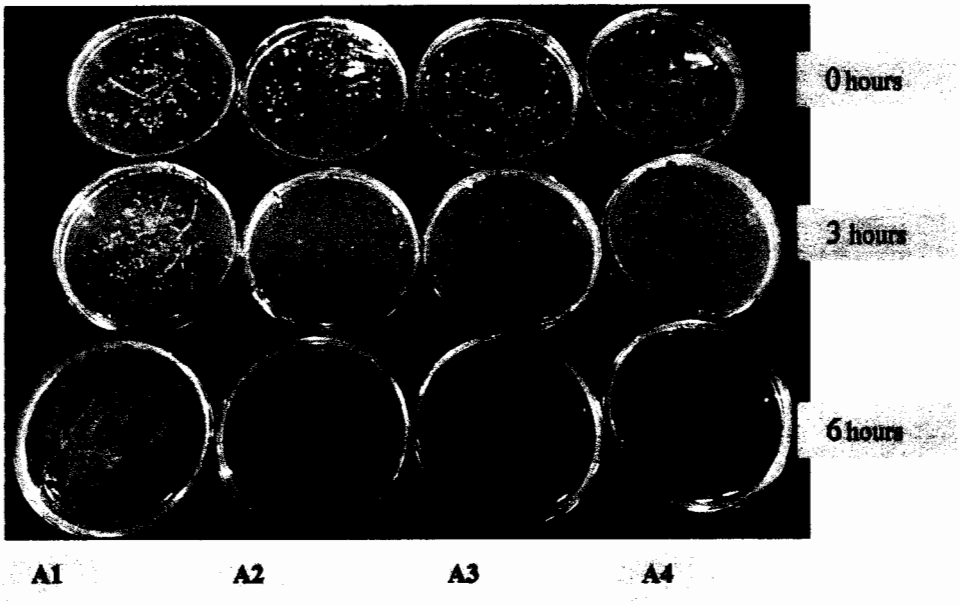


Figure 4.3 The result of bacterial growth inhibition

Picture show result of bacterial growth inhibition at 0hour – 3hour, *P. aeruginosa* 268 + HBI broth found bacteria increases (A1), *P. aeruginosa* 268 + phageWJ9 found bacteria decrease (A2-A4)

Table 4.6 The result of bacterial growth

TIME		Control (*P. aeruginosa* 268 + BHI)									Test (*P. aeruginosa* 268 + bacteriophage WJ9)								
0	Dilution	10^{-4}			10^{-5}			10^{-6}			10^{-4}			10^{-5}			10^{-6}		
	Repeat	1	2	2	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	Colony	186	187	190	25	20	29	5	3	2	-	-	-	-	-	-	-	-	-
	Average	187.6			24.6			3.3			-			-			-		
	SD	1.53			3.13			1.1			-			-			-		
	CFU/ml	$1.8x10^7$			$2.4x10^7$			$3.3x10^7$			-			-			-		
											-								
3	Dilution	10^{-4}			10^{-5}			10^{-6}			10^{-4}			10^{-5}			10^{-6}		
	Repeat	1	2	2	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	Colony	>300	>300	>300	112	96	67	10	18	22	10.3	25	11.3	1	3	0.5	0.5	0.5	0
	Average	>300			91.6			16.6			15.5			1.5			0.3		
	SD	N.D.			16.46			4.46			6.3			1			0.13		
	CFU/ml	TNTC			$9.16x10^7$			$1.66x10^8$			$1.5x10^6$			$1.5x10^6$			$3x10^6$		
6	Dilution	10^{-4}			10^{-5}			10^{-6}			10^{-4}			10^{-5}			10^{-6}		
	Repeat	1	2	2	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	Colony	>300	>300	>300	200	220	200	26	30	21	11	18.3	11	3.6	1.3	1	0	0	0
	Average	>300			206.6			25.6			13.4			1.96			0		
	SD	N.D.			8.93			3.13			3.23			1.08			0		
	CFU/ml	TNTC			$2.0x10^8$			$2.56x10^8$			$1.34x10^6$			$1.96x10^6$			0		
	Average	$\geq 2.28x10^8$																	

0 hour = Control (*P. aeruginosa* 268 + BHI)

3 hour = *P. aeruginosa* 268 + BHI

6 hour = *P. aeruginosa* 268 + BHI

Test (*P. aeruginosa* 268 + bacteriophageWJ9)

Test (*P. aeruginosa* 268 + bacteriophageWJ9)

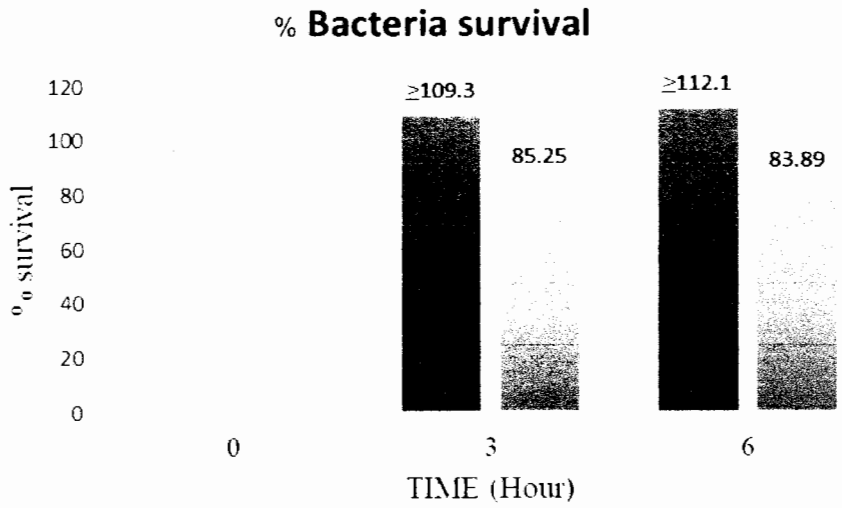


Figure 4.4 Percentage of *P. aeruginosa* 268 Survival

This graph show % Survival of *P. aeruginosa* 268, for control (*P. aeruginosa* 268 + BHI) at 0hour – 6 hour found bacteria continue to cell division but test (*P. aeruginosa* 268 + bacteriophage WJ9) at 6 hour found bacteria decrease cell division 83.89%.

- Control (*P. aeruginosa* 268 + BHI broth)
- *P. aeruginosa* 268 + BHI broth
- Test (*P. aeruginosa* 268 + bacteriophage

4.7 Bacteriophage genome analysis

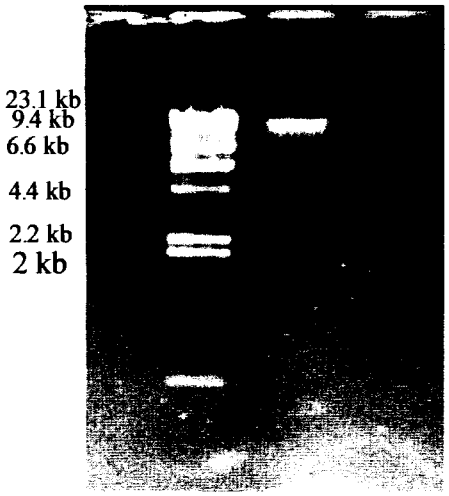


Figure 4.5 the bacteriophage phageWJ9 genome extracted

To analyse bacteriophage genome, bacteriophage genome extraction was firstly performed and followed by restriction endonuclease analysis. As shown in Figure 4.5, the bacteriophage phageWJ9 genome was successfully extracted and verified by agarose gel electrophoresis. Based on this as assay. Lane 1 lambda DNA digested with *Hind*III marker, Lane 2 phage WJ9 nucleic acid.

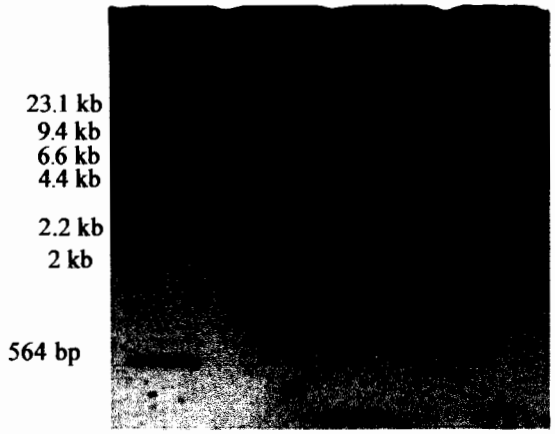


Figure 4.6 Agarose gel electrophoresis of phage WJ9 nucleic acid

Figure 4.6 Ethidium bromide staining gel of the bacteriophage phageWJ9 genome. Lane 1 = marker DNA ladder with size in bp (left side). Lane 2 = digested by RNase, Lane 3 digested by = DNase and Lane 4 = uncut (arrow indicates the size of the amplified product). Agarose gel electrophoresis shown nucleic acid of phage WJ9 were digested by DNase but not RNase as shown in Figure 4.6. Therefore, phage WJ9 have nucleic acid type as DNA.

As shown in Figure 4.7 phageWJ9 genome was digested with different enzymes. It was found that the nucleic acid was not digested with *Eco*RI. The ladder-like bands was obtained after digestion the phage nucleic acid with *Hind*III and *Nco*I. In addition, the nucleic acid of phage WJ9 was digested with DNase, but not for RNase. Therefore, nucleic acid of phage WJ9 was likely to be double stranded DNA because it was digested by *Hind*III and *Nco*I, a restriction enzyme that digest only double stranded DNA sequence. Agarose gel electrophoresis of phage WJ9 nucleic acid after digested by restriction enzyme.

Figure 4.7 PhageWJ9 was digested with different enzymes

Lane 1 lambda DNA digested with *Hind*III marker, Lane 2 phage WJ9 nucleic acid digested with *EcoR*I, Lane 3 phage WJ9 nucleic acid digested with *Hind*III, Lane 4 phage WJ9 nucleic acid digested with *Nco*l, Lane 5 uncut of phage WJ9 nucleic acid. Results of the digestion of nucleic acid isolated from phage WJ9 with different enzymes are shown in Figure 4.7. The nucleic acid was tolerant to *Eco*RI, perhaps it is because nucleic acid of phage WJ9 don' t have site area for digest by *Eco*RI, while it was cut by *Hind*III, and *Nco*I.

CHAPTER 5
DISCUSSION AND CONCLUSION

Antibiotics have been used to treat many infectious diseases caused by pathogens for long period of time. However, the emerging of pathogenic bacteria that are able to resist a variety of antibiotics has been occurred (Parisien and et al, 2008). Recently, multi-drug-resistant (MDR) *P. aeruginosa* have emerged as important nosocomial pathogens that display not only intrinsic resistance to many antibiotics, but also a remarkable ability to develop novel mechanisms of resistance during treatment (Falagas and Kopterides, 2006). This problem has urged scientists to look for alternative therapeutic approaches to against a MDR *P. aeruginosa* infection. One of the promising approaches is bacteriophage therapy, a strategy to inhibit bacterial reproduction by causing bacterial cell lysis. Bacteriophages have high specificity for bacteria genera and species and have an effective selective against multidrug resistant pathogenic bacteria. This bacteriophage's property indicates that they do not harm the normal microflora. Some reports have been shown the human phage therapy trials and *in vivo* studies in animals of using bacteriophage therapy to control bacterial infectious diseases (Inal, 2003). In present study, we found a lytic phage against MDR *P. aeruginosa* in sewage water collected from wastewater treatment plant of Sanprasitthiprasong hospital, Ubon Ratchathani, Thailand. This result is in accordance with the finding of bacteriophage in different place from which its specific bacterial host was isolated is not an unusual result because it has been known for a long time that bacteriophages are wide spread in the environment. Some reports showed the role of phages in bacterial pathogenicity and evolution because phages contribute to bacterial pathogenicity and evolution by multiple mechanisms, including by generating genetic diversity through horizontal gene transfer (Salmond and Fineran, 2015). These are cautions for bacteriophage therapy to treat antibiotic resistant bacteria infection disease because bacteriophage may transfer antibiotic resistant genes form antibiotic resistant bacteria exits to other bacteria strains in the environment.

As a result, the epidemic of resistant genes in bacterial populations has increased. Thus, bacteriophage for treat antibiotic resistant bacteria infection disease should have high specificity for bacteria. In this study, phageWJ9 was shown to be active against approximately at 70% (7/10) of the tested MDR *P. aeruginosa* strains but was not for other tested bacteria. Therefore, phageWJ9 had proper host specificity for being used as a therapeutic agent to control *P. aeruginosa* infectious disease. Bacteriophage classification requires at least two characteristics of phageWJ9 which are type of nucleic acid and morphology. From our study, the nucleic acid of phageWJ9 was likely to be double stranded DNA because it could be digested by *Hind*III, *Nco*I and DNase, but the nucleic acid was tolerant to *Eco*RI, perhaps it is because nucleic acid of phage WJ9 do not have any recognition sites for digest by *Eco*RI, a restriction enzyme digesting only double stranded DNA, but not by RNase, an enzyme digesting only RNA. Transmission electron microscopy revealed that the phage was a tailed phage with an icosahedral head and a long noncontractile tail. Based on these two characteristics and according to the International Committee on Taxonomy of Viruses, phageWJ9 can be classified as a member of the family *Myoviridae*, order *Caudovirales*. This order contains three families, icosahedral head all families, difference only tail, namely, the *Myoviridae* (with long, contractile tail), the *Siphoviridae* (with long, noncontractile tail), and the *Podoviridae* (with short tail), icosahedral head all families (Harper and Enright, 2011). For sensitivity of phageWJ9, several studies documented that chemical resistance of phages varied depending on types of phage (Verma and et al., 2009), for bacteriophageWJ9 could not tolerate in alcohol solution, thus, it is possible to use alcohol to eliminate phageWJ9 if phageWJ9 make it worse treatment outcomes than benefit. However, phageWJ9 was tolerated in 0.85 % normal saline, thus, it may be used in combination with the compounds to improve its therapeutic efficacy. In addition, bacterial inhibition shown that phageWJ9 was shown to be active against *P. aeruginosa* 268, because the percentage of bacteria survival was dramatically decreased after 6 h of incubation, compare with control (without phageWJ9), it is indicated that phageWJ9 has high efficiency for destroying target bacteria. In conclusion, phageWJ9 could be successfully isolated from Wastewater treatment plant, Sanprasitthiprasong hospital, Ubon Ratchathani. It was classified as a member of the family *Myoviridae* in *Caudovirales* order, it showed

high specificity for *P. aeruginosa* genus, the study of the chemical sensitivity of phageWJ9 showed that phageWJ9 was not tolerated in alcohol solution but could be tolerated in 0.85 % normal saline. Bacterial growth inhibition demonstrated that phageWJ9 has high efficiency for destroying target bacteria. Therefore, phageWJ9 is suitable for the next advanced study. Further research is needed to evaluate the potential therapeutic use of the phage to control the MDR *P. aeruginosa* infection in animal and also in human models.

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1. List of chemicals

No.	Chemicals and Reagents	Company
1	Absolute ethanol	AMRESCO®
2	Agarose	AMRESCO®
3	Amikacin	HIMEDIA®
4	Ampicillin	HIMEDIA®
5	DNAse	PanReacAppliChem
6	Ethidime Bromide	AMRESCO®
7	Ceftazidime	HIMEDIA®
8	Ciprofloxacin	HIMEDIA®
9	phenol: chloroform: isoamyl alcohol (1:1:24)	Invitrogen™
10	Isoamyl alcohol	BDH PROLABO®
11	*Eco*RI, *Hind*III and *Nco*I restriction enzyme	Thermo Scientific®
12	RNAse	PanReac AppliChem

2. Media preparation

2.1 Brain Heart Infusion (BHI) agar

Brain Heart Infusion (BHI)	52 g
Agar powder	15 g
Distilled water	1,000 mL

Autoclave and store at 4 °C

2.2 Brain Heart Infusion (BHI) soft agar

Brain Heart Infusion (BHI)	52 g
Agar powder	3 g
Distilled water	1,000 mL

Autoclave and store at 4 °C

2.3 Double strength Brain Heart Infusion (BHI)

Brain Heart Infusion (BHI)	104 g
Distilled water	1,000 mL

Autoclave and store at 4 °C

2.4 Brain Heart Infusion (BHI) broth

Brain Heart Infusion (BHI)	52 g
Distilled water	1,000 mL

Autoclave and store at 4 °C

To prepare 0.5 M EDTA pH 8.0

$Na_2 \cdot EDTA \cdot 2H_2O$	186.1 g
Distilled water	800 mL

Adjust to pH 8.0 with NaOH and adjust volume to 1,000 mL with distilled water, autoclave and store at room temperature.

To prepare Tris-EDTA (TE) buffer

1 M Tris-HCl	10 mL
0.5 EDTA pH 8.0	2 mL
Distilled water	980.8 mL

Autoclave and store at room temperature.

3.6 Tris-acetate-EDTA buffer (TAE)

To prepare 0.5 M EDTA pH (8.0)

$Na_2 \cdot EDTA \cdot 2H_2O$	186.1 g
Distilled water	800 mL

Adjust to pH 8.0 with NaOH and adjust volume to 1,000 mL with distilled water, autoclave and store at room temperature.

To prepare Tris-acetate-EDTA buffer (TAE)

Tris base	4.84 g
Glacial acetic acid	1.142 mL
0.5 EDTA (pH 8)	2 mL
Distilled water	800 mL

adjust volume to 1,000 mL with distilled water.

4. List of instruments

No.	Instruments	Company
1	Class II, Biological Safety Cabinets	Bio-Clean Air Devices & Services
2	MPW-380R refrigerated laboratory centrifuge	MPW MED. INSTRUMENTS
3	Digital dry bath	Labnet International Inc.
4	UVP's ChemiDoc-ItTS2 Imagers	Analytik Jena
5	Heating / drying ovens	MEMMERT
6	High-pressure steam sterilizer	WiseClave - autoclave
7	Incubator	Contherm digital series
8	pH Meter	SI Analytics
9	PowerPac™ Basic Power Supply	Mupid®-One
10	Transmission Electron Microscope	JEOL Ltd.

สารบัญ

การคัดแยก จำแนกและศึกษาคุณลักษณะของแบคเทอริโอเฟจที่จำเพาะต่อ Pseudomonas aeruginosa

[redacted]

บทคัดย่อ

เชื้อ Pseudomonas aeruginosa เป็นแบคทีเรียแกรมลบรูปแท่ง เป็นสาเหตุหนึ่งของโรคติดเชื้อในโรงพยาบาล และมักเป็นสายพันธุ์ที่ดื้อยาปฏิชีวนะในระดับสูงและหลายชนิด การนำมาใช้การรักษาโรคติดเชื้อแบบใหม่เพื่อทดแทนการใช้ยาปฏิชีวนะจึงเป็นสิ่งที่น่าสนใจ โดยเฉพาะอย่างยิ่งการรักษาด้วยแบคทีริโอเฟจ การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อแยกและศึกษาคุณสมบัติของแบคทีริโอเฟจที่จำเพาะต่อเชื้อ P. aeruginosa สายพันธุ์ดื้อยา และการศึกษาประสิทธิภาพในการแยกแบคทีริโอเฟจจากแหล่งน้ำเสีย โรงพยาบาลจากโรงพยาบาลต่าง ๆ ในจังหวัดอุบลราชธานี ที่จำเพาะต่อเชื้อ P. aeruginosa สายพันธุ์ดื้อยาปฏิชีวนะได้ การศึกษาคุณสมบัติของแบคทีริโอเฟจ ได้แก่ การทนต่อสารเคมี ความทนต่อแบคทีริโอเฟจในสภาวะทนต่อสารละลายเกลือต่าง ๆ และความสามารถในการละลายน้ำเลี้ยงได้ การจำแนกชนิดของแบคทีริโอเฟจด้วยวิธีการตัดสารพันธุกรรมและการตัดด้วยเอนไซม์ตัดจำเพาะ ECoRI พบว่าแบคทีริโอเฟจเป็นไวรัสที่มีสารพันธุกรรมเป็นดีเอนเอ สิ่งนี้การศึกษาครั้งนี้แสดงให้เห็นประสิทธิภาพของแบคทีริโอเฟจการทำลายแบคทีเรียที่ดื้อยาปฏิชีวนะได้อย่างจำเพาะ จึงจะเป็นข้อมูลพื้นฐานในการประยุกต์ใช้ในอนาคต

คำสำคัญ Pseudomonas aeruginosa, Bacteriophage, Multidrug-resistant bacteria, Bacteriophage therapy

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