รายงานวิจัยฉบับสมบูรณ์

โครงการ: การพัฒนาวิธีตรวจวัด *Bacteroides*-infecting bacteriophages ด้วยเทคนิคทางดีเอ็นเอเพื่อการบ่งชี้การปนเปื้อนของแหล่งน้ำจากสิ่งปฏิกูลจากคน

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มิถุนายน 2555

สัญญาเลขที่ MRG5380297

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา สำนักงานกองทุนสนับสนุนการวิจัย และสถาบันวิจัยจุฬาภรณ์

บทคัดย่อ

รหัสโครงการ: MRG5380297

ชื่อโครงการ: การพัฒนาวิธีตรวจวัด *Bacteroides*-infecting bacteriophages ด้วยเทคนิคทางดีเอ็นเอเพื่อการบ่งชี้การปนเปื้อนของแหล่งน้ำจากสิ่งปฏิกูลจากคน

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บทคัดย่อ: ด้วยข้อจำกัดของการใช้แบคทีเรียชี้แนะกลุ่มดั้งเดิม (Traditional Fecal Indicators) เป็นมาตรฐานทางชีววิทยาของคุณภาพน้ำ จึงมีการศึกษาจุลินทรีย์กลุ่มอื่นๆที่สามารถนำมาใช้เป็นตัวบ่งชี้คุณภาพน้ำทางชีววิทยาได้ดี หนึ่งในนั้นคือเฟจของแบคทีรอยดีส (bacteriophages of *Bacteroides)* งานวิจัยชิ้นนี้มีวัตถุประสงค์เพื่อพัฒนาวิธีการตรวจหาดีเอ็นเอของเฟจของแบคทีรอยดีส สำหรับใช้ตรวจสอบการปนเปื้อนของสิ่งปฏิกูลจากคนและมูลสัตว์ในแหล่งน้ำของประเทศไทย จากการตรวจหาเฟจชนิด ATCC 51477-B1 และ ATCC 700786-B1 ในน้ำเสียที่เกิดจากคนและสัตว์ในประเทศไทย พบเฟจ ATCC 51477-B1 ในน้ำที่มีสิ่งปฏิกูลจากคนปนเปื้อน โดยตรวจไม่พบหรือพบเฟจชนิดนี้ในปริมาณต่ำมากในตัวอย่างน้ำที่ได้จากสัตว์ แสดงให้เห็นว่าเฟจชนิด ATCC 51477-B1 มีความจำเพาะกับคน ส่วนเฟจชนิด ATCC 700786-B1 ตรวจพบได้บ่อยกว่าในน้ำ จึงจำเป็นต้องพัฒนาวิธีที่ใช้แยกเฟจชนิด ATCC 51477-B1 ออกจากเฟจชนิด ATCC 700786-B1 ด้วยการหาลำดับเบสที่จำเพาะกับเฟจชนิด ATCC 51477-B1 โดยการตัดด้วยเอนไซม์ และการวิเคราะห์หาลำดับเบส จากนั้นทำการพัฒนาวิธี PCR ขึ้นใหม่ และนำมาทดสอบกับน้ำเสียตัวอย่างจากคนและจากสัตว์ พบว่า วิธี PCR ที่พัฒนาขึ้นใหม่นี้ไม่สามารถตรวจพบเฟจ ATCC 51477-B1 ได้แม้ในตัวอย่างที่มีปริมาณเฟจ 257 PFU ต่อ 100 มิลลิลิตร นอกจากนี้ การศึกษานี้ไม่พบความสัมพันธ์ของปริมาณแบคทีเรียโคลิฟอร์มทั้งหมด (total coliforms) และอีโคไล (*E. coli*) กับเฟจชนิด ATCC 51477-B1 กล่าวคือในน้ำเสียตัวอย่างเดียวกัน พบแบคทีเรียดังกล่าวจำนวนมาก แต่กลับไม่พบเฟจ ATCC 51477-B1 เลยทั้งในการวัดด้วยวิธีเพาะเลี้ยงและวิธี PCR ผลการศึกษานี้เป็นการต่อยอดองค์ความรู้ในด้านการตรวจหาที่มาของการปนเปื้อนของแหล่งน้ำด้วยจุลินทรีย์ (microbial source tracking) และเป็นประโยชน์ต่อการจัดการน้ำและการควบคุมมลพิษในแหล่งน้ำต่อไป

คำหลัก : การปนเปื้อนสิ่งปฏิกูล, คุณภาพน้ำ, การตรวจหาที่มาของการปนเปื้อนของแหล่งน้ำด้วยจุลินทรีย์, เฟจของแบคทีรอยดีส, เทคนิคพีซีอาร์

Abstract

Project Code: MRG5380297

Project Title: Development of a DNA-based method for bacteriophages of *Bacteroides* for tracking human-derived fecal pollution in water sources

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Project Period: 2 years

Abstract: Traditional fecal indicators, including a group of coliforms (total and fecal), *E. coli*, and enterococci have many shortcomings that have directed researchers and authoritative parties to discovering new group(s) of indicators. There have been efforts to study several organisms as new groups of fecal indicators, among of which are bacteriophages of *Bacteroides*. The ultimate goal of this research project was to develop a new and rapid DNA-based method to measure phages of *Bacteroides* for a purpose of identifying human-derived fecal pollution in water sources. Firstly, to determine the presence of *Bacteroides* phages in Thailand, the strains ATCC 51477-B1 and ATCC 700786-B1 were enumerated in wastewater samples. Phage ATCC 51477-B1 was positively detected in some human-derived wastewater samples, but was found at very low levels, if not absent, in animal-derived samples. This implies that phage ATCC 51477-B1 is specific to human sources. Secondly, since a non-specific strain (ATCC 700786-B1) was found more prevalently in water, it is important for the newly-designed DNA-based assay not to pick up this non-specific phage strain as well. The DNA regions of phage strain ATCC 51477-B1 that were not present in phage strain ATCC 700786-B1 were therefore determined using molecular techniques such as restriction enzyme digestion, DNA sequencing and sequence analysis. After that, the PCR primers were designed, the PCR reaction components and cycling conditions were optimized, and the PCR method detection limit was determined. Thirdly, the newly-designed PCR method was used with human- and animal-derived wastewater samples to detect phage ATCC 51477-B1. The results indicated that the amount of phage ATCC 51477-B1 of up to 257 PFU/100 ml in wastewater samples was not detected by the PCR assay. Lastly, traditional fecal indicators, i.e., total coliforms and *E. coli*, enumerated in the same set of water samples showed high concentrations, while phage strain ATCC 51477-B1 was not detected in most samples by both the culture method and the PCR assay. Therefore, no correlation was observed. This information helps to advance the knowledge in the field of microbial source tracking that will eventually facilitate in water management and pollution control of water sources.

Keywords: Fecal contamination, water quality, microbial source tracking, bacteriophages of *Bacteroides*, PCR

Executive Summary

The needs to obtain new fecal pollution indicators and/or to develop new detection methods have been emphasized worldwide. Especially in the United States, with increasing evidences that current fecal indicator bacteria (FIB) is not a good indicator for fecal pollution due to regrowth and non-host specific indication, U.S. EPA is on its way to look for another new fecal indicator organisms, in order to revise and set new recreation water quality criteria. Many promising indicator organisms have been studied and proposed as alternative indicator groups. Among these are a group of bacteriophages that specifically infect bacteria in the genus *Bacteroides*. These types of viruses, together with their anaerobic bacterial hosts, reside in the gastrointestinal tract of warm blooded animals, including: humans, pigs, cows, horses, and dogs. Bacteriophage strain ATCC 51477-B1, which infects *B. fragilis* strain HSP40 (ATCC 51477), has been reported to be found only in water samples contaminated with human-derived fecal materials. On the other hand, the strain ATCC 700786-B1, which infects *B. fragilis* strain RYC2056 (ATCC 700786), was shown to be detected in fecally contaminated water samples of both human and animal origins. Human-derived fecal pollution may carry human pathogens that pose higher risk to public health than does fecal contamination of animal origins. Therefore, an application of microorganisms for fecal source identification, a so-called Microbial Source Tracking (MST), is of beneficial use.

The ultimate goal of this research project was to develop a new and rapid DNA-based method to measure phages of *Bacteroides* for a purpose of identifying human-derived fecal pollution in water sources. The specific objectives were to (1) detect human-specific phages of *Bacteroides* in Thailand, (2) design a PCR assay for detection of human-specific phages of *Bacteroides*, (3) validate the *Bacteroides* phage PCR assay with wastewater samples, and (4) compare the human-specific *Bacteroides* phage PCR assay with other fecal indicators.

Firstly, to determine the presence of *Bacteroides* phages in Thailand, the strains ATCC 51477-B1 and ATCC 700786-B1 were enumerated in wastewater samples. Raw sewage samples from influents of wastewater treatment facilities were collected by varying types of facility types and populations served, however, of at least 100 inhabitants. The types of facilities included office buildings, hospitals and residential buildings in Bangkok and nearby provinces. Animal-derived fecal wastewater samples were collected from floor cleaning runoff, which contained feces of swine and cattle from farms near Bangkok. *B. fragilis* strains HSP40 (ATCC 51477) and RYC2056 (ATCC 700786) were used as bacterial hosts. A double layer agar method with incubation under anaerobic conditions was used for

phage quantification. Both types of phages were detected in wastewater samples from hospitals, but not from residential and office facilities. Phage 700786-B1 was more frequently detected and present in higher concentrations in both human- and animal-influenced samples. Phage 51477-B1 was positively detected in some human-derived wastewater samples, but was found at very low levels or was absent in animal-derived samples. This implies that phage 51477-B1 is specific to human sources.

Secondly, a PCR assay for detection of human-specific phages of *Bacteroides* was designed. DNA sequence differences between bacteriophage strains ATCC 51477-B1 and ATCC 700786-B1 were checked using HindIII and Eco72I restriction enzyme digestion. The results showed the different restriction enzyme digestion patterns. Since a non-specific strain (ATCC 700786-B1) was found more prevalently in water, it is important for the newly-designed DNA-based assay not to pick up this non-specific phage strain as well. The DNA regions of phage strain ATCC 51477-B1 that were not present in phage strain ATCC 700786-B1 were therefore determined. The whole genomic sequencing results were performed with Ion Torrent Personal Genome Machine System. The resulting DNA sequences were assembled and analyzed with de novo genome assembly software and Blastn program. The four regions were found as follows: node 13 with 866 bp in length and 99.75% coverage, node 79 with 443 bp in length and 86.20% coverage, node 9 with 941 bp in length and 86.26% coverage, and node 43 with 440 bp in length and 97.91% coverage. Four nodes of DNA of bacteriophage strain ATCC 51477-B1 DNA were therefore used to design PCR primers using the Primer-BLAST tool. The primer pairs that could match DNA of only bacteriophage strain ATCC 51477-B1 were selected for the subsequent PCR designing steps. These primer pairs included BT3759-BT3760, BT3761-BT3762, and BT3763-BT3764 from DNA node 13, and BT3765-BT3766 from DNA node 79. Next, the primer pairs were tested with purified DNA of bacteriophage ATCC 51477-B1 and ATCC 700786-B1. Primer pairs BT3765-BT3766 was selected for further steps due to the absence of non-specific bands. PCR reaction components and cycling conditions were optimized. Method detection limit (MDL) for DNA template concentrations of bacteriophage ATCC 51477-B1 DNA was 0.002 ng in 10 µl PCR reaction. PCR primers, reaction components and cycling conditions are summarized below:

Summary of PCR reaction components and PCR cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	Volume (µl)	PCR step	Cycle	Temp (^{0}C)	Time
Template (at least 0.001 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (5 µM)	1	Annealing		55	20 sec
Reverse primer (5 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold		16	∞

Thirdly, the newly-designed PCR method was used with wastewater samples to detect phage ATCC 51477-B1. In order to account for DNA loss during DNA extraction step, known amount of DNA of another organism was spiked into the water samples before the DNA extraction step. DNA of *Acinetobacter sp*. ADP1 was used. PCR primers for *Acinetobacter sp*. ADP1 were designed and PCR reaction components and cycling conditions were optimized. PCR primers, reaction components and cycling conditions for *Acinetobacter sp*. ADP1 are summarized below.

Summary of PCR reaction components and PCR cycling conditions of *Acinetobacter sp*. ADP1 DNA with primers BT3714-BT3715

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	Volume (µl)	PCR step	Cycle	Temp (^{0}C)	Time
Template (at least 0.01 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (5 µM)	1	Annealing		55	20 sec
Reverse primer (5 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold		16	∞

The PCR detection methods of bacteriophage ATCC 51477-B1 and *Acinetobacter sp.* ADP1 were tested with 22 wastewater samples from the influent of municipal wastewater treatment facilities, the influent of residential buildings, and farm slurries. Results showed no positive detection when wastewater samples contained the concentration of bacteriophage ATCC 51477-B1 up to 257 PFU/100 ml and spiked concentrations of *Acinetobacter sp*. ADP1 DNA of 100 ng.

Lastly, total coliforms and *E. coli* were measured by the membrane filtration method in 22 wastewater samples: 7 samples were farm slurries collected from animal farms, while the rest were human-derived wastewater samples. There were relatively high values of the total coliforms in the range of 520,000-35,350,000 CFU/100 ml and *E. coli* in the range of 170,000-29,550,000 CFU/100 ml. On the other hand, phage strain ATCC 51477-B1 was not detected in most samples by both the culture method and the PCR assay. Therefore no correlation analysis was observed due to many negative results in phage ATCC 51477-B1 detection.

Chapter 1: Research Objectives

The ultimate goal of this research project was to develop a new and rapid DNA-based method to measure phages of *Bacteroides* for a purpose of identifying human-derived fecal pollution in water sources.

The specific objectives are presented in chronological order as follows.

1. Detection of human-specific phages of *Bacteroides* in Thailand
2. Designing a PCR assay for detection of human-specific phages of *Bacteroides*
3. *Bacteroides* phage PCR assay validation with wastewater samples
4. Comparison of the human-specific *Bacteroides* phages PCR assay with other fecal indicators

The research strategies, including specific objectives and their corresponding tasks, are illustrated in **Figure 1.1**.

The specific tasks corresponding to the research objectives are presented as follows.

Objective 1: Detection of human-specific phages of *Bacteroides* in Thailand

Specific task:

1. Detection of phages of *B. fragilis* strain ATCC 51477 from wastewater samples in Thailand

Objective 2: Designing a PCR assay for detection of human-specific phages of *Bacteroides*

Specific tasks:

1. Locating specific regions of DNA of a reference phage strain ATCC 51477-B1
2. Designing primers and assay conditions for a PCR assay
3. Determination of the detection limit of the assay

Objective 3: *Bacteroides* phage PCR assay validation with wastewater samples

Specific tasks:

1. Designing primers and assay conditions for a PCR assay of the spiked *Acinetobacter sp*. ADP1 to estimate DNA loss
2. Determination of the detection limit of the *Acinetobacter sp*. ADP1 PCR assay
3. Detection of bacteriophage DNA using the *Bacteroides* phage PCR assay in human- and animal-derived wastewater samples

Objective 4: Comparison of the human-specific *Bacteroides* phages PCR assay with other fecal indicators

Specific tasks:

1. Measurement of traditional fecal indicators in human- and animal-derived fecal materials and wastewaters

2. Correlation analysis

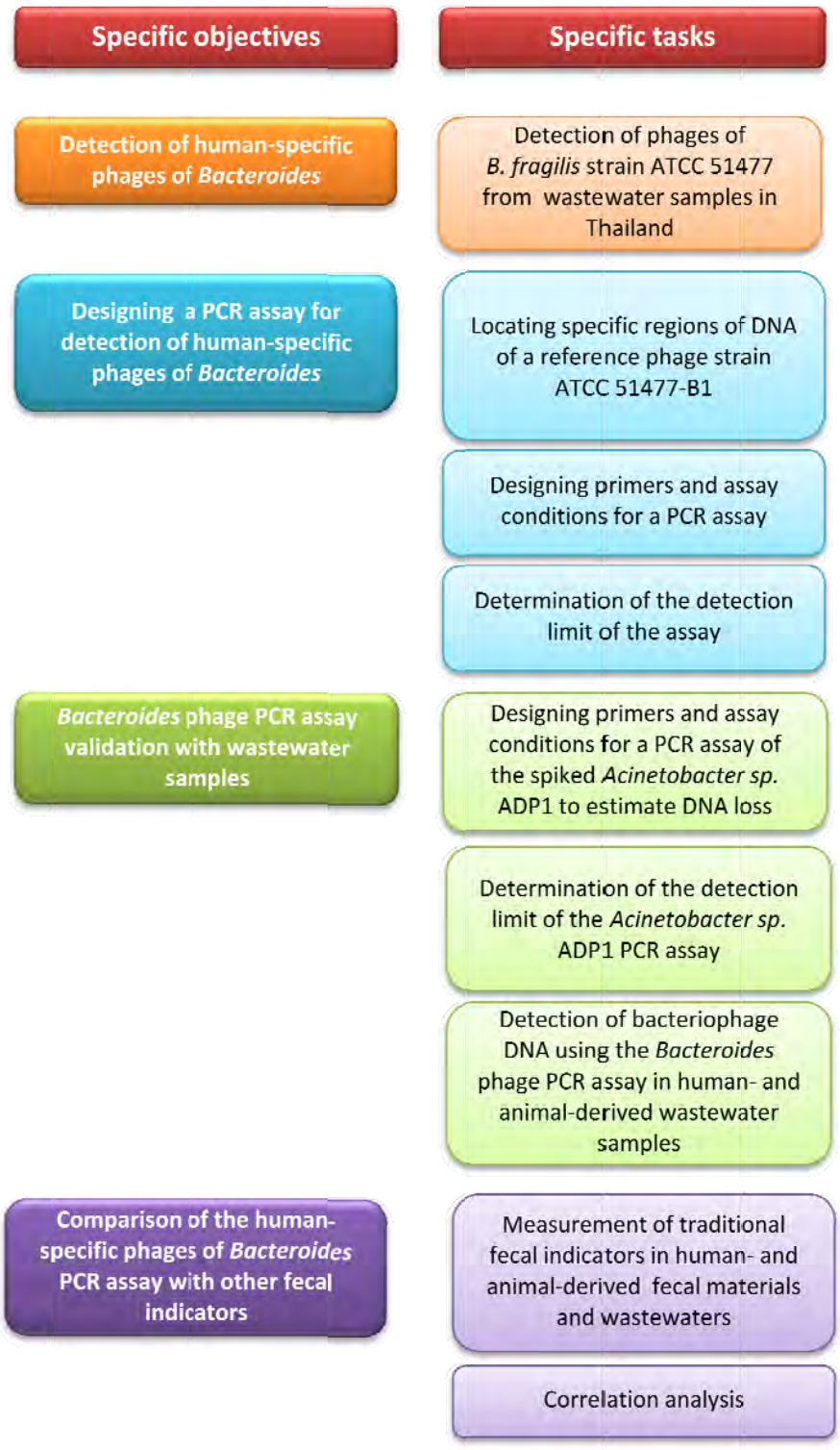

Figure 1.1 The research strategies

Chapter 2: Research Methodology

2.1 Experimental plan

Table 2.1 Experimental plan: specific objectives, specific tasks and methodology

No.	Specific objectives	Specific tasks	Methodology
1	Detection of human-specific phages of *Bacteroides* in Thailand	1.1 Detection of phages of *B. fragilis* strain ATCC 51477 from wastewater samples in Thailand	2.3.1 Water sampling 2.3.2 *Bacteroides* host strains and bacteriophage 2.3.3 Phage enumeration by a double agar overlay plaque assay
2	Designing a PCR assay for detection of human-specific phages of *Bacteroides*	2.1 Locating specific regions of DNA of a reference phage strain ATCC 51477-B1	2.3.4 Concentration and purification of phages 2.3.5 DNA extraction of phage by phenol-chloroform extraction 2.3.6 Locating homologous DNA regions of phages with PCR primers 2.3.7 Restriction enzyme digestion 2.3.8 Whole genome sequencing 2.3.9 Whole genomic DNA assembly and sequence analysis
		2.2 Designing primers and assay conditions for a PCR assay	2.3.10 PCR primer designing 2.3.11 PCR assay optimization

Table 2.1 (Cont'd) Experimental plan: specific objectives, specific tasks and methodology

No.	Specific objectives	Specific tasks	Methodology
		2.3 Determination of the detection limit of the assay	2.3.12 Determination of the detection limit
3	*Bacteroides* phage PCR assay validation with wastewater samples	3.1 Designing primers and assay conditions for a PCR assay of the spiked *Acinetobacter sp*. ADP1 to estimate DNA loss	2.3.13 Culture of *Acinetobacter sp.* ADP1 2.3.10 PCR primer designing 2.3.11 PCR assay optimization
		3.2 Determination of the detection limit of the *Acinetobacter sp*. ADP1 PCR assay	2.3.12 Determination of the detection limit
		3.3 Detection of bacteriophage DNA using the *Bacteroides* phage PCR assay in human- and animal-derived wastewater samples animal-derived fecal wastewaters	2.3.14 Recovery bacteriophage of *Bacteroides* from wastewater samples 2.3.2 *Bacteroides* host strains and bacteriophage 2.3.3 Phage enumeration by a double agar overlay plaque assay
4	Comparison of the human-specific *Bacteroides* phages PCR assay with other fecal indicators	4.1 Measurement of traditional fecal indicators in human- and animal-derived fecal materials and wastewaters	2.3.15 Measurement of traditional fecal indicators in human- and animal-derived fecal materials and wastewaters
		4.2 Correlation analysis	2.3.16 Correlation analysis

2.2 Materials

2.2.1 Microorganisms

Bacteroides fragilis strains ATCC 51477 and ATCC 700786, bacteriophage strains ATCC 51477-B1 and ATCC 700786-B1, and *Acinetobacter baylyi* strain ADP1

2.2.2 Chemicals, enzymes and biological materials

QIAamp DNA Mini Kit , QIAquick® Gel Extraction Kit, QIAprep® Spin Miniprep Kit, meat peptone , tryptone, yeast extract, glucose, sodium chloride, magnesium sulfate·$7H_2O$, monohydrated L-cysteine HCl, calcium chloride, hemin, sodium hydroxide, sodium carbonate, hydrochloric acid , kanamycin, nalidixic acid, paraffin oil, glycerol, Albumin-bovine, sucrose, , PEG M_r8000, sodium chloride, Tris-base, EDTA , phenol, chloroform, ethanol, sodium acetate, 8-hydroxyquinoline, HindIII restriction enzyme, agarose gel, ethidium bromide, agar, acetic acid, Agarose gel, 2x Taq PCR master mix, DMSO

2.3 Methods

2.3.1 Water sampling

Wastewater samples from buildings serving at least 100 inhabitants (Blanch et al., 2006) were collected from the Bangkok area and nearby provinces. The types of facilities sampled included: office buildings, hospitals, and residential buildings. Animal-derived fecal wastewater samples were collected from floor cleaning runoff, which contained feces of swine and cattle from farms near Bangkok. Grab samples of water were performed in 50-ml sterile centrifuge tubes and were stored on ice or at 4°C until used for analysis.

2.3.2 *Bacteroides* host strains and bacteriophages

Bacteroides fragilis strains HSP40 (ATCC 51477) and RYC2056 (ATCC 700786), and bacteriophage strains B-40 (ATCC 51477-B1) and B56-3 (ATCC 700786-B1) were shipped from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) in freezed-dried forms. Upon arrival, the bacterial strains were rehydrated with *Bacteroides* phage recovery medium (BPRM), containing meat peptone, 10 g; casein peptone, 10 g; yeast extract, 2 g; NaCl, 5 g; monohydrated l-cystein, 0.5 g; glucose, 1.8 g; $MgSO_4.7H_2O$, 0.12 g; $CaCl_2$ solution (0.05 g/ml), 1 ml; hemin, 10 ml of a 0.1% (w:v) solution made up in NaOH 0.02%; 1M Na_2CO_3, 25 ml; and water up to 1 l, with an addition of hemin and Na_2CO_3 and pH

adjustment with 35%HCl to 6.8±0.5 after sterilization, and supplementing 100 µg of kanamycin and 100 µg of nalidixic acid per ml of media before use (Araujo *et al*., 2001). The bacterial cultures were anaerobically grown to reach a log phase at a final concentration of approximately 1 x 10^9 CFU/ml. The cultures were then mixed with a ratio of 1:1 with filtered-sterile Bovine Serum Albumin (BSA) – sucrose solution, containing 10% bovine serum albumin factor V and 20% sucrose in distilled water (Araujo et al., 2001), aliquoted into 1-ml portions and stored at -80°C until use. On the day of experiment, one vial of the frozen bacterial culture was thawed, then suspended in 9-ml BPRM broth and incubated at 37°C in anaerobic conditions for 6-7 hours to reach a concentration of approximately 1x10^8 CFU/ml. The culture is then ready for use in phage enumeration assay.

The freeze-dried *Bacteroides*-infecting bacteriophage ATCC 51477-B1 and ATCC 700786-B1 were rehydrated with the BPRM broth and propagated on their corresponding bacterial host strains. Briefly, the phage suspension was mixed with BPRM soft agar, containing BPRM broth with an addition of 7 g of agar per liter of media, and added on bacterial lawn in a petri dish, following anaerobic incubation at 37°C. After 24 hr incubation, 10-ml BPRM broth was added and the soft agar was scraped off the surface and centrifuged at 1,000 rpm for 25 min to sediment the cellular debris and agar. The supernatant was subsequently filtered with a 0.22 um syringe filter, aliquoted and stored at 4°C until use.

2.3.3 Phage enumeration by a double agar overlay plaque assay

The double agar layer method is used for phage enumeration by following Ebdon *et al*. (2007). To summarize, the *Bacteroides* host strain was grown in BPRM broth to the proper optical density. The impurities in water samples were filtered with 0.22 µm polyvinylidene difluoride (PVDF) membrane. Then, 1 mL of the filtered water sample and 1 mL of the *Bacteroides* host strain were mixed with 5.0 mL of BPRM soft agar. The mixture was poured onto the surface of BPRM agar, containing BPRM broth with 15 g of agar per liter of media, and was subsequently allowed to solidify. The plates were incubated for 18±2 h at 36±2°C in anaerobic conditions. The number of phages was counted as plaque forming units (PFU).

2.3.4 Concentration and purification of phages

Three ml of *Bacteroides* working stock was grown in 27 ml of BPRM broth, and incubated anaerobically by adding a top layer of paraffin oil, at 37°C for 18-24 h. After that, 80 ml of BPRM broth was added with 8 ml of the overnight *Bacteroides* culture, approximately 10^7 PFU of phage suspension, and a top layer of sterile paraffin oil. The

culture was incubated at 37°C for 18-25 h, until clear, lysed bacterial cells in the suspension were observed. A control sample was also prepared by adding 8 ml of the same overnight *Bacteroides* culture, with no phage suspension, and a top layer of sterile paraffin oil to 80 ml of BPRM broth. The control sample was then incubated along with the working culture in order to indicate clear, lysed bacterial cells in the working suspension. Next, the suspension was aliquoted into 3 of 50-ml centrifuge tubes and centrifuged at 8,000 x g for 10 min at room temperature. The cell debris at the bottom of the centrifuge tubes was discarded. The supernatant containing phage was centrifuged one additional time to remove existing cell debris and in the suspension. Then 250 µl mixture of 25% polyethylene glycol (PEG; M_r 8,000) and 2.5 M filtered-sterile sodium chloride was added per 1.25 ml of the supernatant. The suspension was continuously stirred in a 4°C refrigerator for at least 30 min. Then the suspension was aliquoted into 3 of 50-ml centrifuge tubes, followed by centrifugation at 8,000 xg for 15 min at 4ºC. The supernatant was discarded. The pellet containing phage particles were resuspended with 2-10 ml Tris-EDTA (TE) buffer (10:1 Tris:EDTA, pH 8.0). The resulting purified phage particles were stored at 4°C until use (adapted from Srifah, 1991).

2.3.5 DNA extraction of phage by phenol-chloroform extraction

1 volume of purified phage in TE buffer (10:1 Tris:EDTA, pH 8.0) was mixed with an equal volume of TE-saturated phenol (pH 8.0, see section 3.3.2.3). The mixture was vortexed for 10 s, and rested on ice for 5 min, followed by another 10-s vortexing. The solution was centrifuged at 5,000 xg for 3 min at room temperature. The upper layer of the solution was transferred to another tube. The solution was added with 0.5 volume of TE-saturated phenol (pH 8.0) and 0.5 volume of chloroform. The mixture was vortexed for 10 s, and rested on ice for 5 min, followed by another 10-s vortexing. The solution was centrifuged at 5,000 xg for 3 min at room temperature. The upper layer was transferred to another tube. 2.5 volume of cool absolute ethanol and 0.1 volume of 3 M sterile sodium acetate was added to the solution. The mixture was then incubated at -20ºC overnight. Next, the suspension was centrifuged at 8,000 xg for 15 min at 4°C. The supernatant was discarded. The pellet was mixed with 2 volume of 80% ethanol and incubated at room temperature for 10 min. The suspension was centrifuged at 8,000 xg for 15 min at 4ºC. The supernatant was discarded. The pellet was left dried at room temperature for 2-3 h, before resuspended with 400 µl of TE buffer (10:1 Tris: EDTA, pH 8.0). The purified DNA was stored at -20°C until use (adapted from Srifah, 1991). DNA concentration was measured by a Nanodrop 2000 spectrophotometer (Thermo Scientific). TE-saturated phenol (pH 8.0) was prepared by the

following steps: phenol (Merck) was melted at 65°C before an addition of 8-hydroxyquinoline to a final concentration of 0.1% (w/v). An equal volume of 1 M Tris-HCl (pH 8.0) was added and incubated at 4ºC for several days. Once the pH of the solution reached 8.0, an equal volume of TE buffer (10:1 Tris:EDTA, pH 8.0) was added and incubated at 4°C for several days (Sambrook and Russell, 2001).

2.3.6 Locating homologous DNA regions of phages with PCR primers

DNA extracts of bacteriophage ATCC 51477-B1 and ATCC 700786-B1 were amplified with PCR primers as shown in **Table 2.2** (Puig *et al*., 2000). Originally, these primers were designed to use for bacteriophage ATCC 51477-B1. Therefore, using these primers can detect a homologous region between this strain and the strain ATCC 700786-B1.

Table 2.2 Primer sequences for DNA amplification of phage ATCC 51477-B1 and ATCC 700786-B1 (Puig *et al*., 2000)

Primer	Sequence (5'→ 3')	Length (base pair)
pH 5-1 forward	GGG AAA GCA CAC AAG CG	17
pH 5-3 reverse	CAG AAC ATT AGT TTT ACG G	19
npH 5-4 forward	GTG GCA CGT GAA CTT CCT TC	20
npH 5-7 reverse	CGT TTT GCA TGG CAT CCG	18

A final concentration of a 50-µl PCR reaction contained 10 µl of Phusion HF buffer, 200 µM dNTPs, 0.4 µM of forward and reverse primers, 1 µl of template DNA, 3% DMSO, and 1 U of Phusion DNA polymerase (Finnzymes). The amplification conditions were composed of an initial denaturation at 98ºC for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 59ºC for npH primer pair and at 53.2°C for pH primer pair for 30 s, and extension at 72ºC for 15 s, followed by a final extension held at 4°C in the last cycle. The PCR products were run on 1% agarose gel with ethidium bromide staining and imaged by the Gel Doc XR System (Biorad). PCR products of interest were purified by QIAquick PCR Purification Kit (Qiagen) and subsequently submitted for DNA sequencing. DNA sequences were analyzed using the Chromas Pro program (Technelysium Pty Ltd.).

Moreover, new sets of primers were designed in this study and PCR conditions were optimized, in order to identify homologous regions between phage strains ATCC 51477-B1 and 700786-B1 (**Table 2.3**). The primers were designed with the Basic Local Alignment

Table 2.3 Sequences of primers designed to amplify different parts of phage genome

Primer	Sequence (5'→ 3')	Length (bp)	Product Size (bp)	DNA region[1]	Product sequence position[1]	Annealing temperature (ºC)
BT3530 forward	CTA ACA AGC ACG ATG ATC CGG C	22	950	Major tail protein 2	37176-38125	N/A[2]
BT3531 reverse	CTC CGG TAT ATG CCA CCC TCC AT	23				
BT3532 forward	AGG GTG GCA TAT ACC GGA GGA C	22	1,880	Putative capsid MP3	38107-39986	58.5
BT3533 reverse	ACC GGG ACG CTG ATT CTT TGC C	22				
BT3534 forward	ACA AGA TCG CAG ATT TGC CAA CAG A	25	1,405	Capsid protein MP1	41134-42538	N/A[2]
BT3535 reverse	GCT TTC TCG TTA CGC TCC GCC A	22				
BT3559 forward	TCC GGA GGC GGA CAG GGT AAC	21	1,017	Capsid protein MP1 first half	40977-41993	63
BT3560 reverse	CTG CAC GGT CTA CAC GGG TC	20				
BT3561 forward	TGC ACC CGA TGT TCC GTG CAA A	22	906	Capsid protein MP1 second half	41748-42653	63
BT3562 reverse	TGC CGC CAT CCT ACG AGC G	19				

Remarks [1]DNA region and sequence position were based on the whole genome sequence of phage strain ATCC 51477-B1 as deposited in Genbank with accession number FJ008913 (Hawkins *et al*., 2008)

[2]PCR amplification conditions followed a 2-step protocol

Search Tool (BLAST; NCBI) based on whole genome sequence of phage strain ATCC 51477-B1 as deposited in Genbank with accession number FJ008913 (Hawkins *et al*., 2008). A 50-µl PCR reaction contained similar contents as aforementioned. PCR amplification cycles were performed with an initial denaturation at 98ºC for 30 s, followed by 35 cycles of a denaturation at 98ºC for 10 s, an annealing step for 30 s at temperature as indicated in **Table 2.3**, and an extension step at 72ºC for 30-55 s, and followed by a final extension of 72ºC for 10 min. The PCR products were run on 1% agarose gel with ethidium bromide staining and imaged by the Gel Doc XR System (Biorad). PCR products of interest were

purified by QIAquick PCR Purification Kit (Qiagen) and subsequently submitted for DNA sequencing. DNA sequences were analyzed using the Chromas Pro program (Technelysium Pty Ltd.).

2.3.7 Restriction enzyme digestion

Known genomic sequence of bacteriophage ATCC 51477-B1was analyzed on a web-based, webcutter 2.0 silico-restriction enzyme digestion program (Webcutter, 1997) in order to find restriction enzymes that can cut the phage with reasonable number of fragments and their sizes. Appropriate restriction enzymes, e.g., HindIII and Eco72I, were selected to cut DNA of both phages ATCC 51477-B1 and ATCC 700786-B1, following instructions enclosed with the enzymes. The reaction components of restriction enzyme are shown in **Tables 2.4 and 2.5.** Fifty µl reaction was chosen for appropriate volume. After that, the 50 µl volume was reduced by DNA precipitation (1/10v 3 M NaOAc pH 6.5, 2v Absolute cold ethanol, incubation at -20^{0}C for 1 hr, centrifugation at 12,000 rpm at 4^{0}C for 15 min, washing the pellet with 70% ethanol, centrifugation at 12,000 rpm at 4^{0}C for 3-5 min, air drying the pellet, adding 10-20 µl SDW) before running all of DNA restriction enzyme digests into gel electrophoresis.

Table 2.4 HindIII restriction enzyme digestion, 50 µl reaction

Component	ATCC 51477-B1 DNA		ATCC 700786-B1 DNA	
	Uncut (µl)	Digestion (µl)	Uncut (µl)	Digestion (µl)
10X buffer FD	5	5	5	5
DNA 2 µg	4.9 [1]	4.2 [2]	4.9 [1]	4.2 [2]
HindIII (FD)	0	1	2	3
Sterile distilled water	40.1	39.1	40.8	39.8
Total [3]	50	50	50	50

Remarks [1] DNA concentration was 407 ng/µl

[2] DNA concentration was 479 ng/µl

[3] Incubation at 37^{0}C for 1.5 h, Inactivation at 80^{0}C for 15 min

Table 2.5 Eco72I restriction enzyme digestion, 50 µl reaction

Component	ATCC 51477-B1 DNA		ATCC 700786-B1 DNA	
	Uncut (µl)	Digestion (µl)	Uncut (µl)	Digestion (µl)
10X Tango BF	5	5	5	5
DNA 2 µg	4.9 [1]	4.2 [2]	4.9 [1]	4.2 [2]
Eco72I (FD)	0	1	2	3
Sterile distilled water	40.1	39.1	40.8	39.8
Total [3]	50	50	50	50

Remarks [1] DNA concentration was 407 ng/µl

[2] DNA concentration was 479 ng/µl

[3] Incubation at 37^0C for 1.5 h, Inactivation at 80^0C for 15 min

Gel electrophoresis was run to show restriction fragments of each sample. Restriction enzymes that provide different restriction patterns between two phages were used for further study of the differences in DNA sequence between the two phages. Restriction fragments of interest were extracted from an agarose gel using the QIAquick® Gel Extraction Kit (QIAGEN).

2.3.8 Whole genome sequencing

The wet laboratory of bacteriophage strains ATCC 51477-B1 and ATCC 700786-B1 were sequenced using the Ion Torrent Personal Genome Machine™ System (Ion Torrent Systems, Inc.). Three major steps were followed. First, the high-quality DNA fragments of around 180-120 bp in size were prepared. The Ion Shear Reagents that contained DNA enzyme were used to shear all genome to short sizes. Then, the adapters were ligated to short DNA fragments and the DNA fragments were size-selected by gel electrophoresis, beads magnetization and nick translation. All steps were checked for quantity of DNA fragments by Bioanalyzer. Second step, the enrichment of DNA fragments was prepared by Ion Xpress template kit, by diluting the sample library of DNA fragment to $280x10^6$ molecules per 18 µl and mixing with oil in IKA DT-20 solution until emulsified. Ion Sphere beads were mixed before PCR amplification overnight. The washing step was followed and the best beads were selected by performing the template-positive Ion Sphere Particle (ISP) enrichment. Quality control of ISP was performed by a Qubit 2.0 Fluorometer. Lastly, DNA fragment sequences, the enrichment template-positive ISP (prepared in the second step) and primers were inserted to Ion 314 Chip. Then, the protocol of PGM sequencer was followed.

In conclusion, the unpaired short reads of 180-210 bp in size for both strains were produced in fastq files (Elliott *et al*., 2012).

2.3.9 Whole genomic DNA assembly and sequence analysis

Short reads of base content in DNA region from wet laboratory were analyzed by using the genomic software analysis. The million copied data from whole genome sequencing PGM sequencer were analyzed by Velvet de novo genomic assembler software (Zerbino and Birney, 2008). Then, the difference of DNA assemblies of bacteriophage DNA were compared with sequence alignment in Blastn program (Altschul *et al*., 1990). Recommended ranges of regions were 400 bps to 1 Kbps **(Figure 2.1).**

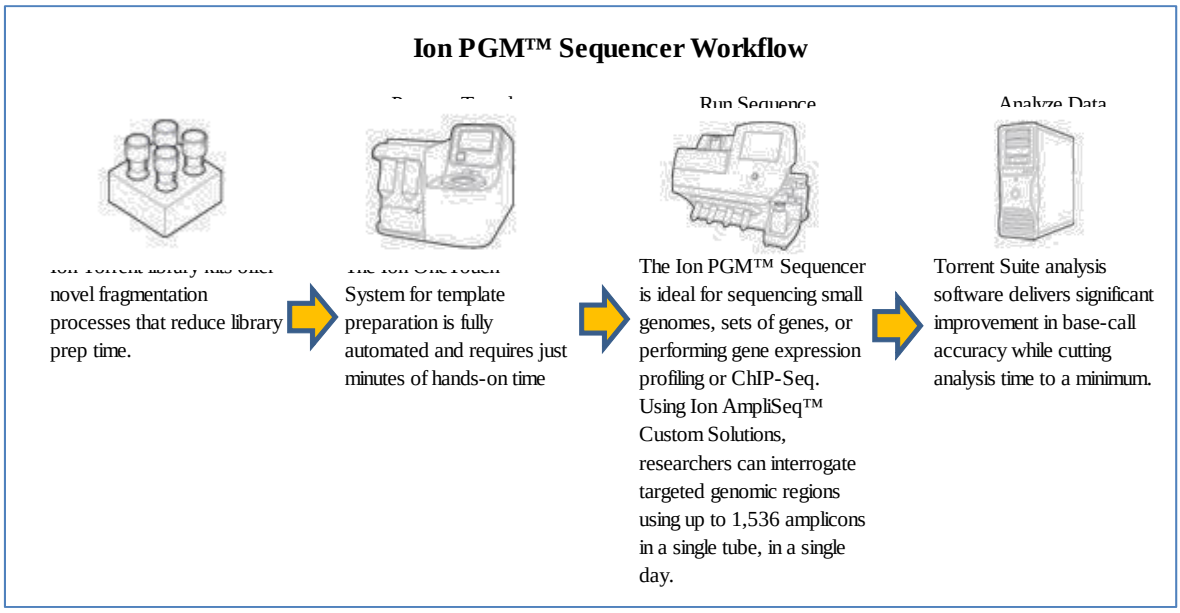

Figure 2.1 Ion personal genome machine sequencer workflow (Life Technologies Corporation, 2012)

2.3.10 PCR primer designing

PCR primers, typically of 15-30 bases long were designed using the Primer-BLAST genomic online software for determining primers specific to the DNA template (NCBI, 2012a).

2.3.11 PCR assay optimization

The PCR reaction components and cycling conditions were optimized. The DNA templates were double-stranded DNA. DMSO was added for unfolding DNA templates of complex structure. DNA denaturation was the critical step in the PCR process. The practical

range of denaturation temperatures for most samples was 94°C -96 °C. One cycle at 5 min denaturation time was routinely used. Optimal annealing temperatures and primer concentrations were determined empirically. When Taq polymerase was used, the annealing temperature was calculated by subtracting 3-5°C to the least T_m (melting temperature) of the primer pair. The PCR products were analyzed by agarose gel electrophoresis and the imaging of DNA fragments was observed by the UV transillumination of ethidium bromide-stained gel.

2.3.12 Determination of the detection limit

The lowest amount of DNA templates to be detectable by the PCR assay, a so-called detection limit, was determined. First, PCR assay was performed to determine the minimum concentration of bacteriophage DNA (ng/µl) by running various concentrations of DNA templates with the PCR assay. Next, the Method Detection limit (MDL) was determined, which is a concentration that can produce at least one positive result (Walker *et al*., 1998).

2.3.13 Culture of *Acinetobacter sp.* ADP1

Acinetobacter sp. ADP1 was cultured Luria–Bertani (LB) medium (Bacto-Tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g, add RO to1 l, adjust pH to 7.0 and autoclave at 121°C (15-lb pressure) for 15 minutes). The culture was incubated at 37°C in aerobic conditions overnight with 100 rpm shaking. Next, the culture was stocked at -80^{O}C in 15% glycerol final concentration (Fuangthong *et al*., 2010).

2.3.14 Recovery of bacteriophage of *Bacteroides* from wastewater samples

Community building and animal farming wastewater samples were sampled by grab method. 3.33 ml of 3% Na_2CO_3 was added in 1 liter of wastewater sample to quench any available free chlorine. Wastewater samples were preserved at 4^{O}C during transportation time. Bacteriophage was recovered from 35 ml of wastewater samples by adding 5 ml 0.4 M $MgCl_2$ (Lukasik *et al*., 2000) and briefly mixing before centrifuging at 6500 xg 20 min. After that, 6.4 ml beef extract eluant pH 7.0 (10 g beef extract, 1.34 g $Na_2HPO_4.7H_2O$, 0.12 g citric acid and 90 ml RO adjust to 100 ml) was added into the tube of remaining pellet. Vortex more than 1 minute, time duration depends on volume of pellet (Eaton *et al*., 2005, Field and Samadpour, 2007, Mendez *et al*., 2004). The supernatant from above step was added to the elute. Half of the solution was spiked with 100 ng of *Acinetobacter sp*. ADP1

before extracted the DNA of wastewater samples was extracted using QIAamp® DNA Maxi Kit (QIAGEN) by following the instruction manual.

2.3.15 Measurement of traditional fecal indicators in human- and animal-derived fecal materials and wastewaters

Water samples were stored on ice or at 4°C until used for analysis within 6 h after sample collection. Total coliforms and *E. coli* in colony forming units (CFU)/100 mL of water are analyzed by following the EPA Method 1604: Total Coliforms and *Escherichia coli* in Water by Membrane Filtration Using a Simultaneous Detection Technique (MI Medium) (US EPA, 2002).

2.3.16 Correlation analysis

The results of phages of *Bacteroides* detected with the newly developed PCR assay and with the traditional plaque assay were compared. Moreover, correlation analysis of the quantitative results of traditional fecal indicators (i.e., total coliforms and *E. coli*) and phages of *Bacteroides* were performed, with parametric method (e.g., Pearson's correlation) or non-parametric mean (e.g., Kendall's tau-b method).

Chapter 3: Results and Discussion

3.1 Detection of phages of *B. fragilis* strains ATCC 51477 and ATCC 700786

B. fragilis strain ATCC 51477 was used to screen and isolate phages present in sewage samples, as this strain has been shown to isolate phages specific to human pollution (Tartera and Jofre, 1987). Since phages were detected in Thailand's sewage samples, no further isolation of other *Bacteroides* host strains was performed. Moreover, *B. fragilis* strain ATCC 700786 was simultaneously used to isolate phages as it has been reported to detect phages at higher concentration but with less specificity to human pollution (Payan et al., 2005). The phage counts from each water sample are reported in **Table 3.1**.

Analysis of phages 51477-B1 and 700786-B1 was performed in 24 wastewater samples collected from human-related facilities, comprising 2 samples from office buildings, 5 samples from residential buildings and hotels, and 17 samples from hospitals **(Figure 3.1).** The wastewater samples taken were grabbed from wastewater sumps that collected wastewater from lavatories of each facility. Of all samples being analyzed, 4 samples were positively detected for phage 51477-B1, 12 samples showed positive detection for phage 7007086-B1, and 3 samples demonstrated positive detection for both phages. Interestingly, all positive samples were collected from hospital facilities. A detection limit for both phages is 1 PFU/ml. Detectable concentrations of phage 51477-B1 ranged from 2 to 15 PFU/ml with a median of 7 PFU/ml, while those of phage 700786-B1 were in a range of 1 to more than 300 PFU/ml with a median of 22 PFU/ml. As presented in this study, phage 700786-B1 was positively detected in wastewater samples more frequently and at higher concentrations than phage 51477-B1, which is in consistent with those reported by Blanch et al. (2006).

Wastewater samples from swine farms and cattle farms were collected from floor cleaning, which contains animal feces. The tests were conducted in 2 wastewater samples from swine farms, and 3 samples from cattle farms **(Figure 3.2).** For swine wastewater samples, phage 51477-B1 was detected in one of two samples at a low concentration of 3 PFU/ml, while phage 700786-B1 was presented in samples at 126 and 180 PFU/ml. The cattle wastewater samples presented no phage 51477-B1 detection, and one positive 700786-B1 sample at a concentration of 5 PFU/ml. More frequent detection and higher concentration of phage 700786-B1 than phage 51477-B1 were observed with animal wastewater samples, similar to those exhibited in human wastewater samples.

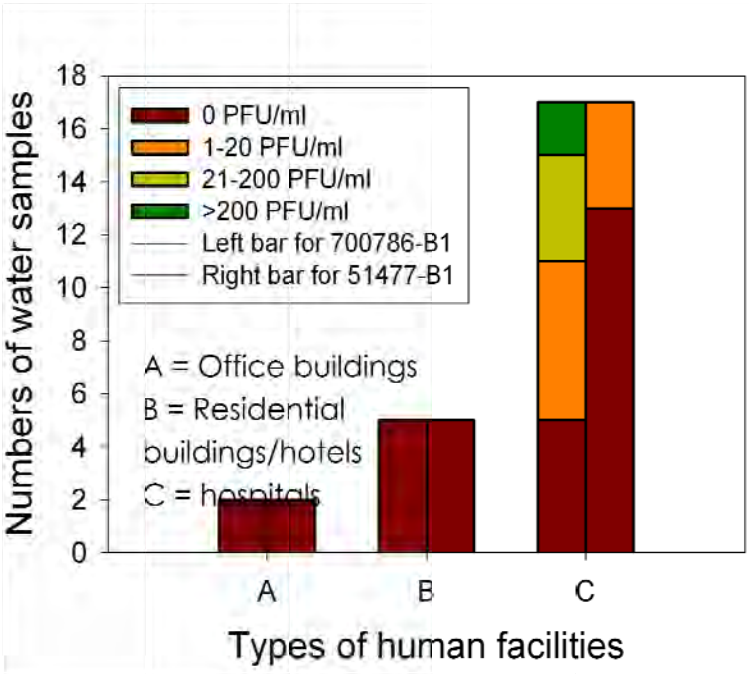

Figure 3.1 Detection of bacteriophage strains 51477-B1 (right bar) and 700786-B1 (left bar) in wastewaters from human-related facilities, including office buildings (A), residential buildings/hotels (B), and hospitals (C)

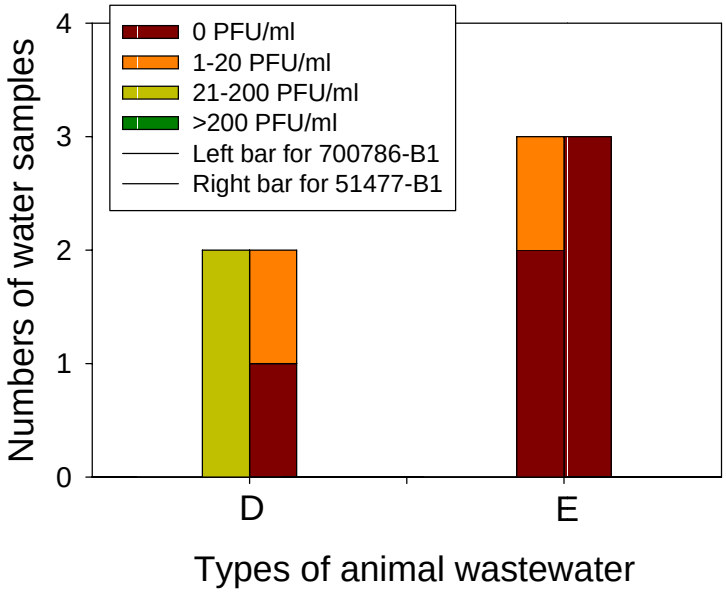

Figure 3.2 Detection of bacteriophage strains 51477-B1 (right bar) and 700786-B1 (left bar) in wastewaters from animal facilities, including swine farms (D) and cattle farms (E)

3.2 Locating specific regions of DNA of a reference phage strain ATCC 51477-B1

3.2.1 Locating specific regions of DNA of phage ATCC 51477-B1 with PCR primers

PCR amplification of the npH primer pair as designed by Puig *et al*. (2000) showed PCR products of between 300-400 bp in size of both bacteriophage strains ATCC 51477-B1 and 700786-B1, being in agreement with 328 bp amplicon as reported by Puig *et al*. (2000). Amplification of the pH primer pair, however, showed no band in either strain. DNA sequence analysis of the PCR products showed similarity to that of ATCC 51477-B1 deposited in Genbank with accession number FJ008913 (Hawkins *et al*., 2008). This indicated similarity in DNA sequences of this 328-bp amplicon between these 2 reference strains.

In order to search for DNA region of different sequence between strains ATCC 51477-B1 and 700786-B1, this study designed primers and optimized for PCR cycle conditions. PCR products were detected for both phage strains when amplified with the primer pair BT3530 and BT3531, and the primer pair BT3532 and BT3533. On the other hand, the primer pair BT3534 and BT3535 could only amplify DNA of phage strain ATCC 51477-B1 but not that of strain ATCC 700786-B1. DNA sequence analysis of the PCR products amplified by primer pair BT3530 and BT3531 showed similarity between that of strain ATCC 51477-B1 and strain 700786-B1. For PCR products amplified with the primer pair BT3532 and BT3533 of both phage strains, subsequent DNA sequencing resulted in similarity between these 2 phage strains. The phage strain ATCC 700786-B1 was amplified by 2 primer pairs: BT3559-BT3562, in order to amplify the capsid protein MP1 region. The PCR products showed the correct sizes. However, the products were not able to be purified due to the presence of non-specific bands. Therefore, the PCR products could not be submitted for DNA sequencing. Consequently, this approach was not yet successful in locating different sequences between strains ATCC 51477-B1 and ATCC 700786-B1.

We, therefore, followed our prior plan of using restriction enzyme digestion technique to find differences of DNA sequences between the two strains. Our previous obstacle of being unable to propagate phages at high concentrations has been overcome with the current technique (see section 2.3.5).

3.2.2 Restriction enzyme digestion

Determination of DNA sequence differences between 2 phage strains was performed by restriction enzyme digestion. The whole genome sequence of 44,929 bp, double-stranded, linear DNA of bacteriophage ATCC 51477-B1 (Hawkins *et al*., 2008) was cut with many

restriction enzymes in silico (Webcutter, 1997). The suitable restriction enzymes were chosen to study further, by following the criteria: (1) they can produce the different lengths that can be separated by visible observation in gel electrophoresis, and (2) they are commonly used in biotechnological laboratory and have high possibility for receiving good results, e.g. 37°C incubation temperature, long incubation time, and less star activity that can lead to random cut. The computer-based (Webcutter, 1997) restriction enzyme digestion results showed that DNA of phage ATCC 51477-B1 with Genbank accession no. FJ008913 (Hawkins *et al.,* 2008) can be cut with Eco72I to provide 8 restriction fragments of the following sizes: 21510, 12836, 4257, 2923, 1509, 677, 674 and 543 bp **(Table 3.1).**

In laboratory, DNA of phage ATCC 700786-B1 was extracted and cut with the Eco72I restriction enzyme. Also uncut conditions were used for being the control of these studies. The uncut reaction of DNA of two phages was incubated at the same conditions and reaction components but without Eco72I restriction enzyme. Restriction patterns of phage ATCC 700786-B1 were observed with distinct sizes compared to those of phage ATCC 51477-B1. This demonstrated that genome sequences were different between the two phage types **(Figure 3.3).**

Additionally, the agarose gel electrophoresis results of DNA of phage ATCC 51477-B1 when cut with Eco72I showed the following approximate DNA sizes: 21510, 12836, 5200, 4900, 4257 and 570. These did not match with the results from computer-based information: 21510, 12836, 4257, 2923, 1509, 677, 674 and 543 bp **(Table 3.1 and Figure 3.3).** The computer-based cutting results were performed by using the whole genomic data of bacteriophage ATCC 51477-B1 Genbank accession no. FJ008913 (Hawkins *et al.,* 2008). The unmatched bands of gel electrophoresis result were 5200 and 4900 bp. The lost bands were 2923 and 1509 bp. Also, the invisible bands were 677, 647 and 543 bp that were predicted for the sizes of nearly 570 bp **(Figure 3.3).** The small bands of 677, 647 and 543 bp in size may be shown in the same level at 570 bp **(Figure 3.3).**

Moreover, the whole genome of two strains of bacteriophages were cut with HindIII restriction enzyme. The DNA of phage ATCC 51477-B1 showed different DNA sizes when compared with DNA of phage ATCC 700786-B1 **(Figure 3.4).** The computer-based results showed that DNA of phage ATCC 51477-B1 can be cut with HindIII to provide 8 restriction fragments of the following sizes: 11806, 11547, 10087, 3465, 3060, 2718, 1164 and 582 bp **(Table 3.2).**

Table 3.1 Eco72I restriction enzyme digestion of bacteriophage ATCC 51477-B1 and ATCC 700786-B1 DNA

Eco72I computer-based cutting at CAC/GTG of bacteriophage ATCC 51477-B1 (FJ008913)		Gel electrophoresis	
Cutting point	**Computer-based cutting size (bp)**	**ATCC 51477-B1 estimated band size (bp)**	**ATCC 700786-B1 estimated band size (bp)**
2,923	2,923	21,510	19,000
3,600	677	12,836	12,800
4,143	543	5,200	4,900
16,979	12,836	4,900	3,600
17,653	674	4,257	3,100
21,910	4,257	570	1,100
43,420	21,510		
44,929	1,509		
Total	**44,929**	**49,273**	**44,500**

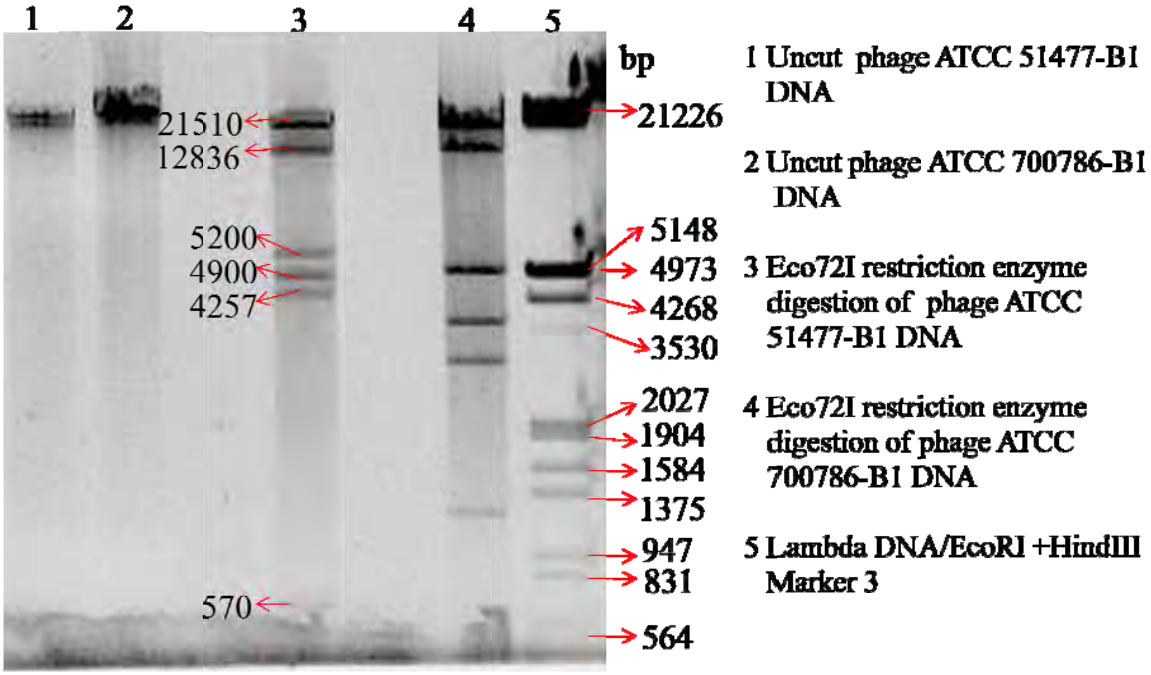

Figure 3.3 Comparison of Eco72I restriction enzyme digestion of DNA of bacteriophage strains ATCC 51477-B1 and ATCC 700786-B1

Moreover, the total summation of band sizes in **Table 3.1** showed different values of phage ATCC 51477-B1 as report by Hawkins *et al*., 2008 (44,929 bp) and gel electrophoresis result (49,273 bp). This could because of the following reasons: (1) the band sizes in gel electrophoresis are estimated, (2) gel electrophoresis cannot separate more than 1 band with slightly different sizes and (3) bacteriophage ATCC 51477-B1 used in this study could be the different strain from ATCC 51477-B1 in Hawkins study.

Table 3.2 HindIII restriction enzyme digestion of bacteriophage ATCC 51477-B1 and ATCC 700786-B1 DNA

HindIII computer-based cutting at A/AGCTT of bacteriophage ATCC 51477-B1 (FJ008913)		Gel electrophoresis	
Cutting point	Computer-based cutting size (bp)	ATCC 51477-B1 estimated band size (bp)	ATCC 700786-B1 estimated band size (bp)
3,465	3,465	11,806	12,000
6,525	3,060	11,547	10,000
18,331	11,806	10,087	4,900
21,049	2,718	3,060	3,100
22,713	1,664	2,718	2,700
34,260	11,547	1,584	1,300
34,842	582	1,164	1,100
	10,087	582	
Total	44,929	42,548	35,100

There are many outstanding results of HindIII restriction enzyme digestion. The unexpected bands of HindIII restriction enzyme digestion of bacteriophage strain ATCC 51477-B1 DNA in lane 4 showed approximately of 1584 and 1164 bp in size **(Figure 3.4).** These band sizes were not found in the result of computer-based restriction enzyme cutting by using whole genome sequencing data of bacteriophage ATCC 51477-B1 with Genbank accession no. FJ008913 (Hawkins *et al*., 2008).

Moreover, DNA of bacteriophages ATCC 51477-B1 DNA and ATCC 700786-B1 were compared by using HindIII fast digestion enzyme and gel electrophoresis to check for the DNA sizes. The two strains of bacteriophages showed the different visible bands in gel electrophoresis. This indicated that two strains showed differences in DNA sequences. The total size of bacteriophage ATCC 700786-B1 that was cut by HindIII was approximately 44,548 bp, while that cut by Eco72I was approximately 35,100 bp.

In summary, the DNA restriction enzyme results of phage ATCC 51477-B1 with Eco72I and HindIII showed the different band sizes when compared with those of phage

ATCC 51477-B1 whole genomic data (Genbank accession no. FJ008913, Hawkins *et al*., 2008). Moreover, DNA sequence of two strains of bacteriophage ATCC 51477-B1 and ATCC 700786-B1 had the different DNA sequence as shown by different restriction enzyme digestion patterns.

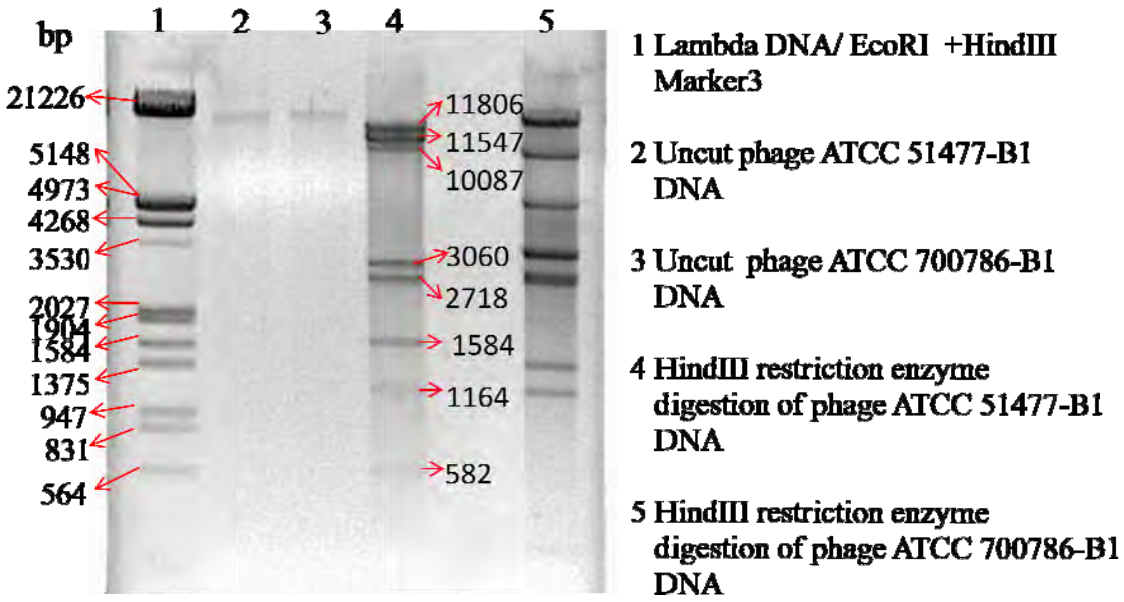

Figure 3.4 Comparison of HindIII restriction enzyme digestion of DNA of bacteriophage strains ATCC 51477-B1 and ATCC 700786-B1

3.2.3 Whole genome sequencing, DNA assembly and sequence analysis

The whole genome sequencing of phage strains ATCC 51477-B1 and ATCC 700786-B1 was performed using the Ion 314 chip-PGM sequencer machine (Personal Genome Machine™ (PGM™) System, Ion Sequencing Kit v2.0 Ion 314™ Chip). The results showed that the total numbers of bases obtained from the PGM sequencer were 9.43 and 7.06 Mbp for bacteriophage ATCC 51477-B1 DNA and 20.53 and 15.44 Mbp for bacteriophage ATCC 700786-B1, respectively (**Table 3.3**).

Normally, the acceptable result of total bases are over 10 Mbp from the Ion 314 chip PGM sequencer. For the reason that the phage genome was smaller in size than the bacterial genome, the whole genome sequencing results of phage ATCC 51477-B1 and ATCC 700786-B1 were large amount of fold in total short read DNA sequence (180-210 bp). Moreover, two strains of phage genomes are approximately only 5 Kbp in size, leading to a high quality of whole genome sequencing result. However the total number of bases of

bacteriophage ATCC 51477-B1 were not over 10 Mbp. Then, the sequencing of each bacteriophage was conducted twice as shown in **Table 3.3.**

Table 3.3 Summary report of whole genome sequencing of Ion 314 chip-PGM sequencer

Parameters	Decription	Genome sequencing			
		Round 1		Round 2	
		51477-B1	700786-B1	51477-B1	700786-B1
Total Number of Bases (Mbp)	Number of filtered and trimmed million base pairs reported in the SFF and FASTQ files	9.43	20.53	7.06	15.44
-Number of Q17 Bases (Mbp)	Number of filtered and trimmed million base pairs contained in read segments that extend from the read start to the 3'-most base at which the total read accuracy is 98% or greater (Q17)	7.88	18.41	6.14	14.03
-Number of Q20 Bases (Mbp)	Number of filtered and trimmed million base pairs contained in read segments that extend from the read start to the 3'-most base at which the total read accuracy is 99% or greater (Q20)	7.24	17.4	5.71	13.31
Total Number of Reads	Total number of filtered and trimmed reads independent of length reported in the SFF and FASTQ files	85,644	212,603	64,224	160,629
Mean Length (bp)	Average length, in base pairs, of all filtered and trimmed library reads reported in the SFF and FASTQ files	110	97	110	96
Longest Read (bp)	Maximum length, in base pairs, of all filtered and trimmed library reads either reported in the file	202	202	200	201

The million copies of short read DNA sequences (180-210 bp) were accomplished by PGM sequencer machine. Predicted quality of DNA sequence included Q20, corresponding to an error rate of 1% (one error in 100 bp) and Q17, corresponding to an error rate of 2% (one error in 50 bp).

The resulting DNA sequences were assembled and analyzed using de novo genome assembler program and Blastn program (Zerbino and Birney 2008, Altschul *et al*., 1990). The appropriate DNA sizes for designing PCR assay were 400 – 1 Kbps. Then the process of DNA analysis required the DNA region of about 400 – 1 Kbps in size, as well as the high quality of coverage percentage. Coverage percentage is a percentage of average number of times that a base was independently sequenced and aligned to the reference genome. The high coverage percentage means that the result carries high accuracy of base alignment.

The alignment results showed four regions of DNA of bacteriophage ATCC 51477-B1 that were not in bacteriophage ATCC 700786-B1 in the length of 400 bps-1 Kbps: node 9

with a length of 941 bp **(Figure 3.5)**, node 13 with a length of 866 bp **(Figure 3.6)**, node 43 with a length of 440 bp **(Figure 3.7)**, and node79 with a length of 443 bp **(Figure 3.8).** Node 13 demonstrated the highest coverage presentation of base pairing in genomic program analysis, which accounted for 99.74 %. However, nodes 43, 9 and 79 had the relatively high quality of base pair coverage percentages, which were 97.91, 86.26 and 86.19 percentage, respectively. This available information was used to design primers for PCR detection method that was restricted only to the bacteriophage ATCC 51477-B1.

NODE_9.ref_length_941_cov_86.261424

AAACTTAACTCCGTTAATAACTTCATCCCGCTTGTTCACAAGTCCACTTTCTACGAGTCGCTTTACAACCAAA
CGCGCAGTTTGCTTACTTGTATTTAACCATTCTGCAATATAAGACAAAGAACCCTTAAATTCAGTTTCACCGT
CTTGGCTAAATCCATATATTAAAGCATAACATATTAGATCGTTTCCGGTTAGATTCAACTCCTTAACCATCCA
AGCCTGTATATTGATATAATTTTTATTACTTAATACCATAATATTTAGTTTTATAGGCGGGTATTACCCCGCCA
TGTTATTTATTTCTTACCTAAAAACTTATTGATAAAATACTGCTGTCCTCGACCCGTTACCATCGTAGTGTTAT
AAACCTTAGTTTCTCCCCCGTCAATAATTACACGTTTGCGAATCTCAAACAGACCCATGTTCATGTATGTTTG
GGTAGGCTGATTTCTTGACTCTCCTACACTGCACAAGTATCCGGCTTTTCTGAGACGTTCATACAGTTGCTTT
TCTCCGATCTGATAACCGTTTTGAGTGATTATTTTTGCAAGTTCACGGATAAGAATAGACTTCTTAGACGCTT
CGACTGCTTCGCTA AATAGAACTTTCGGTTTATCAGCCTCGATCTTCGCCTCCGCTTGCTTTCTCTTTTCCTGC
TCGTTTTTCAACTGTGTAGCCAACTGGATAATAAAGTCAGGTGATGTTAGAGTTTTCTCTATTGCTTCCGGTG
TCATGTATGCACCATGTTTACGGATAGACGGTAAAACATCTCCGCAAACCCAATCTTGAAAAGCCTCCGCTT
CAGGTTTATCTGAACGCATTATAGCCTTATACAAGTTCTTTTCGTTAACATACACAATATTCTGCATACCTCCA
TTAGTAGGGGTATTAATCAAACTAACACCCTTTTCATCCAATCTGTTTTTTGTAGCTCCCGGTTGTAAATTTA
GAATTTTA C

Figure 3.5 DNA region of phage ATCC 51477-B1 (node 9), with 941 bp in length and 86.26% coverage

NODE_13.ref_length_866_cov_99.747113

CAGGTATCGGTCAAAGATGGGGAAACCCCTCTATTACACCGTCTGCAACTAAATTATCAACTGGTAGATATA
GAGTAACTATATCCGGCGTATCATCAAACGGGTTTATACCTATGGTTACCGCTTGCAATAGTTCAAAATGGG
TAAACGCATGTATAGAGGAATACAGTTTCACATCTAATTATTTCACCGTTAAATTTATGGACGTTAATACTG
GCATGATTGATACTGATTTTGTTTTTTATATTTGCGGTTGGGTATAACATTAATTTTAACGGTAAGTTGGTTT
TATCCTTCTTACCGTTTACCTTTGTGGCAAATAAATTATTCATTCACAATTAAATATTAATTTTTATGGCAACA
AAAGCAGCTATTTTTGATTTGGAGAGTGTCAAATACTCAAAAGAGACACAAATTTTAGATTATAGTTTTGAG
ACTGATAACGGTCTCTTCAAAGGTCAGATCACAATCGTACAGCAACCCGATCAAGTAAAGCAGATCACACA
CTGTACAGCGGAGGTATCAGTTAAAGAAATGGTTCAAGTACCAAGCGCAGAAAGCGAACCTACCATGCAG
GAACAATATGTGCAATTAGGCACTTTGTCGATGTCGCAGGCTCGTTTTGAACTGAATCAGTTCCCCGTGCAC
GAAAAGACACCCGCTTTGCTTGCAGACTTTCAAAACTGACAATTTGCGCTAACTAAAAAGCAGTAATCATGT
CAGTATCACAAGAACAATTAAGATTGTTGATAGTCTCAACGTGTAGTCCGATTCTTGCATTCTTGACACCTA
CGTCCGGATTCTTAACGGCACTTGTATTCATGTTTGTGTTTAACATAATTTGCGGTATGCGTGCGGACGGTG
TTAGTGTGTCGGTTAGAGGCAT

Figure 3.6 DNA region of phage ATCC 51477-B1 (node 13), with 866 bp in length and 99.75% coverage

NODE_43.ref_length_440_cov_97.911364

AAGATCGCAAAGGACGCAGCAGATCAAGCCGCACAGGACGCAGCGAACGCAGCACAATCAGCACAGGA
GGCGAAAGACAGGCTTAATAAATGGCTGATGATGGACTTATCTCACCGACTGAGAAACCTGCATTGATTG
ACGAAGGGAAGCGAATTCAAGCGGAGTATCTGCAAATTAAAGCGAATGCGGACAAATACGGTGTGTCTG
TAACTGAATACACAGAGGCGTATAACAATTACCTTAACGAACTACGTTATCATTCGGCTACTACACCTGAA
AATATTGTCGTGCGTCCAGAATTGGCACAGAGCCAAACGGCTTACTACGACAAACGTAATGGAGCGTTGA
ATGCAATTGCTACGGCTTCAAAAGAATATGTAGATAATGCTGACAAAAAGTTAAAGGAATACTTAGATAC
TG AGATAACTGCTATTCCCGGTAAGATTGAACTTGCTGTACG

Figure 3.7 DNA region of phage ATCC 51477-B1 (node 43), with 440 bp in length and 97.91% coverage

NODE_79.ref_length_443_cov_86.198646

TAGCTCCGGACACCGATAGCCAGAATTGACTGTGATAGGTTTACCGTAAATCGCTCTCAGAGGGTCTAATA
CGTTATCAACCAAAGCCGTTAAATTACGCTCTACCTCCGGTGTTGGAGTGTTATCTATTCCTTTCGCCTCAG
CCGTTGAGGACTTTATAAGTTCATTAATCGTAAAATACTTCATAATCTAAATGTTTAAAGGGGGTATTATAC
CCCATTGTGAATACCCTGTTTTTCTTTCTCTTCTTGCTCTTCAATAATCTTAGCCGCATGTTCACCCATTAGAC
GCTTAAACTCGAAACGGATAATGTGATATATCAGTTTAAAGCCGTTGTTCTTCGGGTATGTGGTACAAAGA
TTCCTAAAGCCATTGCATAAGTAGACGTACATAAATACGTAGGTGATAGTCTTTGCGCACATTATTGCAGC
GTCTTTATCTCCCATCTTAGTGACAGTCGAAA

3.3 Designing a PCR assay for detection of bacteriophage strain ATCC 51477-B1

3.3.1 Designing PCR primers of bacteriophage strain ATCC 51477-B1 regions

The primers specific to DNA of bacteriophage ATCC 51477-B1, and not to that of bacteriophage ATCC 700786-B1, were designed from DNA regions of 4 nodes using NCBI primer-BLAST (NCBI, 2012a) as shown in **Figure 3.9**. Five primer pairs were designed for each node. Their characteristics are presented in **Tables 3.4-3.7**. Primer pairs that can match only to *Bacteroides* phage B40-8 culture collection ATCC 51477-B1 were selected for use in further steps. These included BT3759- BT3760 from node 13, BT3761-BT3762 from node 13, BT3763-BT3764 from node 13 and BT3765-BT3766 from node 79.

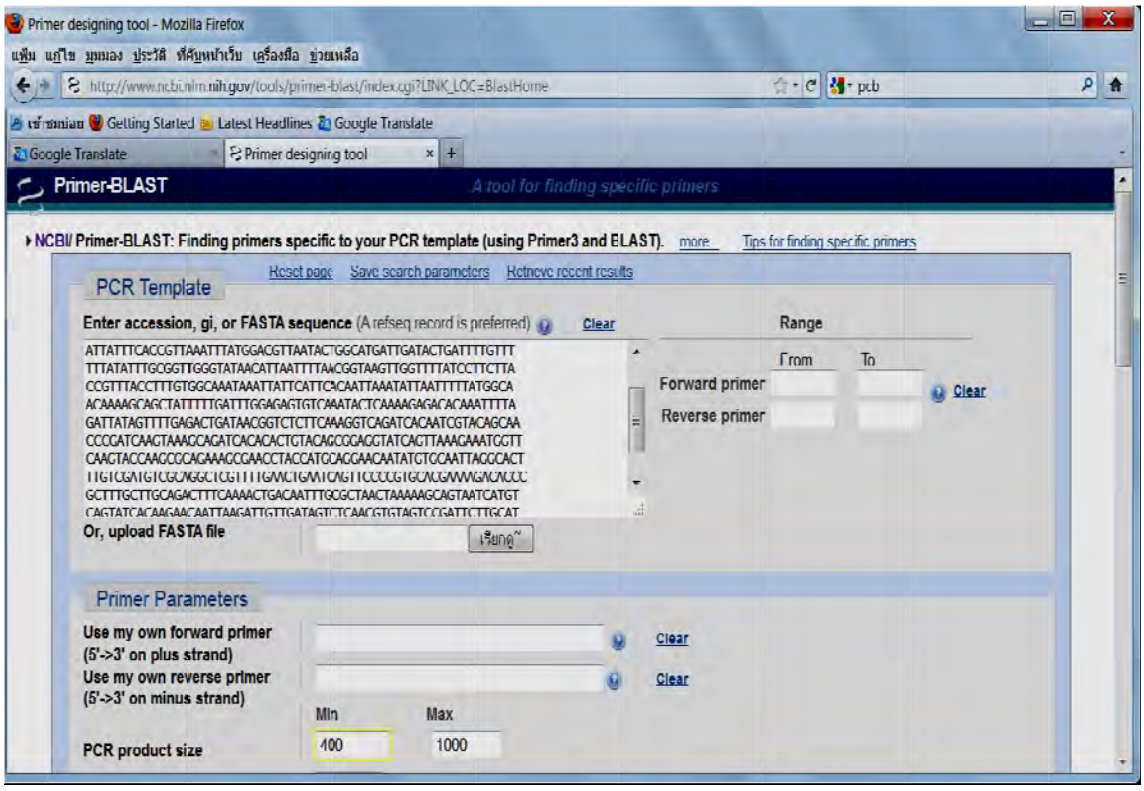

Figure 3.9 An example of the NCBI Primer-BLAST web page (NCBI, 2012a)

Table 3.4 Summary of primer pairs that were designed from DNA region node 9 and their characteristics

Description	NODE_9.ref_length_941 bp_cov_86.26%				
	Primer pair 1	Primer pair 2	Primer pair 3	Primer pair 4	Primer pair 5
Primer length (bp)	22, 20	21, 21	23, 20	22, 21	23, 20
Start point	58, 641	59, 646	57, 645	59, 640	58, 640
Stop point	79, 622	79, 622	79, 626	80, 620	80, 621
Product length (bp)	584	588	589	582	583
Tm (ºC)	58.56, 59.98	57.5, 59.6	59.5, 58.5	58, 59	59, 58
GC%	50, 70	38 ,90	52, 65	54.5 , 66.67	52, 65
Self complementarity	4,4	0, 0	4, 4	4, 4	4, 4
Self 3'complementarity	2,2	2, 3	2, 3	2, 1	2, 3
Organisms matched with primer pairs	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1
	Sinorhizobium fredii HH103	*Sinorhizobium fredii* HH103	*Sinorhizobium fredii* HH103	*Xanthomonas oryzae pv. oryzae* PXO99A	*Xanthomonas oryzae pv. oryzae* PXO99A
	Ostreococcus lucimarinus CCE9901	*Schistosoma mansoni* strain Puerto Rico	*Geobacter bemidjiensis* Bem	*Xanthomonas oryzae pv. oryzae* MAFF 311018	*Xanthomonas oryzae pv. oryzae* MAFF 311018
	Micromonas sp. RCC299	*Pantoea vagans* C9-1	*Stackebrandtia nassauensis* DSM 44728	*Xanthomonas oryzae pv. oryzae* KACC10331	*Xanthomonas oryzae pv. oryzae* KACC10331
	Halorhodospira halophila SL1	-	*Burkholderia cenocepacia* J2315	*Xanthomonas oryzae pv. oryzicola* BLS256	*Ostreococcus lucimarinus* CCE9901
	Methylocella silvestris BL2	-	*Schistosoma mansoni* strain Puerto Rico	*Granulicella sp.* MP5ACTX9	*Xanthomonas oryzae pv.* oryzicola BLS256
	Rahnella sp. Y9602	-	-	*Methylocella silvestris* BL2	*Micromonas sp.* RCC299
	Rahnella aquatilis CIP 78.65	-	-	-	*Methylocella silvestris* BL2
Primer name	-	-	-	-	-

Table 3.5 Summary of primer pairs that were designed from DNA region node 43 and their characteristics

Description	NODE_43.ref_length_440 bp_cov_97.91%				
	Primer pair 1	Primer pair 2	Primer pair 3	Primer pair 4	Primer pair 5
Primer length (bp)	21, 25	21, 23	22, 23	20, 23	21, 23
Start point	52, 459	51, 457	58, 457	52, 457	53, 475
Stop point	72, 435	71, 435	79, 435	71, 435	73, 435
Product length (bp)	408	407	400	406	405
Tm (ºC)	58.7, 56	57.4, 55.2	58.2, 55.2	56.2, 55.2	57.1, 55.2
GC%	62, 48	57, 48	55, 83.5	60, 48	57, 47.8
Self complementarity	2, 5	2, 5	2, 5	2, 5	2, 5
Self 3'complementarity	2, 3	2, 3	0, 3	2, 3	0, 3
Organisms matched with primer pairs	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1
	Oryza sativa Japonica Group chromosome 5 clone P0617H07	*Bacteroides* phage B124-14	*Oryzias latipes* DNA, MHC class I region, strain: HNI	*Bacteroides* phage B124-14	*Bacteroides* phage B124-14
	Oryza sativa Japonica Group chromosome 5 clone P0473H02	*Oryza sativa Japonica* Group chromosome 5 clone P0617H07	*Oryzias latipes* DNA, MHC Class I Region, strain: Hd-rR	*Oryza sativa* Japonica Group chromosome 5 clone P0617H07	*Desulfobulbus propionicus* DSM 2032
	Oryza sativa Japonica Group chromosome 5 clone P0473H02	*Oryza sativa Japonica* Group chromosome 5 clone P0473H02	*Lotus japonicus* genomic DNA, chromosome 5, clone: LjT03E08, TM1696	*Oryza sativa Japonica* Group chromosome 5 clone P0473H02	Human chromosome 14 DNA sequence BAC C-3104H21
	-	*Desulfobulbus propionicus* DSM 2032	*Leishmania* major strain Friedlin	*Desulfobulbus propionicus* DSM 2032	*Erwinia billingiae* strain Eb661
	-	*Erwinia billingiae* strain	*Erwinia sp.* Ejp617	*Erwinia billingiae* strain	-
	-	-	*Erwinia pyrifoliae* DSM	-	-
	-	-	*Erwinia pyrifoliae* strain Ep1/96	-	-
	-	-	*Chlorella vulgaris* retrotransposon Zepp DNA	-	-
	-	-	*Oryzias latipes* DNA, MHC class I region, strain: cab	-	-
Primer name	-	-	-	-	-

Table 3.6 Summary of primer pairs that were designed from DNA region node 13 and their characteristics

Description	NODE_13.ref_length_866 bp_cov_99.75%				
	Primer pair 1	Primer pair 2	Primer pair 3	Primer pair 4	Primer pair 5
Primer length (bp)	22, 22	21, 21	21, 20	21, 21	24, 21
Start point	84, 675	85, 675	84, 672	83, 647	7, 676
Stop point	205, 655	105, 655	104, 653	105, 655	30, 656
Product length (bp)	593	591	589	592	670
Tm (ºC)	58.4, 59.5	57.3, 58.5	57, 57	58.5, 57.4	59.6, 58.2
GC%	54.5, 54.5	57, 57	57, 55	52, 55	54, 57
Self complementarity	6, 4	6, 4	6, 4	6, 4	5, 4
Self 3'complementarity	2, 1	2, 1	2, 1	2, 1	3, 0
Organisms matched with primer pairs	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1
	-	-	*Lactobacillus* ruminis ATCC 27782	-	*Pan troglodytes* BAC clone CH251-3G8
	-	-	*Paenibacillus polymyxa* E681	-	Homo sapiens genomic DNA, chromosome 11 clone:CMB9-51L7,
	-	-	-	-	Homo sapiens genomic DNA, chromosome 11q clone:RP11-691F15
Primer name	BT3759-BT3760	BT3761-BT3762	-	BT3763-BT3764	-

Table 3.7 Summary of primer pairs that were designed from DNA region node 79 and their characteristics

Description	NODE_79.ref_length_443 bp_cov_86.20%				
	Primer pair 1	Primer pair 2	Primer pair 3	Primer pair 4	Primer pair 5
Primer length (bp)	21, 24	20, 25	21, 23	20, 24	22, 25
Start point	2, 433	3, 433	3, 432	2, 432	2, 434
Stop point	22,410	22, 409	23, 410	21, 409	23, 410
Product length (bp)	432	431	430	431	433
Tm (ºC)	59.4, 58.8	58.3 , 59.6	59 ,57.6	58, 58.5	60, 59.4
GC%	90, 83	65, 48	66.7, 47.8	65, 50	63.6, 48
Self complementarity	6, 6	6, 6	6, 6	6, 6	6, 6
Self 3'complementarity	2, 2	2, 1	2, 2	2, 1	2, 2
Organisms matched with primer pairs	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1	*Bacteroides* phage B40-8 culture-collection ATCC:51477-B1
	Human DNA sequence from clone RP11-151A11	Human DNA sequence from clone RP11-151A11	Human DNA sequence from clone RP11-151A11	Human DNA sequence from clone RP11-151A11	-
	-	*Yarrowia lipolytica* strain CLIB122	*Yarrowia lipolytica* strain CLIB122	-	-
Primer name	-	-	-	-	BT3765-BT3766

3.3.2 Optimization of PCR reaction components and cycling conditions for detecting bacteriophage ATCC 51477-B1

There were four pairs of selected primers of *Bacteroides* phage ATCC 51477-B1 that were produced. The characteristics are shown in **Table 3.8**.

Table 3.8 PCR primers used for detection of bacteriophage ATCC 51477-B1

No.	Name	Type	Sequence (5'-->3')	Length (bp)	T_m calculated from GC contents (°C)	Gene amplified	Organism	Product size (bp)
1	BT3759	For.	TTC-GGC-GTA-TCA-TCA-AAC-GGG-T	22	54.8	16s rRNA	Bacteriophage ATCC 51477-B1	593
2	BT3760	Rev.	AGT-CTG-CAA-GCA-AAG-CGG-GTG-T	22	56.7	16s rRNA		
3	BT3761	For.	CCG-GCG-TAT-CAT-CAA-ACG-GGT	21	56.3	16s rRNA	Bacteriophage ATCC 51477-B1	591
4	BT3762	Rev.	GTC-TGC-AAG-CAA-AGC-GGG-TGT	21	56.3	16s rRNA		
5	BT3763	For.	ATC-CGG-CGT-ATC-ATC-AAA-CGG-GT	23	57.1	16s rRNA	Bacteriophage ATCC 51477-B1	592
6	BT3764	Rev.	TCT-GCA-AGC-AAA-GCG-GGT-GT	20	53.8	16s rRNA		
7	BT3765	For.	AGC-TCC-GGA-CAC-CGA-TAG-CCA-G	22	60.4	16s rRNA	Bacteriophage ATCC 51477-B1	433
8	BT3766	Rev.	GAC-GCT-GCA-ATA-ATG-TGC-GCA-AAG-A	25	57.7	16s rRNA		

Melting temperature (T_m) of primers were used to calculate annealing temperature (T_a) for PCR cycling conditions. When Taq polymerase was used, the annealing temperature was calculated by subtracting 3-5 $^{\circ}$C from the least value of T_m of each primer pair. For example, primer BT3759-BT3760 showed T_m 54.8°C and 56.7°C, the PCR annealing temperature was 54.8-3 and 54.8-5 that is 51.8 and 49.8°C. Therefore, the annealing temperature of PCR cycling condition for bacteriophage ATCC 51477-B1 was approximately at 49 - 52°C **(Table 3.8).**

There are several formulas for calculating melting temperatures (T_m). In this study, T_m calculations provided a good starting point for determining appropriate annealing temperatures. After that, a precise optimum annealing temperature must be determined empirically.

The basic T_m calculations follow the formula below for primers longer than 14 bp (Bolton and McCarthy 1962; Promega Corporation, 2012).

T_m = 64.9°C + 41°C x (no. of G's and C's in the primer – 16.4)/N

Where N is the length of the primer.

For example, forward primer of bacteriophage strain ATCC 51477-B1 (BT3765) is (AGC-TCC-GGA-CAC-CGA-TAG-CCA-G) 22mer composed of 2 T's, 6 A's, 8 C's, and 6 G's. Thus, its melting temperature is calculated as

64.9°C + ((41°C x (14 – 16.4))/22 = 60.4°C

Reverse primer of bacteriophage strain ATCC51477-B1 (BT3766) is (GAC-GCT-GCA-ATA-ATG-TGC-GCA-AAG-A) 25mer composed of 4 T's, 9 A's, 5 C's, and 7 G's. Thus, its melting temperature is calculated as

64.9°C + ((41°C x (12 – 16.4))/25 = 57.7°C

Moreover, one concern of non-optimum T_a was non-specific PCR bands. The non-specific PCR products will occur when using too low annealing temperature (T_a less than 50°C). This is because low T_a could cause a high number of base pair mismatches, meaning that one or both primers will anneal to other sequences that are not the true target DNA. Furthermore, too high annealing temperature (T_a more than 60°C) will produce deficient primer-template hybridization resulting in low PCR product yield.

Figure 3.10 showed PCR products run by gel electrophoresis after running with forward primer BT3759 and reverse primer BT3760 testing with 20 ng of phage ATCC 51477-B1 DNA and phage ATCC700786-B1 DNA and their phage particles. The PCR reaction components and cycling conditions are present in **Table 3.9**. The results showed that DNA of phage ATCC 51477-B1 was amplified at both 49.9°C and 55.5°C of annealing temperatures. Moreover, DNA of bacteriophage ATCC 700786-B1 and their particles were not amplified. However, these results were rejected because non-specific bands were presented under the expected band (593 bp).

Table 3.9 PCR reaction components and cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3759-BT3760

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3- 5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		49.9, 55.5	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

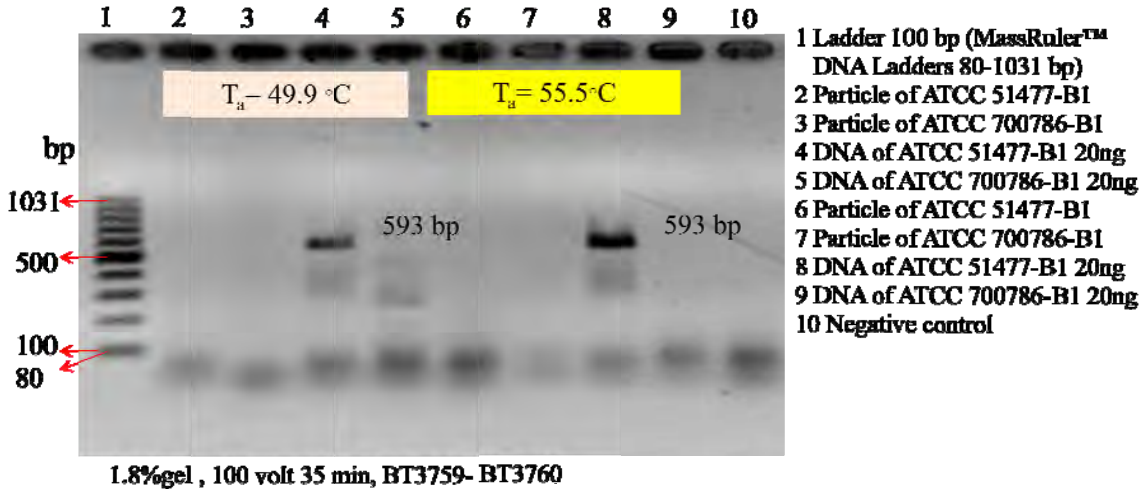

Figure 3.10 Gel electrophoresis results showing PCR products amplified with forward primer BT3759 and reverse primer BT3760 and 20 ng DNA template of bacteriophage strain ATCC 51477-B1

Next, forward primer BT3761 and reverse primer BT3762 were tested with 20 ng of phage ATCC 51477-B1 DNA and phage ATCC700786-B1 DNA and their particles (**Table 3.10**). The results showed bands of phage ATCC 51477-B1 DNA being amplified better at 55.5°C than at 49.9°C of annealing temperatures**.** This study indicated that 55.5°C is better than 49.9°C of annealing temperatures for PCR cycling conditions of primers BT3761-

BT3762. Moreover, DNA of bacteriophage ATCC 700786-B1 and their particle were not amplified. However, non-specific bands were presented other than 591 bps expected band **(Figure 3.11)**.

Table 3.10 PCR reaction components and cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3761-BT3762

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		49.9, 55.5	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

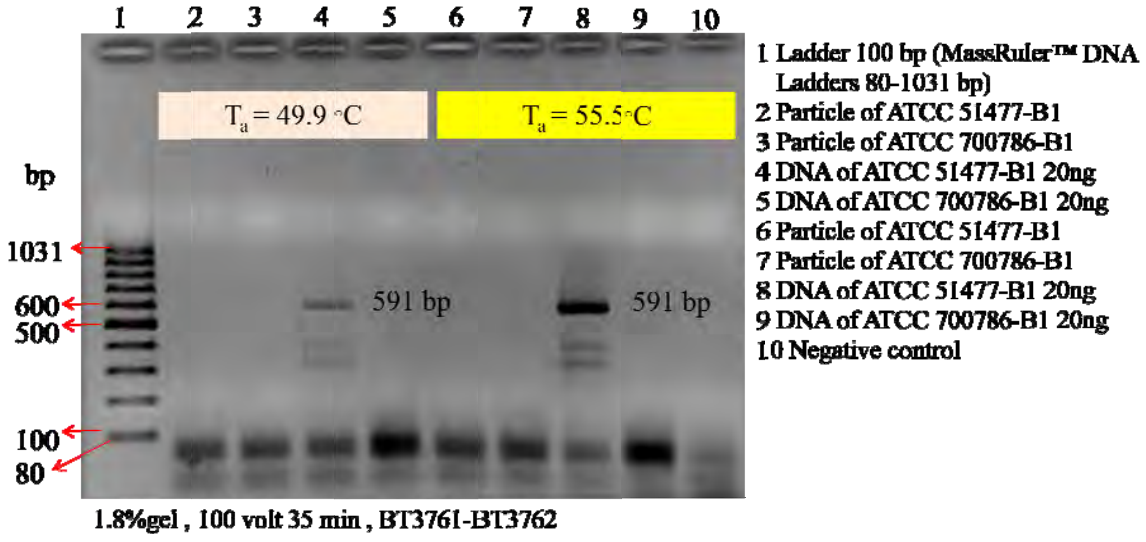

Figure 3.11 Gel electrophoresis results showing PCR products amplified with forward primer BT3761 and reverse primer BT3762 and 20 ng DNA template of bacteriophage strain ATCC 51477-B1

Next, forward primer BT3763 and reverse primer BT3764 were tested with 20 ng of phage ATCC 51477-B1 DNA and phage ATCC 700786-B1 DNA and their particles **(Table 3.11).** The expected bands with 592 bp in size were presented at both annealing temperatures of phage ATCC 51477-B1 **(Figure 3.12)**. Moreover, DNA of bacteriophage ATCC 700786-B1 and their particles were not amplified. However, non-specific bands were also presented.

Table 3.11 PCR reaction components and cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3763-BT3764

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		52.7, 55.4	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

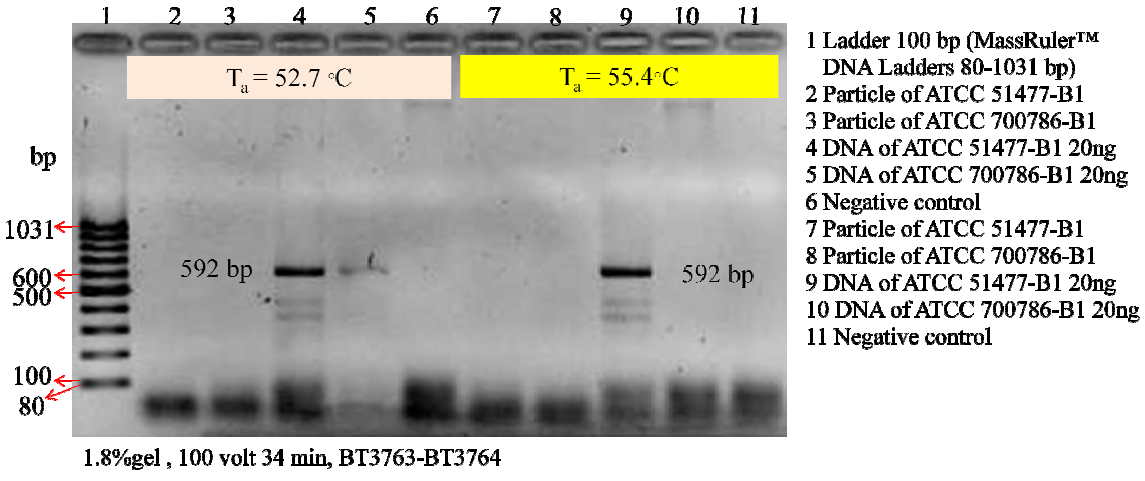

Figure 3.12 Gel electrophoresis results showing PCR products amplified with forward primer BT3763 and reverse primer BT3764 and 20 ng DNA template of bacteriophage strain ATCC 51477-B1

The last primer pair BT3765-BT3766 was tested with 20 ng of phage ATCC 51477-B1 DNA and phage ATCC 700786-B1 DNA and their particles **(Table 3.12)**. The expected bands with 433 bp in size were presented at both annealing temperatures of phage ATCC 51477-B1 **(Figure 3.13)**. Large amount of PCR product was amplified by using annealing temperatures at 52.7°C and 55.4°C. Moreover, DNA of bacteriophage ATCC 700786-B1 and their particles were not amplified. Non-specific bands did not appear. Therefore, this primer pair was used in further step.

Table 3.12 PCR reaction components and cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		52.7, 55.4	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

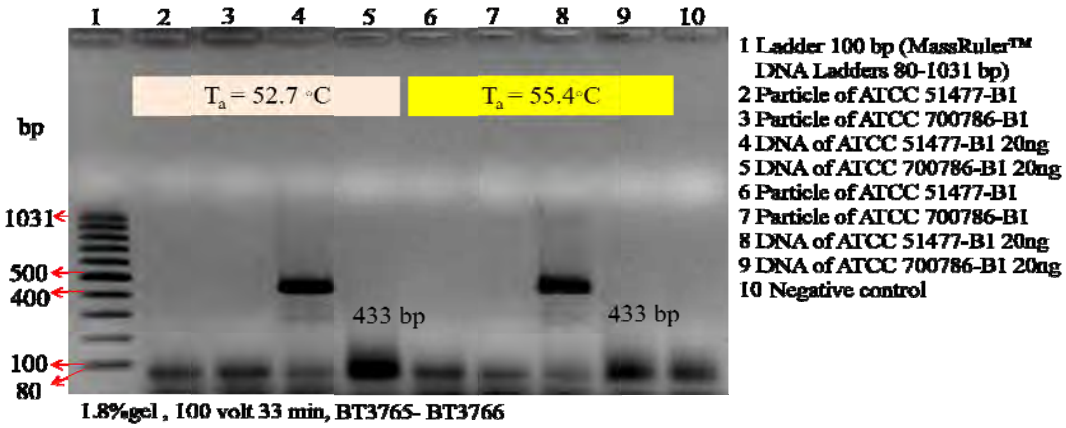

Figure 3.13 Gel electrophoresis results showing PCR products amplified with forward primer BT3765 and reverse primer BT3766 and 20 ng DNA template of bacteriophage strain ATCC 51477-B1

In summary, the best primer pair for detecting the bacteriophage strain ATCC 51477-B1 was the primer pair BT3765 and BT3766 with Taq polymerase enzyme. Next, the annealing temperatures were set as gradient PCR reaction to find an optimum annealing temperature **(Table 3.13)**.

Table 3.13 PCR reaction components and cycling conditions for gradient PCR reactions of annealing temperatures of bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3- 5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		Gradient 48-58	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

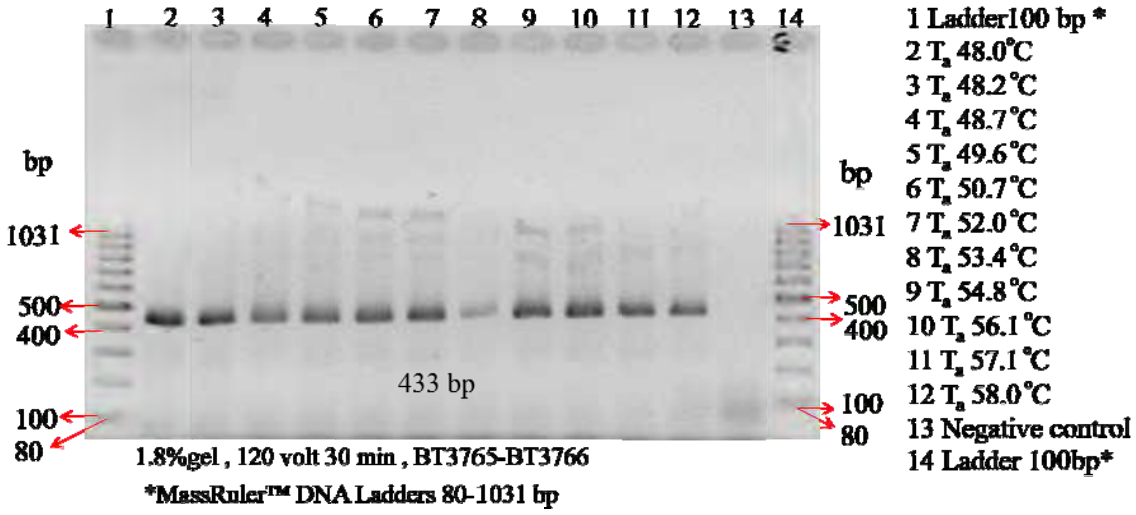

Figure 3.14 Gel electrophoresis showing gradient PCR results at varied annealing temperatures of 48.0-58.0ºC of bacteriophage ATCC 51477-B1 DNA with primer pair BT3765-BT3766

Figure 3.14 showed visible bands of expected size (433 bp) when amplified with annealing temperatures in the range of 48.0–58.0°C. Therefore, this indicated that all annealing temperatures in this range were appropriate for amplification of bacteriophage ATCC 51477-B1 by using primer pair BT3765 and BT3766 **(Figure 3.14).**

Primer concentrations were optimized for the PCR assay with primers BT3765-BT3766. PCR reaction components and cycling conditions are shown in **Table 3.14**. The result showed that primer concentration of at least 5 µM was enough to see product bands **(Figure 3.15).** However, the band intensity when run PCR with 5 µM primer concentration appeared to be lower than that of 10 µM primer concentration. Therefore, 10 µM primer concentration was used in further experiments.

Table 3.14 PCR reaction components and cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766 at varied primer concentrations

PCR reaction component		**PCR cycling condition**			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (various conc.)	1	Annealing		55	20 sec
Reverse primer (various conc.)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

Next, the 433 bp PCR products were extracted from gel electrophoresis and submitted for DNA sequencing. Fifty-microliter PCR reaction was performed **(Table 3.15** and **Figure 3.16).**

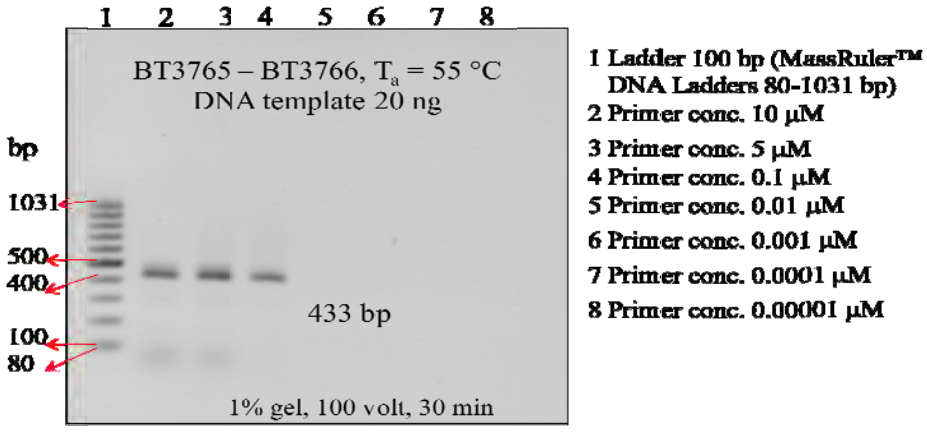

Figure 3.15 Gel electrophoresis showing PCR products of bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766 at varied primer concentrations

Table 3.15 PCR reaction components and cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766 for PCR product sequencing

PCR reaction component		PCR cycling condition			
Ingredient of reaction 50 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	3	Initial denaturation	1	95	3- 5 min
2x PCR MM (Taq polymerase)	25	Denaturation	35	95	20 sec
Forward primer (10 µM)	2	Annealing		55	20 sec
Reverse primer (10 µM)	2	Extension		72	30 sec
DMSO	2	Final extension	1	72	10 min
Sterile distilled water	16	Hold	1	16	∞
Total	50				

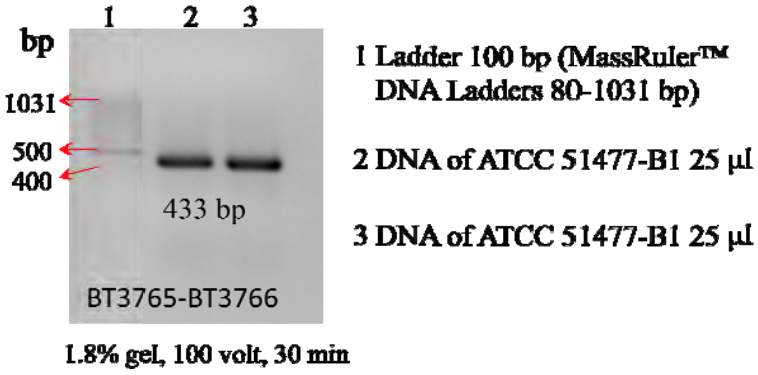

Figure 3.16 Gel electrophoresis showing PCR product of bacteriophage ATCC 51477-B1 with primers BT3765-BT3766 for DNA sequencing

Table 3.16 shows three results of alignment as follows: (1) alignment results of forward and reverse sequencing results showed the high quality result of identity over 97 %, (2) alignment results of 433-bp region from node 79 with forward and reverse sequencing results showed identity of 99% in both (3) alignment results of whole genome of phage ATCC 51477-B1 (Genbank accession no. FJ008913) with forward and reverse sequencing results showed 98% identity in both. Therefore, the results can be interpreted that the good quality of PCR product was obtained. **(Table 3.16).**

Table 3.16 PCR product sequencing and their alignment results of bacteriophage ATCC 51477-B1 DNA

PCR amplification		Primer	**For. BT3765**	**Rev. BT3766**
		Organism	Bacteriophage ATCC 51477-B1	
		PCR product [1] (bp)	433	
Sequencing product (bp) [2]			417	409
Alignment [3]	**Sequencing results of forward and reverse primers**	Length (bp)	414	
		Score (bits)	625 bits (338)	
		Identities	367/397 (97%)	
		Gaps	9/379 (2%)	
	Node 79 (433bp) and sequencing results of each primer	Length (bp)	419	413
		Score (bits)	713 bits (386)	713 bits (386)
		Identities	400/406 (99%)	401/407 (99%)
		Gaps	4/406 (1%)	5/407 (1%)
	Whole genome ATCC 51477-B1 (FJ008913) and sequencing result of each primer	Length (bp)	419	413
		Score (bits)	702 bits (380)	706 bits (382)
		Identities	398/406 (98%)	401/409 (98%)
		Gaps	4/406 (1%)	5/409 (1%)

Remarks [1]A tool for finding specific primers (NCBI, 2012a)

[2]DNA sequencing (Pacific science, 2012)

[3]Align Sequences Nucleotide BLAST (NCBI, 2012b)

3.4 Determination of the detection limit of the newly–designed PCR assay of bacteriophage strain ATCC 51477-B1

The detection limit of the newly-designed PCR assay was determined with DNA of bacteriophage ATCC 51477-B1 with primers BT3765-BT3766. The primer concentration used was 5 µM in order to reduce the primer dimer at low concentration of DNA template **(Table 3.17)**. The results showed that the least concentration of DNA template that can produce visible band was 0.002 ng **(Figure 3.17)**.

Table 3.17 PCR reaction components and cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3765- BT3766 to determine the detection limit

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (various conc.)	2	Initial denaturation	1	95	3- 5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (5µM)	1	Annealing		55	20 sec
Reverse primer (5µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

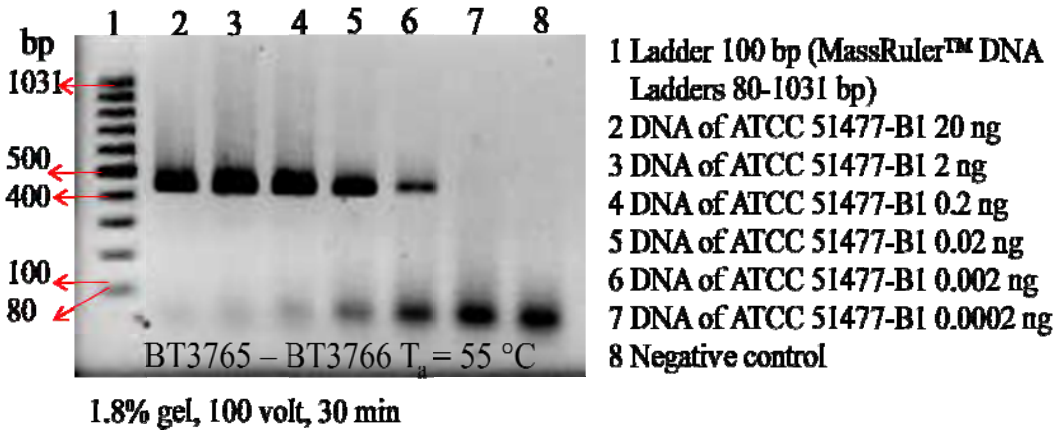

Figure 3.17 Gel electrophoresis showing the detection limit for PCR assay of bacteriophage ATCC 51477-B1 DNA with primers BT3765- BT3766

Next, the method detection limit (MDL) was determined using PCR reaction components and cycling conditions as shown in **Table 3.18**. The results showed that the DNA template of 0.002 ng could produce at least one positive result of bacteriophage ATCC 51477-B1 in 10 µl PCR reaction with Taq polymerase and primers BT3765-BT3766 **(Figure 3.18)**. The result showed that the positive bands were observed at lanes 2, 5, 6, 7, 8, 9, 10 and 11. Therefore, it indicated that the method detection limit of the newly-designed PCR assay for bacteriophage ATCC 51477-B1 DNA was 0.002 ng **(Figure 3.18)**.

Table 3.18 PCR reaction components and cycling conditions for determining the method detection limit (MDL) of 0.002 ng bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766

PCR reaction component		**PCR cycling condition**			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (0.001 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10µM)	1	Annealing		55	20 sec
Reverse primer (10µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

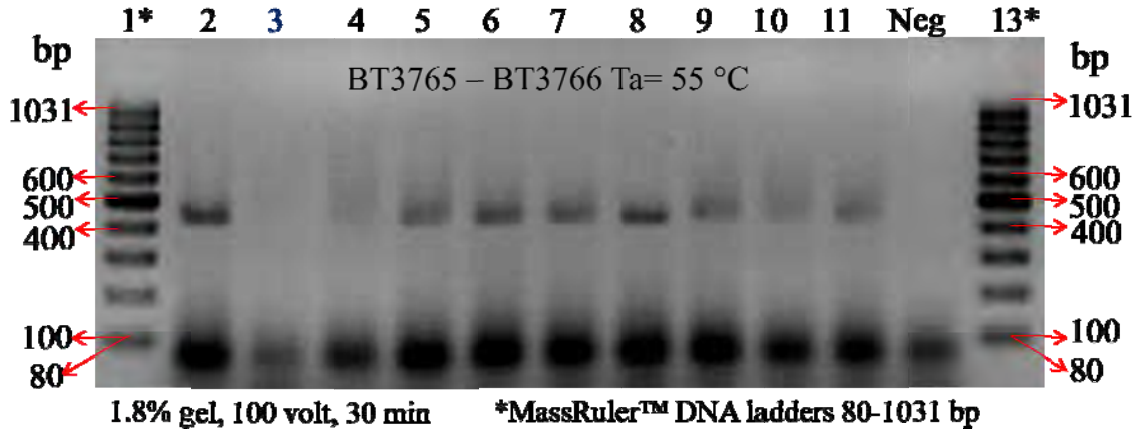

Figure 3.18 Gel electrophoresis showing the PCR method detection limit (MDL) of 0.002 ng of bacteriophage ATCC 51477-B1 DNA with Taq polymerase and primers BT3765-BT3766

3.5 *Bacteroides* phage PCR assay validation with wastewater samples

3.5.1 Determination of DNA loss by spiking *Acinetobacter sp*. ADP1 DNA into wastewater samples

When the newly-designed PCR assay for detection of bacteriophage ATCC 51477-B1 was used in environmental samples, DNA extraction step would be involved. To be able to calculate % loss of phage DNA in the samples, known amount of DNA of another organism needed to be spiked into the samples before the DNA extraction step. This was performed by assuming that % loss of DNA of bacteriophage ATCC 51477-B1 was similar to that of the spike DNA. The criteria for selecting the organism for spiking were as follow: (1) it normally could not be found in wastewater and natural water, and (2) the amount of the spiked DNA was detectable, both before and after DNA extraction step. Therefore, *Acinetobacter sp*. ADP1 was used.

3.5.1.1 Designing PCR primers for *Acinetobacter sp*. strain ADP1

The whole genome sequence of *Acinetobacter sp*. strain ADP1 (Barbe *et al.,* 2004) was used to design the reverse primer using forward primer reported elsewhere (Maslunka *et al*., 2008). Primer characteristics are shown in **Table 3.19**.

Table 3.19 PCR primers used for detection of *Acinetobacter sp*. ADP1

No.	Name	Type	Sequence (5'-->3')	Length (bp)	T_m (°C)	Gene amplified	Organism	Product size (bp)	Reference
1	BT3714	For.	TTC-AAG-AGC-AGA-AAA-GCA-ACG-C	22	54.8	16s rRNA gene	*Acinetobacter sp*. ADP1	417	Maslunka C. *et al*., 2008
2	BT3715	Rev.	GTT-ACC-TTA-GCT-CCA-CAG-CTT	21	51.8	16s rRNA gene			This study

3.5.1.2 Optimization of PCR reaction components and cycling conditions for detecting *Acinetobacter sp*. ADP1

There was formula for calculating melting temperatures (T_m) for primers longer than 14 bp (Bolton and McCarthy 1962; Promega Corporation, 2012). In this study, T_m calculations provided a good starting point for determining appropriate annealing temperatures for PCR cycling conditions. The formula below is used:

$$T_m = 64.9°C + 41°C \times (\text{no. of G's and C's in the primer} - 16.4)/N$$

Where N is the length of the primer.

Therefore, the forward primer of *Acinetobacter sp.* ADP1 (BT3714) is (TTC-AAG-AGC-AGA-AAA-GCA-ACG-C) 22mer is composed of 2 T's, 10 A's, 5 C's, and 5 G's. Thus, its melting temperature is calculated as

$$64.9°C + (41°C \times (10 - 16.4)/22) = 53.0°C$$

The reverse primer of *Acinetobacter sp*. ADP1 (BT3715) is (GTT-ACC-TTA-GCT-CCA-CAG-CTT) 21mer composed of 7 T's, 4 A's, 7 C's, and 3 G's. Thus, its melting temperature is calculated as

$$64.9°C + (41°C \times (10 - 16.4)/21) = 53.0°C$$

The annealing temperature (T_a) of PCR when using Taq polymerase enzyme was normally calculated by subtracting $3^{\circ}C$-$5^{\circ}C$ to the least T_m of the primer pair. Therefore, the annealing temperature (T_a) of primer pair BT3714-BT3715 was $48^{\circ}C$ (53.0-$5^{\circ}C$).

The 10 µl PCR reaction with the Taq polymerase enzyme was used to amplify both *Acinetobacter sp*. ADP1 DNA and bacteriophage ATCC 51477-B1 DNA. Although, Taq polymerase was not the best enzyme but Taq was still used in this study. This was because Taq polymerase is the low cost enzyme that was proper for developing DNA-based detection methods.

The annealing temperature of the PCR assay was optimized by running a gradient PCR at various annealing temperatures **(Table 3.20** and **Figure 3.19)**. The results showed the clear visible bands at annealing temperatures of 50.7, 52.0, 53.4, 54.8, 56.1, 57.1 and $58.0^{\circ}C$. The annealing temperature for PCR cycling conditions of *Acinetobacter sp*. ADP1 DNA with primers BT3714-BT3715 had to use the same conditions as PCR cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766. Therefore, an annealing temperature of $55^{\circ}C$ was used.

Table 3.20 PCR reaction components and cycling conditions for determining optimum annealing temperatures of *Acinetobacter sp.* ADP1 with primers BT3714-BT3715

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		Gradient 48-58	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

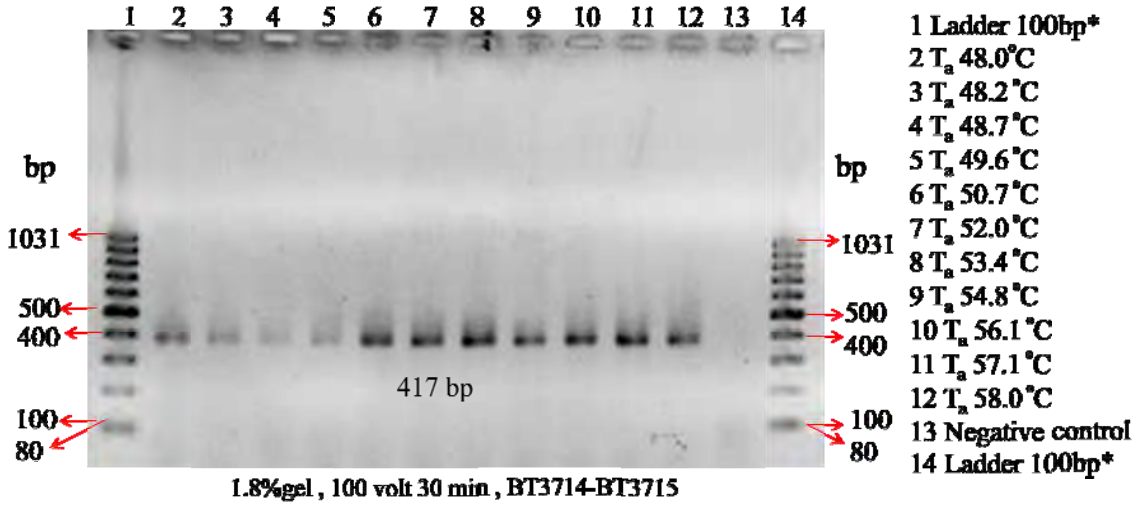

Figure 3.19 Gel electrophoresis showing gradient PCR results at various annealing temperatures of *Acinetobacter sp.* ADP1 with primers BT3714 - BT3715

Next, the primer concentrations of *Acinetobacter sp*. ADP1 were optimized for PCR assay with primers BT3714-BT3715. PCR reaction components and cycling conditions are shown in **Table 3.21**. The results showed that primer concentrations of at least 10 µM produced the visible bands of PCR product. Then, 10 µM primer concentration was used in further step **(Figure 3.20).**

Table 3.21 PCR reaction components and cycling conditions of *Acinetobacter sp.* ADP1 DNA with primers BT3714-BT3715 at varied primer concentrations

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (various conc.)	1	Annealing		55	20 sec
Reverse primer (various conc.)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

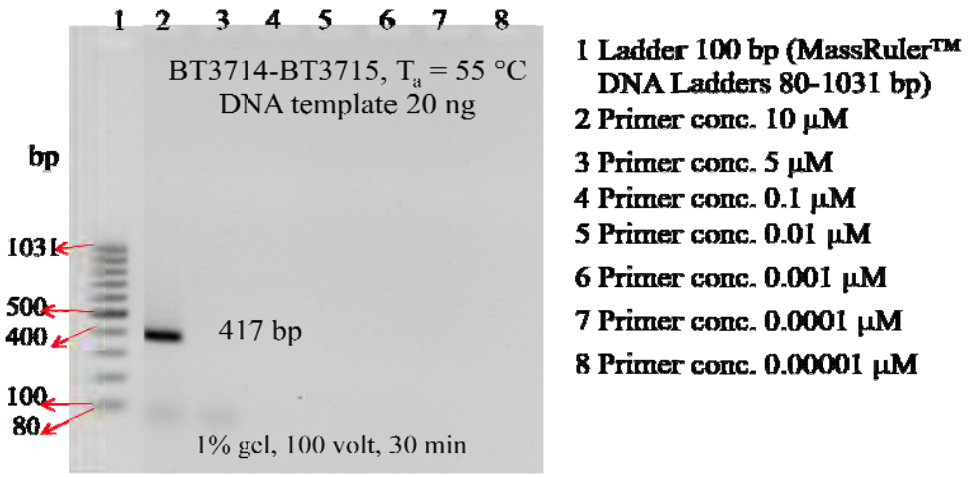

Figure 3.20 Gel electrophoresis showing PCR products of *Acinetobacter sp.* ADP1 DNA with primers BT3714-BT3715 at varied primer concentrations

3.5.1.3 Determination of the detection limit of the newly–designed PCR assay of *Acinetobacter sp.* strain ADP1

The detection limit of the newly-designed PCR assay was determined with DNA of *Acinetobacter sp*. ADP1 with primer pairs BT3714-BT3715 **(Table 3.22)**. The results showed that the least concentration of DNA template that can produce visible band was 0.02 ng **(Figure 3.21)**.

Table 3.22 PCR reaction components and cycling conditions of *Acinetobacter sp.* ADP1 DNA with primers BT3714-BT3715 to determine the detection limit

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		55	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

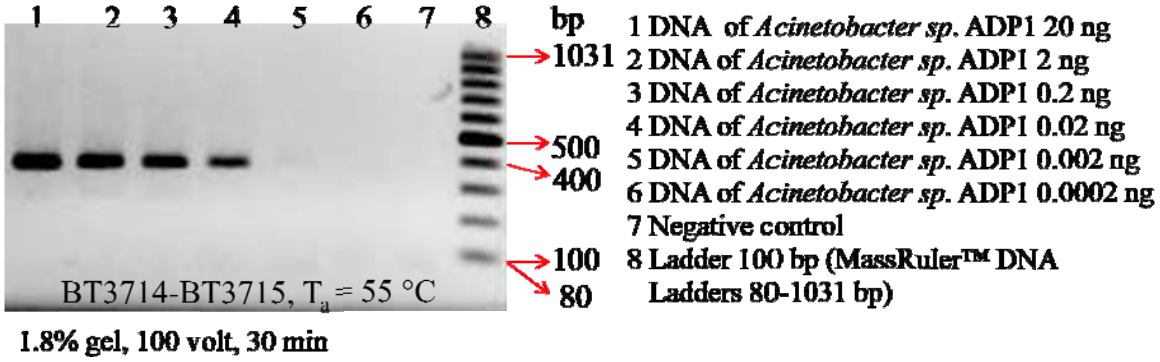

Figure 3.21 Gel electrophoresis showing the detection limit for the PCR assay of *Acinetobacter sp.* ADP1 with primers BT3714-BT3715

Next, method detection limit (MDL) was determined using PCR reaction component and cycling conditions as shown in **Table 3.23**. The results showed that the DNA template of 0.02 ng could produce at least one positive result of *Acinetobacter sp* ADP1 in 10 µl PCR reaction with Taq polymerase and primer pair BT3714-BT3715 **(Figure 3.22).** The result showed that the positive bands were observed at lanes 7, 8, 9 and 11. Therefore, it indicated that the method detection limit of *Acinetobacter sp*. ADP1 DNA was 0.02 ng **(Figure 3.22).**

Table 3.23 PCR reaction components and cycling conditions for determining the method detection limit (MDL) of 0.02 ng *Acinetobacter sp.* ADP1 DNA with primers BT3714-BT3715

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (0.01 ng/µl)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		55	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

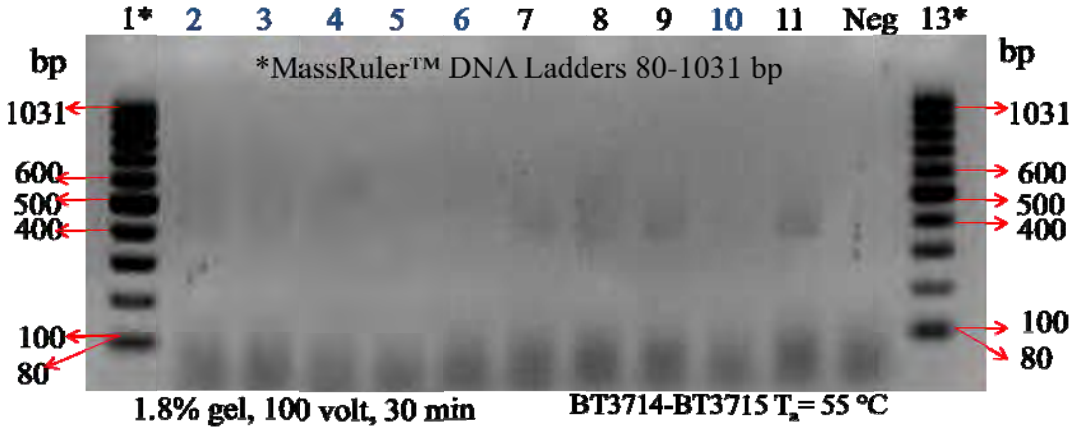

Figure 3.22 Gel electrophoresis showing the PCR method detection limit (MDL) of 0.02 ng *Acinetobacter sp.* ADP1 DNA with Taq polymerase and primers BT3714- BT3715

Next, the 417 bp PCR products of *Acinetobacter sp.*ADP1 were extracted from gel and submitted for DNA sequencing. Fifty-microliter PCR reaction was performed **(Table 3.24** and **Figure 3.23).**

Table 3.24 PCR reaction components and cycling conditions of *Acinetobacter sp.* ADP1 primers BT3714- BT3715 for DNA sequencing

PCR reaction component		PCR cycling condition			
Ingredient of reaction 50 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (10 ng/µl)	3	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	25	Denaturation	35	95	20 sec
Forward primer (10 µM)	2	Annealing		55	20 sec
Reverse primer (10 µM)	2	Extension		72	30 sec
DMSO	2	Final extension	1	72	10 min
SDW	16	Hold	1	16	∞
Total	50				

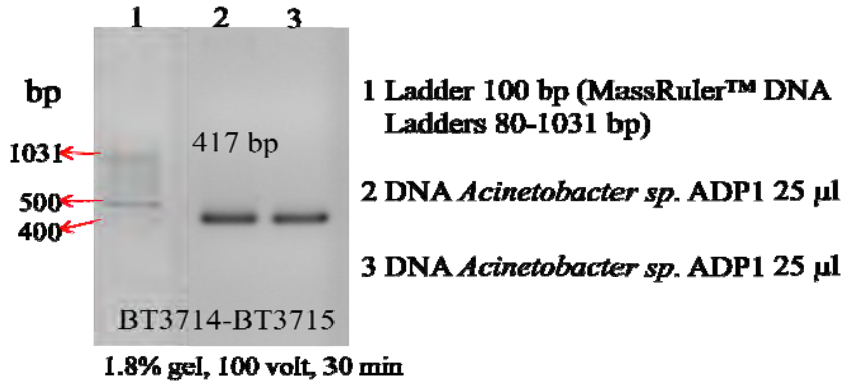

Figure 3.23 Gel electrophoresis showing PCR product *of Acinetobacter sp.* ADP1 with primers BT3714-BT3715 for DNA sequencing

Table 3.25 shows two results as follows: (1) alignment results of forward and reverse sequencing results showed the high quality result of identity over 99 %, (2) alignment results of 417-bp region from Genbank accession no. CR543816 (Barbe *et al*., 2004) with forward and reverse sequencing results showed over 99% identity in both. Therefore the results can be interpreted that the good quality of PCR product was obtained **(Table 3.25)**.

Table 3.25 PCR product sequencing and their alignment results of *Acinetobacter sp.* ADP1 DNA

PCR amplification		Primer	**For. BT3714**	**Rev. BT3715**
		Organism	*Acinetobacter sp*. ADP1	
		PCR product[1] (bp)	417	
Sequencing product (bp) [2]			349	350
Alignment [3]	**Sequencing results of forward and reverse primers**	Length (bp)	303	
		Score (bits)	462 (250)	
		Identities	258/261 (99%)	
		Gaps	3/261 (1%)	
	***Acinetobacter sp.* ADP1 DNA (417bp) and sequencing result of each primer**	Length (bp)	352	352
		Score (bits)	608 bits (329)	601 bits (325)
		Identities	342/347 (99%)	337/342 (99%)
		Gaps	5/347 (1%)	4/342 (1%)

Remarks [1]A tool for finding specific primers (NCBI, 2012a)

[2]DNA sequencing (Pacific science, 2012)

[3]Align Sequences Nucleotide BLAST (NCBI, 2012b)

3.5.2 Detection of bacteriophage DNA using the *Bacteroides* phage PCR assay in human- and animal-derived wastewater samples

The newly-developed PCR assay for detecting bacteriophage ATCC 51477-B1 was used to detect phage in wastewater samples. The characteristics of wastewater samples used are presented in **Table 3.26**.

The result of this study showed negative results of 22 wastewater samples when detecting for bacteriophage ATCC 51477-B1 with primer pair BT3765-BT3766 **(Table 3.27** and **Figure 3.24).** This could be because of the following reasons: (1) low amount of phage particles was present in wastewaters as shown by mostly detection using culture method, (2) the phage DNA was lost during wastewater DNA extraction, and (3) inhibitors were present in environment samples that inhibited the PCR reactions.

Table 3.26 Wastewater samples and their DNA extraction results

No.	Source of wastewater	ATCC 51477-B1 (PFU/100ml)	Date of sampling	WW[1] Volume use (ml)	4M $MgCl_2$ (ml)	Eluant (ml)	*Acinetobacter* spiked DNA (ng)	Volume to extract	Date of DNA extraction	Volume eluted (µl)	DNA Conc. (ng/µl)	A260/[2] A230	A280/[3] A230
1	Influent of hospital A	0	29-Nov-11	40	0	10.00	50	100%	1-Dec-11	600	216.40	1.70	0.65
2	Influent of WWTP A	0	9-Dec-11	35	5	6.40	100	50%	17-Dec-11	600	6.20	1.15	0.87
3	Influent of WWTP B	0	9-Dec-11	35	5	6.40	100	50%	17-Dec-11	600	39.00	0.39	-0.30
4	Night soil treatment plant	0	21-Dec-11	35	5	6.40	100	50%	22-Dec-11	600	94.30	2.31	0.15
5	Influent of WWTP C	0	21-Dec-11	35	5	6.40	100	50%	22-Dec-11	600	36.30	2.68	0.13
6	Farm slurries, sheeps	0	23-Dec-11	35	5	6.40	100	50%	30-Dec-11	600	9.85	1.94	0.21
7	Farm slurries, dairy goats	0	23-Dec-11	35	5	6.40	100	50%	30-Dec-11	600	23.20	1.86	0.25
8	Influent of WWTP D	0	23-Dec-11	35	5	6.40	100	50%	30-Dec-11	600	76.90	2.41	0.15
9	Influent of WWTP E	0	23-Dec-11	35	5	6.40	100	50%	30-Dec-11	600	43.10	2.28	0.17
10	Farm slurries, dairy cattle	0	23-Dec-11	35	5	6.40	100	50%	5-Jan-12	600	33.20	1.38	-0.63
11	Farm slurries, meat cattle	0	23-Dec-11	35	5	6.40	100	50%	5-Jan-12	600	52.70	2.12	0.19
12	Farm slurries, horses	1.33[4]	23-Dec-11	6[5]	5	35.00	100	50%	5-Jan-12	600	223.00	1.83	0.23
13	Farm slurries, meat goats	0	23-Dec-11	35	5	6.40	100	50%	5-Jan-12	600	55.80	2.16	0.17
14	Farm slurries, buffaloes	0	23-Dec-11	35	5	6.40	100	50%	5-Jan-12	600	60.00	1.65	0.52
15	Influent of WWTP F	0	11-Jan-12	35	5	6.40	0	50%	13-Jan-12	600	83.40	2.50	0.15
16	Influent of hospital B[1]	257	11-Jan-12	35	5	6.40	0	50%	13-Jan-12	600	75.60	2.27	0.16
17	Influent of residential buiding A	0	17-Jan-12	35	5	6.40	100	50%	21-Jan-12	600	79.00	2.41	0.15
18	Influent of residential buiding B	0	19-Jan-12	35	5	6.40	100	50%	21-Jan-12	600	67.50	2.36	0.16
19	Influent of WWTP G	0	23-Jan-12	35	5	6.40	100	50%	27-Jan-12	600	8.90	0.96	1.86
20	Influent of WWTP H	0	26-Jan-12	35	5	6.40	100	50%	27-Jan-12	600	57.80	0.15	2.36
21	Influent of WWTP I	0	26-Jan-12	35	5	6.40	100	50%	27-Jan-12	600	48.10	0.16	2.22
22	Influent of WWTP J	0	26-Jan-12	35	5	6.40	100	50%	27-Jan-12	600	45.70	4.21	1.44

Remarks

[1] WW = Wastewater

[2] A260/A230 Absorbance at 260-nm wavelength / absorbance at 230-nm wavelength, indicating DNA purification

[3] A280/A230 Absorbance at 280-nm wavelength / absorbance at 230-nm wavelength, indicating protein and chromosome contamination

[4] PFU/g

[5] g

Table 3.27 PCR reaction components and cycling conditions of bacteriophage ATCC 51477-B1 DNA with primer pair BT3765-BT3766 detected in wastewater samples

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (various conc.)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		55	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold	1	16	∞

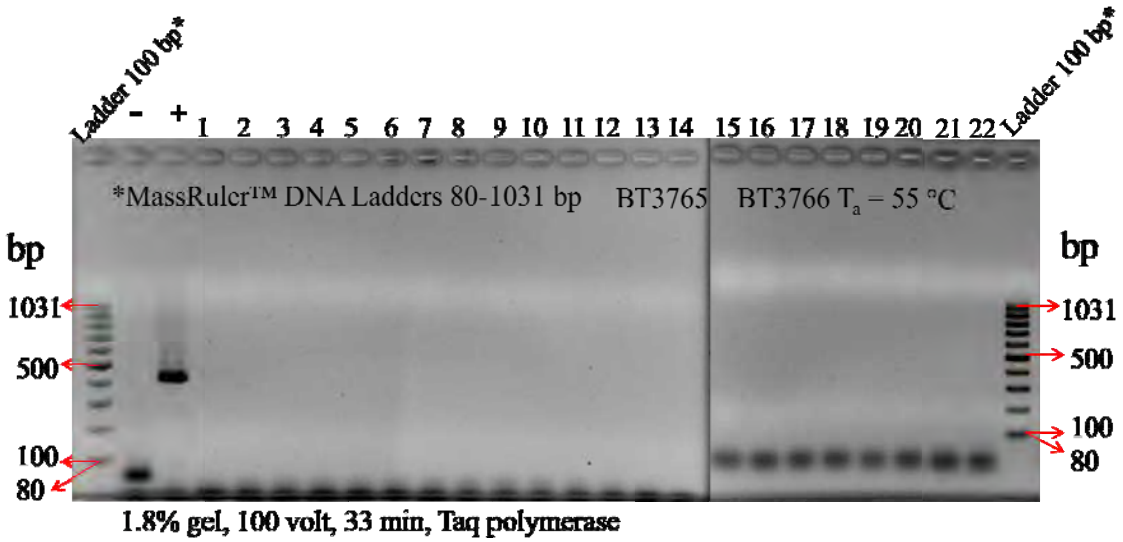

Figure 3.24 Gel electrophoresis showing PCR results of bacteriophage strain ATCC 51477-B1 DNA with primers BT3765-BT3766 detected in wastewater samples

Acinetobacter sp. strain ADP1 was spiked and detected by the PCR assay with primer pair BT3714-BT3715 to determine %loss of DNA after DNA extraction step **(Table 3.28).** The result showed negative detection in all samples **(Figure 3.25).** There were positive explanations for the negative results as follow: (1) the amount of spiked DNA of *Acinetobacter sp.* ADP1 was not enough (2) the DNA was completely lost during wastewater DNA extraction, and (3)

bacterial DNA was harder to amplify than DNA of viruses because bacterial genome contents a lot of G-C base pairs that carry triple bond pairing structure.

However, the results of this study for the new PCR assay for bacteriophages can indicate that bacteriophage ATCC 51477-B1 in the amount less than 257 PFU/100 ml in wastewater samples were not detected by this assay.

Table 3.28 PCR reaction components and cycling conditions of *Acinetobacter sp.* ADP1 with primers BT3714- BT3715 detected in wastewater samples

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	**Volume (µl)**	**PCR step**	**Cycle**	**Temp (^{0}C)**	**Time**
Template (various conc.)	2	Initial denaturation	1	95	3-5 min
2x PCR MM (Taq polymerase)	5	Denaturation	35	95	20 sec
Forward primer (10 µM)	1	Annealing		55	20 sec
Reverse primer (10 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold		16	∞

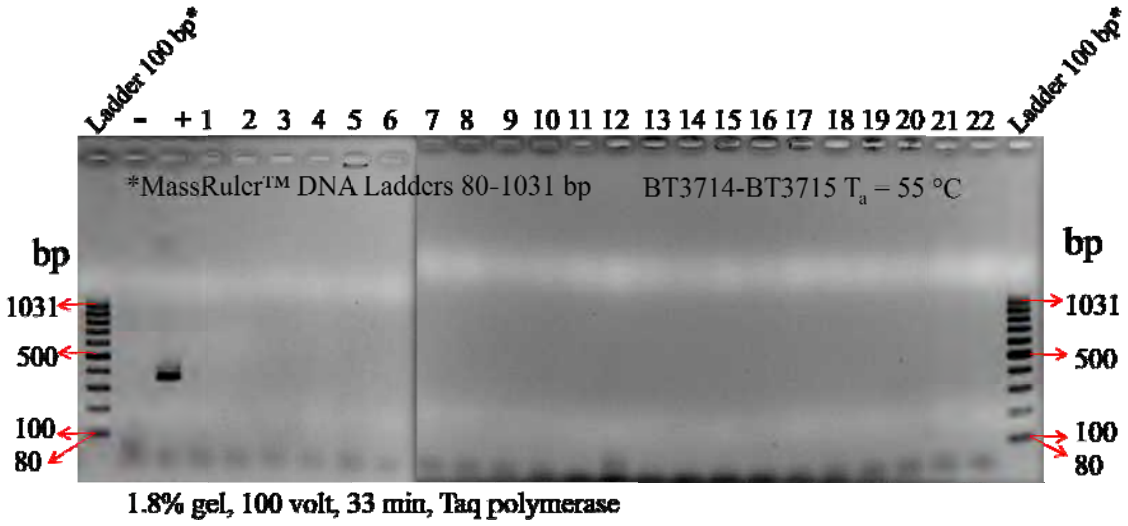

Figure 3.25 Gel electrophoresis showing PCR results of *Acinetobacter sp.* ADP1 DNA with primer BT3714 - BT3715 detected in wastewater samples

3.6 Comparison of the human-specific phages of *Bacteroides* PCR assay with other fecal indicators and correlation analysis

Out of 22 wastewater samples, 7 samples were farm slurries collected from animal farms, while the rest were human-derived wastewater samples from hospitals, municipal wastewater treatment plants, residential buildings and a nightsoil treatment plant. There were relatively high values of the total coliforms in the range of 520,000-35,350,000 CFU/100 ml and *E. coli* in the range of 170,000-29,550,000 CFU/100 ml (**Table 3.29**). However, there were no standard for wastewater influents of those wastewaters in Thailand yet. For 1 animal manure, the samples were collected by solid that were unavailable of the total coliforms and fecal standard.

Phage ATCC 51477-B1 was measured by the culture method and the PCR assay. Results showed that only 2 samples were positively detected by the culture method at 1.33 and 257 PFU/100 ml. All PCR results turned negative. The results of this study for the new PCR assay for bacteriophages can indicate that bacteriophage ATCC 51477-B1 in the amount less than 257 PFU/100 ml in wastewater samples were not detected by this assay. All samples showed high quantity of total coliforms and *E. coli*. No correlation analysis was performed due to many negative results.

Table 3.29 Measurement of phage ATCC 51477-B1, total coliforms and *E. coli* in wastewater samples

No.	Source of Wastewater	ATCC 51477-B1		Total coliforms (CFU/100 ml)	*E. coli* (CFU/100 ml)
		Culture method (PFU/100 ml)	PCR assay		
1	Influent of hospital A	0	negative	2,520,000	2,130,000
2	Influent of [1]WWTP A	0	negative	1,090,000	360,000
3	Influent of WWTP B	0	negative	1,380,000	880,000
4	Night soil treatment plant	0	negative	19,000,000	12,000,000
5	Influent of WWTP C	0	negative	520,000	190,000
6	Farm slurries, sheeps	0	negative	541,000	532,000
7	Farm slurries, dairy goats	0	negative	35,350,000	29,550,000
8	Influent of WWTP D	0	negative	1,350,000	1,090,000
9	Influent of WWTP E	0	negative	1,240,000	820,000
10	Farm slurries, dairy cattle	0	negative	12,300,000	11,600,000
11	Farm slurries, meat cattle	0	negative	3,400,000	3,150,000
12	Farm slurries, horses	[2] 1.33	negative	3,280,000	2,970,000
13	Farm slurries, meat goats	0	negative	690,000	510,000
14	Farm slurries, buffaloes	0	negative	530,000	170,000
15	Influent of WWTP F	0	negative	2,880,000	2,450,000
16	Influent of hospital B	257	negative	2,980,000	2,440,000
17	Influent of residential building A	0	negative	690,000	570,000
18	Influent of residential building B	0	negative	790,000	470,000
19	Influent of WWTP G	0	negative	2,210,000	1,160,000
20	Influent of WWTP H	0	negative	1,540,000	750,000
21	Influent of WWTP I	0	negative	1,780,000	630,000
22	Influent of WWTP J	0	negative	748,000	292,500
	Remarks	[1]WWTP = municipal wastewater treatment plant			
		[2]PFU/g			

Chapter 4: Conclusions and Recommendations

4.1 Conclusions

The conclusions of this study are presented with their corresponding objectives as follows:

Objective 1: Detection of human-specific phages of *Bacteroides* in Thailand

The detection method for phages 51477-B1 and 700786-B1, involving a double agar plaque assay with incubation under anaerobic conditions, is straightforward and can be easily performed in typical water analysis laboratories. Consequently, the technique seems to be suitable for regular monitoring of phages, especially in developing countries. Both types of phages were detected in wastewater samples from hospitals, but not from residential and office facilities. Phage 700786-B1 was more frequently detected and present in higher concentrations in both human- and animal-influenced samples. Phage 51477-B1 was positively detected in some human-derived wastewater samples, but was found at very low levels or was absent in animal-derived samples. This implies that phage 51477-B1 is specific to human sources.

Objective 2: Designing a PCR assay for detection of human-specific phages of *Bacteroides*

Difference in DNA sequences between the human-specific strain of bacteriophage ATCC 51477-B1 and the non-specific strain of phage ATCC 700786-B1 has been observed. First DNA sequence differences between bacteriophage strains ATCC 51477-B1 and ATCC 700786-B1 were checked using HindIII and Eco72I restriction enzyme digestion. The results showed the different restriction enzyme digestion patterns. Second, the whole genome sequencing, genomic assembly and genomic analysis were performed in order to find DNA regions of bacteriophage strain ATCC 51477-B1 which were not found in bacteriophage strain ATCC 700786-B1. The four regions were found as follows: node 13 with 866 bp in length and 99.75% coverage, node 79 with 443 bp in length and 86.20% coverage, node 9 with 941 bp in length and 86.26% coverage, and node 43 with 440 bp in length and 97.91% coverage.

Fore nodes of DNA of bacteriophage strain ATCC 51477-B1 DNA were used to design PCR primers using the Primer-BLAST tool. The primer pairs that could match DNA of only bacteriophage strain ATCC 51477-B1 were selected for the subsequent PCR designing steps. These primer pairs included BT3759-BT3760, BT3761-BT3762, and BT3763-BT3764 from DNA node 13, and BT3765-BT3766 from DNA node 79. Next, the primer pairs were tested with purified DNA of bacteriophage ATCC 51477-B1 and ATCC 700786-B1. PCR reaction

components and cycling conditions were optimized. PCR primers, reaction components and cycling conditions are summarized in **Table 4.1**.

Table 4.1 Summary of PCR reaction components and PCR cycling conditions of bacteriophage ATCC 51477-B1 DNA with primers BT3765-BT3766

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	Volume (µl)	PCR step	Cycle	Temp (^{0}C)	Time
Template (at least 0.001 ng/µl)	2	Initial denaturation	1	95	3- 5 min
2x PCR MM (Taq polymerase)	5	Denaturation	30-35	95	20 sec
Forward primer (5 µM)	1	Annealing		55	20 sec
Reverse primer (5 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold		16	∞

Method detection limit (MDL) for DNA template concentrations of bacteriophage ATCC 51477-B1 DNA was 0.002 ng in 10 µl PCR reaction.

Objective 3: *Bacteroides* phage PCR assay validation with wastewater samples

The DNA-based detection method was designed to measure phage ATCC 51477-B1 DNA in wastewater samples. Therefore, in order to account for DNA loss during DNA extraction step, known amount of DNA of another organism was spiked into the water samples before the DNA extraction step. DNA of *Acinetobacter sp*. ADP1 was used. PCR primers for *Acinetobacter sp*. ADP1 were designed and PCR reaction components and cycling conditions were optimized. PCR primers, reaction components and cycling conditions for *Acinetobacter sp*. ADP1 are summarized in **Table 4.2**.

The PCR detection methods of bacteriophage ATCC 51477-B1 and *Acinetobacter sp.* ADP1 were tested with 22 wastewater samples from the influent of municipal wastewater treatment facilities, the influent of residential buildings, and farm slurries. Results showed no positive detection when wastewater samples contained the concentration of bacteriophage ATCC 51477-B1 up to 257 PFU/100 ml and spiked concentrations of *Acinetobacter sp*. ADP1 DNA of 100 ng.

Table 4.2 Summary of PCR reaction components and PCR cycling conditions of *Acinetobacter sp*. ADP1 DNA with primers BT3714-BT3715

PCR reaction component		PCR cycling condition			
Ingredient of reaction 10 (µl)	Volume (µl)	PCR step	Cycle	Temp (^{0}C)	Time
Template (at least 0.01 ng/µl)	2	Initial denaturation	1	95	3- 5 min
2x PCR MM (Taq polymerase)	5	Denaturation	30-35	95	20 sec
Forward primer (5 µM)	1	Annealing		55	20 sec
Reverse primer (5 µM)	1	Extension		72	30 sec
DMSO	1	Final extension	1	72	10 min
Total	10	Hold		16	∞

Objective 4: Comparison of the human-specific phages of *Bacteroides* PCR assay with other fecal indicators

Total coliforms and E. coli were measured in 22 wastewater samples: 7 samples were farm slurries collected from animal farms, while the rest were human-derived wastewater samples. There were relatively high values of the total coliforms in the range of 520,000-35,350,000 CFU/100 ml and *E. coli* in the range of 170,000-29,550,000 CFU/100 ml. No correlation analysis was performed due to many negative results in phage ATCC 51477-B1 detection.

4.2 Recommendations

The recommendations below are suggested for further studies:

1. In order to obtain large amount of bacteriophage DNA from wastewater samples, better extraction methods are needed. Suggestions are as follows: (1) increasing the volume of wastewater sample for extraction, (2) precipitating the DNA to increase the concentration, (3) add specific chemicals to settle down the suspended phage particle during the step of phage recovery.
2. Because this research project developed the DNA detection method for one strain of phage (ATCC 51477-B1), the other strain (ATCC 700786-B1) still needs the detection method as well. Both strains should be detectable in one test.

3. Once the whole genome sequence of bacteriophage ATCC 51477-B1 is known, the protein functions should be studied. This information will help to identify differences between phages ATCC 51477-B1 and ATCC 700786-B1.

4. The relationship of human-specific bacteriophage strain ATCC 51477-B1 and other microorganisms in fecal materials, such as *Escherichia*, *Enterobacter, Klebsiella*, and *Citrobacter*, should be studied in order to determine the correlation.

5. Because this study designed DNA-based detection method that only visible bands of PCR results could be observed. The future study should involve the development of the DNA detection method for predicting amount of fecal contamination in environment.

6. The PCR assay to be used with environmental samples needs to be optimized to reduce the inhibition cause by inhibitors present in the environment.

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Appendix A:

List of Publications

ผลงานวิจัยที่ผ่านมา ได้มีการนำเสนอผลงานแบบบรรยาย แบบโปสเตอร์ และการตีพิมพ์ในวารสารวิชาการ ดังต่อไปนี้

1. ผลงานตีพิมพ์ในวารสารวิชาการ (แสดงใน Appendix B)

Sirikanchana, Kwanrawee and Mongkolsuk, Skorn (2011) "*Bacteroides*-infecting Bacteriophage in Wastewater as an Indicator of Human- or Animal-derived Fecal Pollution" Thai Environmental Engineering Journal, *accepted*.

2. การนำเสนอผลงานแบบบรรยายในงานประชุมระดับประเทศ (แสดงใน Appendix C)

"*Bacteroides*-infecting Bacteriophage in Wastewater as an Indicator of Human- or Animal-derived Fecal Pollution" at the Environmental Engineering Association of Thailand's 10th National Environmental Conference. March 23-25, 2011. BP Samila Beach Hotel & Resort, Songkla, Thailand.

3. การนำเสนอผลงานแบบโปสเตอร์ในงานประชุมระดับประเทศ (แสดงใน Appendix D)

"Study of *Bacteroides*-infecting Bacteriophage as an Indicator for Human- and Animal-derived Fecal Pollution in Water in Thailand" at the Conference on Frontiers in Environmental Health, Toxicology and Management of Chemicals (Poster presentation). Center of Excellence on Environmental Health, Toxicology and Management of Chemicals (ETM). July 3, 2010, Convention Center, Chulabhorn Research Institute, Bangkok, Thailand.

4. การนำเสนอผลงานแบบบรรยายในงานประชุมนานาชาติ (แสดงใน Appendix E)

"Genomic Differences between Human-specific and Non-specific Strains of Bacteriophages of *Bacteroides fragilis* as Fecal Source Identifiers" at the 1st International Conference on Environmental Science, Engineering and Management. March 21-23, 2012. Polvadol Resort and Spa, Chiang Rai, Thailand.

Appendix B:

Journal Publication

Sirikanchana, Kwanrawee and Mongkolsuk, Skorn (2011) "*Bacteroides*-infecting Bacteriophage in Wastewater as an Indicator of Human- or Animal-derived Fecal Pollution" Thai Environmental Engineering Journal, *accepted*.

การตรวจวิเคราะห์ไวรัสของแบคทีรอยดีสในน้ำเสียเพื่อใช้บ่งชี้การปนเปื้อนของสิ่งปฏิกูลจากคนหรือมูลสัตว์

Bacteroides-infecting Bacteriophage in Wastewater as an Indicator of Human- or Animal-derived Fecal Pollution

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บทคัดย่อ

ในปัจจุบัน การปนเปื้อนของสิ่งปฏิกูลในแหล่งน้ำจืดนั้น นับว่าเป็นปัญหาที่มีความสำคัญมากขึ้น โดยส่งผลกระทบต่อสุขภาพของประชาชนผู้ใช้และสัมผัสน้ำปนเปื้อนนั้น การใช้แบคทีเรียชี้แนะกลุ่มดั้งเดิม (Traditional Fecal Indicators) ซึ่งประกอบไปด้วยแบคทีเรียกลุ่มโคลิฟอร์มทั้งหมด แบคทีเรียกลุ่มฟีคัลโคลิฟอร์ม อีโคไล และ เอ็นเธอโร-คอกคัส เพื่อเป็นมาตรฐานทางชีววิทยาของคุณภาพน้ำนั้น ยังใช้กันอยู่ในปัจจุบัน แต่ด้วยข้อด้อยต่างๆผลักดันให้เกิดการวิจัยที่แสวงหาจุลินทรีย์ชนิดใหม่ที่ทำหน้าที่แบบเดียวกันได้ โดยไวรัสของแบคทีรอยดีส (Bacteroides-infecting Bacteriophages) เป็นจุลินทรีย์ชนิดหนึ่งที่ได้รับความสนใจและมีการวิจัยในประเทศต่างๆทั่วโลก แต่ยังไม่มีรายงานการศึกษาไวรัสชนิดนี้ในประเทศแถบเอเชีย วัตถุประสงค์หลักของงานวิจัยนี้ คือ เพื่อศึกษาความเหมาะสมของการใช้ไวรัสของแบคทีรอยดีส เพื่อเป็นตัวบ่งชี้การปนเปื้อนของสิ่งปฏิกูลจากคนและมูลสัตว์ในแหล่งน้ำในประเทศไทย โดยไวรัสของแบคทีรอยดีสที่ทำการศึกษา ประกอบด้วยชนิด 51477-B1 และชนิด 700786-B1 ซึ่งตรวจวัดได้ด้วยแบคทีรอยดีส แฟรจิลลิส (B. fragilis) ชนิด HSP40 และชนิด RYC2076 ตามลำดับ สำหรับวัตถุประสงค์ย่อยของโครงการ ประกอบด้วย 1) เพื่อ

ตรวจหาชนิดและปริมาณของไวรัสทั้งสองชนิดจากแหล่งกำเนิดน้ำเสียจากคนและสัตว์ (โคและสุกร) และ 2) เพื่อศึกษาความจำเพาะของไวรัสทั้งสองชนิดต่อน้ำเสียที่มาจากสิ่งปฏิกูลจากคนและจากมูลสัตว์ จากการศึกษาพบว่า ตรวจพบไวรัสทั้งสองชนิดในน้ำเสียที่มาจากโรงพยาบาล (น้ำเสียก่อนเข้าระบบบำบัดน้ำเสีย) โดยไม่พบไวรัสทั้งสองชนิดในน้ำเสียจากโรงแรม/อาคารพักอาศัย หรืออาคารสำนักงานเลย และตรวจพบไวรัสชนิด 51477-B1 ในตัวอย่างน้ำเสียจากคนในปริมาณปานกลางถึงสูงในจำนวน 4 ตัวอย่างจากน้ำเสียที่วิเคราะห์ 17 ตัวอย่าง และพบในปริมาณค่อนข้างต่ำในตัวอย่างน้ำเสียจากสัตว์ ในจำนวน 1 ตัวอย่างจากจำนวนที่วิเคราะห์ทั้งหมด 5 ตัวอย่าง ซึ่งจากผลเบื้องต้นดังกล่าว แสดงความเป็นไปได้ว่าไวรัสชนิดนี้อาจแสดงความจำเพาะต่อน้ำเสียจากคน อย่างไรก็ตาม ผลการวิจัยเบื้องต้นนี้ต้องได้รับการยืนยันจากการวิเคราะห์ตัวอย่างด้วยจำนวนที่มากขึ้น และจากแหล่งกำเนิดน้ำเสียหลายๆชนิดก่อน โดยขณะนี้อยู่ระหว่างการทำการวิเคราะห์เพิ่มเติมสำหรับตัวอย่างน้ำจากแม่น้ำและคลองที่ได้รับการปนเปื้อนจากสิ่งปฏิกูลจากคนหรือมูลสัตว์ ผลที่ได้จากงานวิจัยนี้จะเป็นข้อมูลสำคัญในการใช้จุลินทรีย์กลุ่มใหม่ที่สามารถบ่งชี้การปนเปื้อนสิ่งปฏิกูลเพื่อนำมาประยุกต์ใช้ในการจัดการแหล่งน้ำในประเทศไทยต่อไป

คำสำคัญ ไวรัสของแบคทีรอยดีส น้ำเสีย การตรวจหาแหล่งที่มาของการปนเปื้อนสิ่งปฏิกูล มลพิษจากสิ่งปฏิกูล

Abstract

Fecal pollution in freshwater sources is a problem of increasing concern worldwide, as it can deteriorate water quality to a level that threatens human health and limits usage of available water resources. Traditional fecal indicators, such as total and fecal coliforms, E. coli, and enterococci, have many shortcomings that have directed researchers and regulatory agencies to search for new types of indicators of fecal contamination. Bacteroides-infecting phages are one of the new types of fecal indicators that are currently being evaluated in regions outside Asia. The ultimate goal of this study is to assess the suitability of Bacteroides-infecting phages as fecal identifiers in Thailand. This study focuses on the phages, 51477-B1 and 700786-B1, which specifically infect B. fragilis strains HSP40 and RYC2076, respectively. The objectives of this study are to (1) identify and quantify bacteriophages, 51477-B1 and 700786-B1, in wastewater contaminated with human and animal (i.e., swine and cattle) feces in Thailand; and (2) assess phage specificity in detecting fecal sources (human versus animals). Both 51477-B1 and 700786-B1 were detected in wastewater samples from hospitals, but not from residential and office facilities. Phage 700786-B1 was more frequently detected, and present in higher concentrations, in samples contaminated with human- and animal feces. Phage 51477-B1 was detected in 4 out of 17 human-derived wastewater samples. In contrast, it was found at very low level in 1 out of 5 wastewater samples contaminated with animal feces. This implies that phage 51477-B1 may show specificity to human sources. However, analysis of a larger number of samples from multiple sources will be necessary before firm conclusions can be drawn. Additional experiments are also under way to assess the ability to detect phages 51477-B1 and 700786-B1 in river waters

subject to fecal pollution from human and/or animal sources. This information will aid in the identification of a new group of fecal identifiers for use in Thailand.

KEYWORDS: Bacteroides-infecting bacteriophage, wastewater, fecal source tracking, fecal pollution

INTRODUCTION

The use of currently regulated group of fecal indicator bacteria, i.e., coliforms, for evaluating the biological safety of recreational waters has some limitations. One of the limitations is an inability to identify sources of pollution. Bacteriophages that specifically infect bacteria in the genus Bacteroides have been studied regarded their ability to indicate the presence of fecal contaminations and types of sources. Therefore, they have been proposed as a promising new fecal indicator group (Payan et al., 2005; Blanch et al., 2006). These types of viruses, together with their anaerobic bacterial hosts, reside in the gastrointestinal tract of warm blooded animals, including: humans, pigs, cows, horses, and dogs. To date, studies of indigenous Bacteroides-infecting phages have been conducted in a number of geographical areas outside Asia. Before they can be served as a global fecal indicator, Bacteroides-infecting bacteriophages need to be further researched in a wider set of geographical areas, including Thailand. The objectives of this study were to (1) identify and quantify Bacteroides-infecting bacteriophages in wastewaters contaminated with human and animal (i.e., swine and cattle) waste in Thailand; and (2) assess phage specificity in regard to fecal sources (human versus animals).

METHODS

Wastewater samples from buildings serving at least 100 inhabitants (Blanch et al., 2006) were collected from the Bangkok area and nearby provinces. The types of facilities sampled included: office buildings, hospitals, and residential buildings. Animal-derived fecal wastewater samples were collected from floor cleaning runoff, which contained feces of swine and cattle from farms near Bangkok. Water samples were stored on ice or at 4°C prior to analysis. Bacteroides fragilis strains HSP40 (ATCC 51477) and RYC2056 (ATCC 700786) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). A double agar layer method was used for phage enumeration (Ebdon et al., 2007), with a detection limit of 1 PFU/ml.

RESULTS AND DISCUSSION

Analysis of phages 51477-B1 and 700786-B1 was performed in 24 wastewater samples collected from human-related facilities, comprising 2 samples from office buildings, 5 samples from residential buildings and hotels, and 17 samples from hospitals (Figure 1). The wastewater samples taken were grabbed from wastewater sumps that collected wastewater from lavatories of each facility. Of all samples being analyzed, 4 samples were positively detected for phage 51477-B1, 12 samples showed positive detection for phage 7007086-B1, and 3 samples demonstrated positive detection for both phages. Interestingly, all positive samples were collected from hospital facilities. A detection limit for both phages is 1 PFU/ml. Detectable concentrations of phage 51477-B1 ranged from 2 to 15 PFU/ml with a median of 7 PFU/ml, while those of phage 700786-B1 were in a range of 1 to more than 300 PFU/ml with a median of 22 PFU/ml. As presented in this study, phage 700786-B1 was positively detected in wastewater samples more frequently and at higher concentrations than phage 51477-B1, which is in consistent with those reported by Blanch et al. (2006).

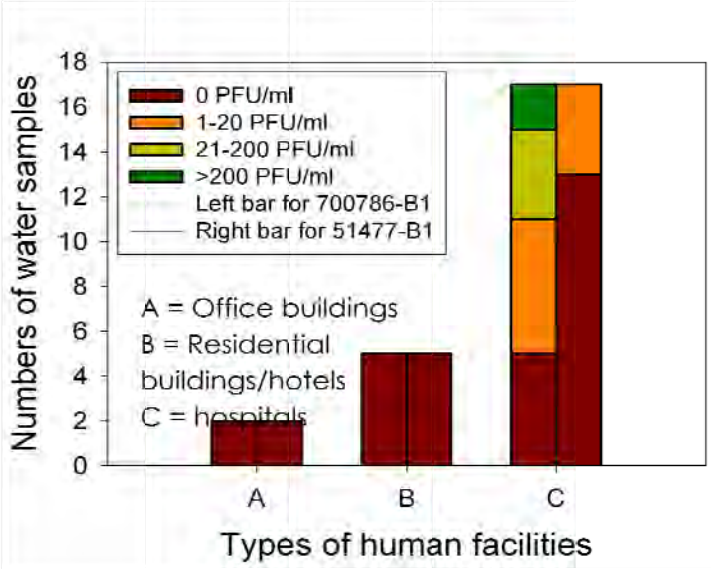

Figure 1. Detection of Bacteroides-infecting bacteriophage strains 51477-B1 (right bar) and 700786-B1 (left bar) in wastewaters from human-related facilities, including office buildings (A), residential buildings/hotels (B), and hospitals (C).

Wastewater samples from swine farms and cattle farms were collected from floor cleaning, which contains animal feces. Preliminary tests were conducted in 2 wastewater samples from swine farms, and 3 samples from cattle farms (Figure 2). For swine wastewater samples, phage 51477-B1 was detected in one of two samples at a low concentration of

3 PFU/ml, while phage 700786-B1 was presented in samples at 126 and 180 PFU/ml. The cattle wastewater samples presented no phage 51477-B1 detection, and one positive 700786-B1 sample at a concentration of 5 PFU/ml. More frequent detection and higher concentration of phage 700786-B1 than phage 51477-B1 were observed with animal wastewater samples, similar to those exhibited in human wastewater samples.

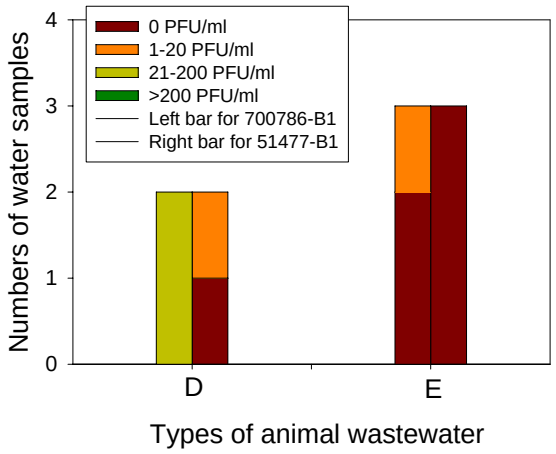

Figure 2. Detection of Bacteroides-infecting bacteriophage strains 51477-B1 (right bar) and 700786-B1 (left bar) in wastewaters from animal facilities, including swine farms (D) and cattle farms (E).

CONCLUSIONS

The detection method for phages 51477-B1 and 700786-B1, involving a double agar plaque assay with incubation under anaerobic conditions, is straightforward and can be easily performed in typical water analysis laboratories. Consequently, the technique seems to be suitable for regular monitoring of phages, especially in developing countries. Both types of phages were detected in wastewater samples from hospitals, but not from residential and office facilities. Phage 700786-B1 was more frequently detected and present in higher concentrations in both human- and animal-influenced samples. Phage 51477-B1 was positively detected in some human-derived wastewater samples, but was found at very low levels or was absent in animal-derived samples. This implies that phage 51477-B1 is specific to human sources. Analysis of a larger number of samples from multiple sources is ongoing and is necessary before firm conclusions can be drawn. This information will aid in the identification of a new group of fecal identifiers for use in Thailand.

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Appendix C:

Proceeding for Oral Presentation

"*Bacteroides*-infecting Bacteriophage in Wastewater as an Indicator of Human- or Animal-derived Fecal Pollution" at the Environmental Engineering Association of Thailand's 10th National Environmental Conference. March 23-25, 2011. BP Samila Beach Hotel & Resort, Songkla, Thailand.

23R2-01

การตรวจวิเคราะห์ไวรัสของแบคทีรอยดีสในน้ำเสีย เพื่อใช้บ่งชี้การปนเปื้อนของสิ่งปฏิกูลจากคนหรือมูลสัตว์

Bacteroides-infecting Bacteriophage in Wastewater as an Indicator of Human- or Animal-derived Fecal Pollution

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บทคัดย่อ

ในปัจจุบัน การปนเปื้อนของสิ่งปฏิกูลในแหล่งน้ำจืดนั้น นับว่าเป็นปัญหาที่มีความสำคัญมากขึ้น โดยส่งผลกระทบต่อสุขภาพของประชาชนผู้ใช้และสัมผัสน้ำปนเปื้อนนั้น การใช้แบคทีเรียชี้แนะกลุ่มดั้งเดิม (Traditional Fecal Indicators) ซึ่งประกอบไปด้วยแบคทีเรียกลุ่มโคลิฟอร์มทั้งหมด แบคทีเรียกลุ่มฟีคัลโคลิฟอร์ม อีโคไล และเอ็นเธอโร-คอกคัส เพื่อเป็นมาตรฐานทางชีววิทยาของคุณภาพน้ำนั้น ยังใช้กันอยู่ในปัจจุบัน แต่ด้วยข้อด้อยต่างๆ ผลักดันให้เกิดการวิจัยที่แสวงหาจุลินทรีย์ชนิดใหม่ที่ทำหน้าที่แบบเดียวกันได้ โดยไวรัสของแบคทีรอยดีส (*Bacteroides*-infecting Bacteriophages) เป็นจุลินทรีย์ชนิดหนึ่งที่ได้รับความสนใจและมีการวิจัยในประเทศต่างๆ ทั่วโลก แต่ยังไม่มีรายงานการศึกษาไวรัสชนิดนี้ในประเทศแถบเอเชีย วัตถุประสงค์หลักของงานวิจัยนี้ คือ เพื่อศึกษาความเหมาะสมของการใช้ไวรัสของแบคทีรอยดีส เพื่อเป็นตัวบ่งชี้การปนเปื้อนของสิ่งปฏิกูลจากคนและมูลสัตว์ในแหล่งน้ำในประเทศไทย โดยไวรัสของแบคทีรอยดีสที่ทำการศึกษา ประกอบด้วยชนิด 51477-B1 และชนิด 700786-B1 ซึ่งตรวจวัดได้ด้วยแบคทีรอยดีส แฟรจิลลิส (*B. fragilis*) ชนิด HSP40 และชนิด RYC2076 ตามลำดับ สำหรับวัตถุประสงค์ย่อยของโครงการ ประกอบด้วย 1) เพื่อตรวจหาชนิดและปริมาณของไวรัสทั้งสองชนิดจากแหล่งกำเนิดน้ำเสียจากคนและสัตว์ (โคและสุกร) และ 2) เพื่อศึกษาความจำเพาะของไวรัสทั้งสองชนิดต่อน้ำเสียที่มาจากสิ่งปฏิกูลจากคนและจากมูลสัตว์ จากการศึกษาพบว่า ตรวจพบไวรัสทั้งสองชนิดในน้ำเสียที่มาจากโรงพยาบาล (น้ำเสียก่อนเข้าระบบบำบัดน้ำเสีย) โดยไม่พบไวรัสทั้งสองชนิดในน้ำเสียจากโรงแรม/อาคารพักอาศัย หรืออาคารสำนักงานเลย และตรวจพบไวรัสชนิด 51477-B1 ในตัวอย่างน้ำเสียจากคนในปริมาณปานกลางถึงสูงในจำนวน 4 ตัวอย่างจากน้ำเสียที่วิเคราะห์ 17 ตัวอย่าง และพบในปริมาณค่อนข้างต่ำในตัวอย่างน้ำเสียจากสัตว์ ในจำนวน 1 ตัวอย่างจากจำนวนที่วิเคราะห์ทั้งหมด 5 ตัวอย่าง ซึ่งจากผลเบื้องต้นดังกล่าว แสดงความเป็นไปได้ว่า ไวรัสชนิดนี้อาจแสดงความจำเพาะต่อน้ำเสียจากคน อย่างไรก็ตาม ผลการวิจัยเบื้องต้นนี้ต้องได้รับการยืนยันจากการวิเคราะห์ตัวอย่างด้วยจำนวนที่มากขึ้น และจากแหล่งกำเนิดน้ำเสียหลายๆชนิดก่อน โดยขณะนี้อยู่ระหว่างการทำการวิเคราะห์เพิ่มเติมสำหรับตัวอย่างน้ำจากแม่น้ำและคลองที่ได้รับการปนเปื้อนจากสิ่งปฏิกูลจากคนหรือมูลสัตว์ ผลที่ได้จากงานวิจัยนี้จะเป็นข้อมูลสำคัญในการใช้จุลินทรีย์กลุ่มใหม่ที่สามารถบ่งชี้การปนเปื้อนสิ่งปฏิกูลเพื่อนำมาประยุกต์ใช้ในการจัดการแหล่งน้ำในประเทศไทยต่อไป

คำสำคัญ : ไวรัสของแบคทีรอยดีส; น้ำเสีย; การตรวจหาแหล่งที่มาของการปนเปื้อนสิ่งปฏิกูล; มลพิษจากสิ่งปฏิกูล

Abstract

Fecal pollution in freshwater sources is a problem of increasing concern worldwide, as it can deteriorate water quality to a level that threatens human health and limits usage of available water resources. Traditional fecal indicators, such as total and fecal coliforms, *E. coli*, and enterococci, have many shortcomings that have directed researchers and regulatory agencies to search for new types of indicators of fecal contamination. *Bacteroides*-infecting phages are one of the new types of fecal indicators that are currently being evaluated in regions outside Asia. The ultimate goal of this study is to assess the suitability of *Bacteroides*-infecting phages as fecal identifiers in Thailand. This study focuses on the phages, 51477-B1 and 700786-B1, which specifically infect *B. fragilis* strains HSP40 and RYC2076, respectively. The objectives of this study are to (1) identify and quantify bacteriophages, 51477-B1 and 700786-B1, in wastewater contaminated with human and animal (i.e., swine and cattle) feces in Thailand; and (2) assess phage specificity in detecting fecal sources (human versus animals). Both 51477-B1 and 700786-B1 were detected in wastewater samples from hospitals, but not from residential and office facilities. Phage 700786-B1 was more frequently detected, and present in higher concentrations, in samples contaminated with human- and animal feces. Phage 51477-B1 was detected in 4 out of 17 human-derived wastewater samples. In contrast, it was found at very low level in 1 out of 5 wastewater samples contaminated with animal feces. This implies that phage 51477-B1 may show specificity to human sources. However, analysis of a larger number of samples from multiple sources will be necessary before firm conclusions can be drawn. Additional experiments are also under way to assess the ability to detect phages 51477-B1 and 700786-B1 in river waters subject to fecal pollution from human and/or animal sources. This information will aid in the identification of a new group of fecal identifiers for use in Thailand.

Keywords : *Bacteroides*-infecting bacteriophage; wastewater; fecal source tracking; fecal pollution

Introduction

The use of currently regulated group of fecal indicator bacteria, i.e., coliforms, for evaluating the biological safety of recreational waters has some limitations. One of the limitations is an inability to identify sources of pollution. Bacteriophages that specifically infect bacteria in the genus *Bacteroides* have been studied regarded their ability to indicate the presence of fecal contaminations and types of sources. Therefore, they have been proposed as a promising new fecal indicator group (Payan et al., 2005; Blanch et al., 2006). These types of viruses, together with their anaerobic bacterial hosts, reside in the gastrointestinal tract of warm blooded animals, including: humans, pigs, cows, horses, and dogs. To date, studies of indigenous *Bacteroides*-infecting phages have been conducted in a number of geographical areas outside Asia. Before they can be served as a global fecal indicator, *Bacteroides*-infecting bacteriophages need to be further researched in a wider set of geographical areas, including Thailand. The objectives of this study were to (1) identify and quantify *Bacteroides*-infecting bacteriophages in wastewaters contaminated with human and animal (i.e., swine and cattle) waste in Thailand; and (2) assess phage specificity in regard to fecal sources (human versus animals).

Methods

Wastewater samples from buildings serving at least 100 inhabitants (Blanch et al., 2006) were collected from the Bangkok area and nearby provinces. The types of facilities sampled included: office buildings, hospitals, and residential buildings. Animal-derived fecal wastewater samples were collected from floor cleaning runoff, which contained feces of swine and cattle from farms near Bangkok. Water samples were stored on ice or at 4°C prior to analysis. *Bacteroides fragilis* strains HSP40 (ATCC 51477) and RYC2056 (ATCC 700786) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). A double agar layer method was used for phage enumeration (Ebdon et al., 2007), with a detection limit of 1 PFU/ml.

Results and Discussion

Analysis of phages 51477-B1 and 700786-B1 was performed in 24 wastewater samples collected from human-related facilities, comprising 2 samples from office buildings, 5 samples from residential buildings and hotels, and 17 samples from hospitals (Figure 1). The wastewater samples taken were grabbed from wastewater sumps that collected wastewater from lavatories of each facility. Of all samples being analyzed, 4 samples were positively detected for phage 51477-B1, 12 samples showed positive detection for phage 7007086-B1, and 3 samples demonstrated positive detection for both phages. Interestingly, all positive samples were collected from hospital facilities. A detection limit for both phages is 1 PFU/ml. Detectable concentrations of phage 51477-B1 ranged from 2 to 15 PFU/ml with a median of 7 PFU/ml, while those of phage 700786-B1 were in a range of 1 to more than 300 PFU/ml with a median of 22 PFU/ml. As presented in this study, phage 700786-B1 was positively detected in wastewater samples more frequently and at higher concentrations than phage 51477-B1, which is in consistent with those reported by Blanch et al. (2006).

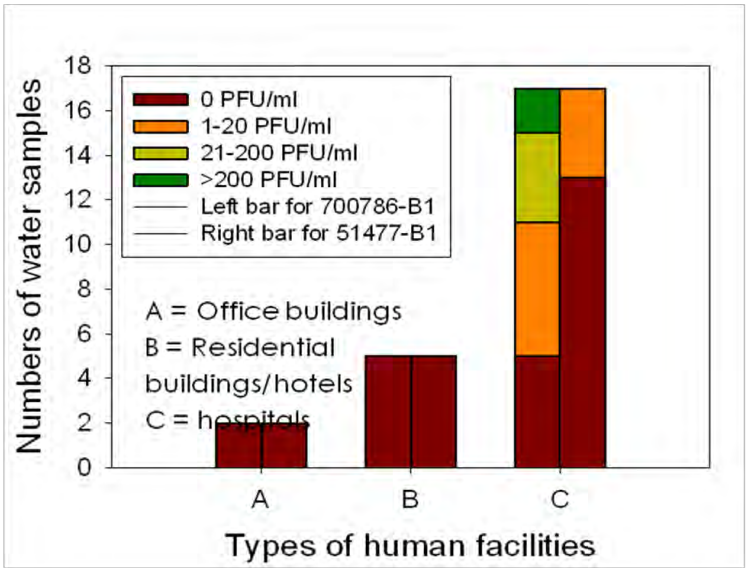

Figure 1. Detection of *Bacteroides*-infecting bacteriophage strains 51477-B1 (right bar) and 700786-B1 (left bar) in wastewaters from human-related facilities, including office buildings (A), residential buildings/hotels (B), and hospitals (C)

Wastewater samples from swine farms and cattle farms were collected from floor cleaning, which contains animal feces. Preliminary tests were conducted in 2 wastewater samples from swine farms, and 3 samples from cattle farms (Figure 2). For swine wastewater samples, phage 51477-B1 was detected in one of two samples at a low concentration of 3 PFU/ml, while phage 700786-B1 was presented in samples at 126 and 180 PFU/ml. The cattle wastewater samples presented no phage 51477-B1 detection, and one positive 700786-B1 sample at a concentration of 5 PFU/ml. More frequent detection and higher concentration of phage 700786-B1 than phage 51477-B1 were observed with animal wastewater samples, similar to those exhibited in human wastewater samples.

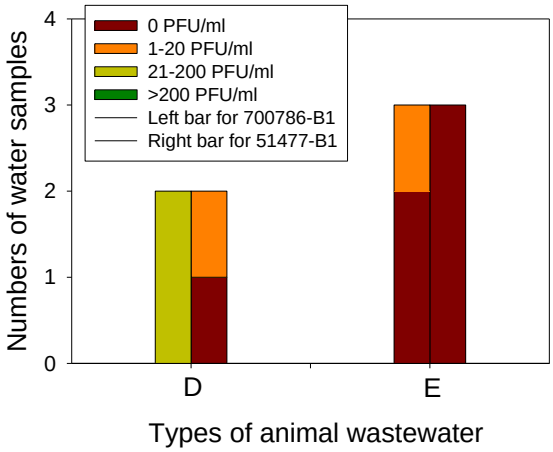

Figure 2. Detection of *Bacteroides*-infecting bacteriophage strains 51477-B1 (right bar) and 700786-B1 (left bar) in wastewaters from animal facilities, including swine farms (D) and cattle farms (E)

Conclusions

The detection method for phages 51477-B1 and 700786-B1, involving a double agar plaque assay with incubation under anaerobic conditions, is straightforward and can be easily performed in typical water analysis laboratories. Consequently, the technique seems to be suitable for regular monitoring of phages, especially in developing countries. Both types of phages were detected in wastewater samples from hospitals, but not from residential and office facilities. Phage 700786-B1 was more frequently detected and present in higher concentrations in both human- and animal-influenced samples. Phage 51477-B1 was positively detected in some human-derived wastewater samples, but was found at very low levels or was absent in animal-derived samples. This implies that phage 51477-B1 is specific to human sources. Analysis of a larger number of samples from multiple sources is ongoing and is necessary before firm conclusions can be drawn. This information will aid in the identification of a new group of fecal identifiers for use in Thailand.

Acknowledgement

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สมาคมวิศวกรรมสิ่งแวดล้อม
แห่งประเทศไทย

ขอมอบใบรับรองนี้เพื่อแสดงว่า

ดร.ขวัญรวี สิริกาญจน

ได้นำเสนอบทความเรื่อง

การตรวจวิเคราะห์ไวรัสของแบคทีรอยดีสในน้ำเสียเพื่อใช้บ่งชี้การปนเปื้อนของสิ่งปฏิกูลจากคนหรือมูลสัตว์

การประชุมวิชาการสิ่งแวดล้อมแห่งชาติครั้งที่ 10
10th National Environmental Conference
วันที่ 23 – 25 มีนาคม 2554
ณ โรงแรม บีพี สมิหลา บีช แอนด์ รีสอร์ท จังหวัดสงขลา

ให้ไว้ ณ วันที่ 23 มีนาคม 2554

ดร.เกษมสันติ์ สุวรรณรัต
นายกสมาคมวิศวกรรมสิ่งแวดล้อมแห่งประเทศไทย

Appendix D:

Extended Abstract and

Poster Presentation

"Study of *Bacteroides*-infecting Bacteriophage as an Indicator for Human- and Animal-derived Fecal Pollution in Water in Thailand" at the Conference on Frontiers in Environmental Health, Toxicology and Management of Chemicals (Poster presentation). Center of Excellence on Environmental Health, Toxicology and Management of Chemicals (ETM). July 3, 2010, Convention Center, Chulabhorn Research Institute, Bangkok, Thailand.

STUDY OF *Bacteroides*-INFECTING BACTERIOPHAGE AS AN INDICATOR FOR HUMAN- AND ANIMAL-DERIVED FECAL POLLUTION IN WATER IN THAILAND

Kwanrawee Sirikanchana

Translational Research Unit, Chulabhorn Research Institute and Center of Excellence on Environmental Health, Toxicology and Management of Chemicals

Abstract:

Fecal pollution in freshwater sources is a problem of increasing concern worldwide, as it can deteriorate water quality to a level that threatens human health and limits usage of available water resources. Traditional fecal indicators, such as total and fecal coliforms, *E. coli*, and enterococci, have many shortcomings that have directed researchers and regulatory agencies to search for new types of indicators of fecal contamination. *Bacteroides*-infecting phages are one of the new types of fecal indicators that are currently being evaluated in regions outside Asia. The ultimate goal of this study is to assess the suitability of *Bacteroides*-infecting phages as fecal identifiers in Thailand. This study focuses on the phages, 51477-B1 and 700786-B1, which specifically infect *B. fragilis* strains HSP40 and RYC2076, respectively. The objectives of this study are to (1) identify and quantify bacteriophages, 51477-B1 and 700786-B1, in wastewater contaminated with human and animal (i.e., swine and cattle) feces in Thailand; (2) assess phage specificity in detecting fecal sources (human versus animals); and (3) correlate the level of phage with the levels total coliforms and *E. coli* in wastewater samples. Both 51477-B1 and 700786-B1 were detected in wastewater samples from hospitals, but not from residential and office facilities. Phage 700786-B1 was more frequently detected, and present in higher concentrations, in samples contaminated with human- and animal feces. Phage 51477-B1 was detected in 4 out of 17 human-derived wastewater samples. In contrast, it was found at very low level in 1 out of 5 wastewater samples contaminated with animal feces. This implies that phage 51477-B1 may show specificity to human sources. However, analysis of a larger number of samples from multiple sources will be necessary before firm conclusions can be drawn. Additional experiments are also under way to assess the ability to detect phages 51477-B1 and 700786-B1 in river waters subject to fecal pollution from human and/or animal sources. This information will aid in the identification of a new group of fecal identifiers for use in Thailand.

Keywords: *Bacteroides*-infecting bacteriophage, Water pollution, Fecal source tracking, Fecal pollution

Introduction:

Currently regulated groups of fecal indicator bacteria, i.e., total and fecal coliforms, *E. coli* and enterococci, have posed limitations, including an inability to identify sources of pollution. These limitations have driven the United States to look for new fecal indicator organisms for their revised and new recreation water quality criteria (USEPA, 2007a,b). A group of bacteriophages that specifically infect bacteria in a genus *Bacteroides* have been studied and proposed as a promising new indicator group (Payan *et al.*, 2005; Blanch *et al.*, 2006). This type of viruses, together with its anaerobic bacteria host, reside in gastrointestinal areas of warm blooded animals, including human, pigs, cows, horses, dogs, etc. Two types of

Bacteroides-infecting phages that have been studied include one that specifically infects *B. fragilis* strain HSP40 (ATCC 51477), a so-called phage 51477-B1, and another that infects *B. fragilis* strain RYC2056 (ATCC 700786), a so-called phage 700786-B1. Specificity of phage 51477-B1 to human-derived fecal sources has promoted its use for tracking sources of fecal pollution (Tartera and Jofre, 1987). However, the application of this type of phages could be challenged by its detection capability that varied by geographical areas (Payan *et al.*, 2005). On the contrary, phage 700786-B1 has been detected in diverse geographical areas. Nonetheless, its presence in both human- and animal-derived fecal sources has weakened its use as a fecal source tracker (Payan *et al.*, 2005). To date, the study of indigenous *Bacteroides*-infecting phages have been conducted in certain geographical areas, namely Mediterranean (Tartera and Jofre, 1987; Duran *et al.*, 2002), Europe (Puig *et al.*, 1999), USA (Kator and Rhodes, 1992) and South Africa (Grabow *et al.*, 1993). Particularly in Asia, the study of locally-isolated *Bacteroides*-infecting phages is yet limited. To purposely serve as a global fecal indicator, *Bacteroides*-infecting bacteriophages need to be studied in other geographical areas, including Thailand, for its presence in wastewater and its specificity to types of fecal pollution.

The objectives of this study are to (1) identify and quantify *Bacteroides*-infecting bacteriophages in wastewaters derived from human and animals (i.e., swine and cattle) in Thailand; (2) assess phage specificity to fecal sources (human versus animals); and (3) correlate the level of phages with the level of water pollution, by comparing with the coliform groups (total coliforms and *E. coli*).

Methodology:

Water samples

Water samples from the influents of the wastewater treatment facilities, serving at least 100 inhabitants (Blanch *et al.*, 2006), are collected from Bangkok area and nearby provinces. Types of facility include, but not limit to, office buildings, hospitals, and residential buildings. Animal-derived fecal wastewater samples are collected from small-, medium-, and large-sized commercial swine and cattle farms near Bangkok. Rivers and canals possibly influenced by fecal contamination from human or animal sources are obtained for this study. Water samples are stored on ice or at 4°C until used for analysis.

Bacteroides host strains and phages

Bacteroides fragilis strains HSP40 (ATCC 51477) and RYC2056 (ATCC 700786) were ordered from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). Phages 51477-B1 and 700786-B1 were also ordered from the ATCC and used as references.

Enumeration of phages, total coliform bacteria and *E. coli*

A double agar layer method is used for phage enumeration by following Ebdon *et al.* (2007). To summarize, the *Bacteroides* host strain is grown in *Bacteroides* phage recovery medium (BPRM) broth to the proper optical density. The impurities in water samples are filtered with 0.22 µm polyvinylidene difluoride (PVDF) membrane. Then, 1 mL of the filtered water sample and 1 mL of the *Bacteroides* host strain are mixed with 5 mL of semi-solid BPRM agar. The mixture is poured onto the surface of BPRM agar and is solidified. The plates are then incubated for 18±2 h at 36±2°C in anaerobic conditions. The number of phages is counted as plaque forming units (PFU) per ml. Total coliforms and *E. coli* are analyzed using the membrane filtration method and MI agar, and enumerated as colony forming units (CFU) per 100 ml (US EPA, 2002).

Correlation analysis

Correlation analysis of the concentrations of traditional fecal indicators (i.e., total coliforms and *E. coli*) and phages infecting *Bacteroides* in water samples are performed with parametric method (e.g., Pearson's correlation) or non-parametric mean (e.g., Kendall's tau-b method).

Results and Discussion:

Preliminary analysis of phages 51477-B1 and 700786-B1 was performed in 24 wastewater samples collected from human-related facilities, comprising 2 samples from office buildings, 5 samples from residential buildings and hotels, and 17 samples from hospitals. The wastewater samples taken were grabbed from wastewater sumps that collected wastewater from lavatories of each facility. Of all samples being analyzed, 4 samples were positively detected for phage 51477-B1, 12 samples showed positive detection for phage 7007086-B1, and 3 samples demonstrated positive detection for both phages. Interestingly, all positive samples were collected from hospital facilities. A detection limit for both phages is 1 PFU/ml. Detectable concentrations of phage 51477-B1 ranged from 2 to 15 PFU/ml with a median of 7 PFU/ml, while those of phage 700786-B1 were in a range of 1 to more than 300 PFU/ml with a median of 22 PFU/ml. As presented in this study, phage 700786-B1 was positively detected in wastewater samples more frequently and at higher concentrations than phage 51477-B1, which is in consistent with those reported by Blanch et al. (2006).

Wastewater samples from swine farms and cattle farms were collected from floor cleaning, which contains animal feces. Preliminary tests were conducted in 2 wastewater samples from swine farms, and 3 samples from cattle farms. For swine wastewater samples, phage 51477-B1 was detected in one of two samples at a low concentration of 3 PFU/ml, while phage 700786-B1 was presented in samples at 126 and 180 PFU/ml. The cattle wastewater samples presented no phage 51477-B1 detection, and one positive 700786-B1 sample at a concentration of 5 PFU/ml. More frequent detection and higher concentration of phage 700786-B1 than phage 51477-B1 were observed with animal wastewater samples, similar to those exhibited in human wastewater samples.

Total coliforms and *E. coli* were measured in 5 human wastewater samples, ranging from 0 to 21.3 x 10^6 CFU/100 ml and 0 to 19.3 x 10^6 CFU/100 ml, respectively. Swine wastewater samples presented 7.1 x 10^6 to 290 x 10^6 CFU/100 ml and 5.5 x 10^6 to 190 x 10^6 CFU/100 ml, for total coliforms and *E. coli*, respectively; while cattle wastewater samples exhibited 2.0 x 10^6 to 9.2 x 10^6 CFU/100 ml and 0.9 x 10^6 to 2.0 x 10^6 CFU/100 ml, for total coliforms and *E. coli*, respectively. No significant correlation was observed between the current fecal indicator group (total coliform and *E. coli*) and two types of phages. Additional samples from human- and animal-related activities will need to be further analyzed for both types of phages and the current fecal indicator group before firm conclusions can be drawn.

Moreover, negative detection of phages 51477-B1 and 700786-B1 in wastewater samples will be analyzed and the detection method for phages 51477-B1 and 700786-B1 will be optimized to reduce the detection limit.

Conclusions:

The detection method for phages 51477-B1 and 700786-B1 involving a double agar plaque assay and incubation under anaerobic conditions can be achieved straightforwardly in typical water analysis laboratories. Consequently, the technique seems to be suitable for regular monitoring of phages, especially in developing countries. Preliminary results showed that both 51477-B1 and 700786-B1 were detected in wastewater samples from hospitals, but not from residential and office facilities. Phage 700786-B1 was more frequently detected, and present in higher concentrations, in samples contaminated with human- and animal feces. Phage 51477-B1 was detected in 4 out of 17 human-derived wastewater samples. In contrast, it was found at very low level in 1 out of 5 wastewater samples contaminated with animal feces. This implies that phage 51477-B1 may show specificity to human sources. However, analysis of a larger number of samples from multiple sources will be necessary before firm conclusions can be drawn. Additional experiments are also under way to assess the ability to detect phages 51477-B1 and 700786-B1 in river waters subject to fecal pollution from human and/or animal sources. This information will aid in the identification of a new group of fecal identifiers for use in Thailand.

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Appendix E:

Proceeding for Oral Presentation

"Genomic Differences between Human-specific and Non-specific Strains of Bacteriophages of *Bacteroides fragilis* as Fecal Source Identifiers" at the 1st International Conference on Environmental Science, Engineering and Management. March 21-23, 2012. Polvadol Resort and Spa, Chiang Rai, Thailand.

Genomic Differences between Human-specific and Non-specific Strains of Bacteriophages of *Bacteroides fragilis* as Fecal Source Identifiers

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ABSTRACT

Bacteroides-infecting bacteriophages are one of the new types of fecal indicators that are currently being evaluated in regions outside Asia. Bacteriophage strain ATCC 51477-B1, which infects *B. fragilis* strain HSP40 (ATCC 51477), has been reported to be found only in water samples contaminated with human-derived fecal materials. On the other hand, the strain ATCC 700786-B1, which infects *B. fragilis* strain RYC2056 (ATCC 700786), was shown to be detected in fecally contaminated water samples of both human and animal origins. The genomic information of phage ATCC 51477-B1 has been fully characterized. However, that of phage ATCC 700786-B1 has not yet been studied. Since a non-specific strain (ATCC 700786-B1) was found more prevalently in water, it is important for the newly-designed DNA-based assay not to pick up this non-specific phage strain as well. Therefore, there is a need to study the regions of difference between these two phage strains. The objective of this study was to determine regions of difference in DNA sequences between bacteriophages strain ATCC 51477-B1, representing a sewage-specific strain, and strain ATCC 700786-B1, representing a non-specific strain, using molecular techniques such as restriction enzyme digestion, DNA sequencing and sequence analysis. Genome sequencing was performed with Ion Torrent Personal Genome Machine System. The resulting DNA sequences were assembled and analysed with de novo genome assembly software and Blastn program. We found 4 regions of length 400bps - 1Kbps of bacteriophage ATCC 51477-B1 that were not in bacteriophage ATCC 700786-B1: node 43 (440 bp), node 13 (866 bp), node79 (443 bp), node 9 (941 bp). Information received from this study will facilitate the development of a DNA-based detection method that can detect the presence and qualitatively estimate levels of human-specific phages infecting *Bacteroides*. Therefore, after being validated with environmental samples, the method will be ready for measuring water quality, tracking human-derived fecal sources, and facilitating in water management and pollution control of water sources.

Keywords : Bacteriophages of *Bacteroides*; fecal source tracking; fecal pollution; water quality; DNA

INTRODUCTION

The needs to obtain new fecal pollution indicators and/or to develop new detection methods have been emphasized worldwide. Especially in the United States, with increasing evidences that current fecal indicator bacteria (FIB) is not a good indicator for fecal pollution due to regrowth and non-host specific indication, US EPA is on its way to look for another new fecal indicator organisms, in order to revise and set new recreation water quality criteria [1,2]. *Bacteroides*-infecting bacteriophages are one of the new types of fecal indicators that are currently being evaluated in regions outside Asia. They are groups of bacterial viruses that specifically replicate in anaerobic, gram negative, rod shape *Bacteroides* genus. The *Bacteroides* hosts and their phages reside in intestinal tracks of human and animals [3]. Bacteriophage strain ATCC 51477-B1, which infects *Bacteroides fragilis* strain HSP40 (ATCC 51477), has been reported to be found only in water samples contaminated with human-derived fecal materials [4]. On the other hand, the strain ATCC 700786-B1, which infects *B. fragilis* strain RYC2056 (ATCC 700786), was shown to be detected in fecally contaminated water samples of both human and animal origins [5, 6]. The ability to identify sources of fecal contamination will facilitate in water management and pollution control of water resources.

The genomic information of phage ATCC 51477-B1 has been fully characterized [7]. However, that of phage ATCC 700786-B1 has not yet been studied. The genome sequence, once available, can be used to design a DNA-based detection method that is restricted only to the human-specific phage. However, to achieve such an application, DNA sequences specific only to the human-specific phage are needed to be identified. Since a non-specific strain (ATCC 700786-B1) was found more prevalently in water [5], it is important for the newly-designed DNA-based assay not to pick up this non-specific phage strain as well.

Therefore, there is a need to study the regions of difference between these two phage strains. The objective of this study was to determine regions of difference in DNA sequences between bacteriophages strain ATCC 51477-B1, representing a sewage-specific strain, and strain ATCC 700786-B1, representing a non-specific strain, using molecular techniques such as restriction enzyme digestion, DNA sequencing and sequence analysis.

METHODOLOGY

Bacteroides reference host strains and bacteriophages

B. fragilis strains ATCC 51477 and ATCC 700786 were used as host strains for detection of bacteriophages. Both strains were ordered from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) in a freeze-dried form. Upon arrival, *Bacteroides* lyophilized vials were rehydrated with 10 ml *Bacteroides* Phage Recovery Medium (BPRM) broth [8] by following the enclosed instruction. Bacteriophage strains ATCC 51477-B1 and ATCC 700786-B1 were used as references for phages detected with *B. fragilis* strains ATCC 51477 and ATCC 700786, respectively. Both phages were ordered from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) in a freeze-dried form. Upon arrival, bacteriophage lyophilized vials were rehydrated and propagated by following the ATCC instruction. The double agar overlay plaque method was used for phage enumeration [8]. The number of phages was counted as plaque forming units (PFU). The detection limit was 100 PFU/100 ml.

DNA extraction of bacteriophages

DNA extraction of phage was performed by phenol-chloroform extraction by following the standard protocol [10]. The final pellet was resuspended with 400 µl of TE buffer (10:1 Tris: EDTA, pH 8.0). The purified DNA was stored at -20°C until use. DNA concentration was measured by a Nanodrop 2000 spectrophotometer (Thermo Scientific).

Restriction enzyme digestion

Known genomic sequence of bacteriophage ATCC 51477-B1 was analyzed on a web-based, webcutter 2.0 program (http://users.unimi.it/~camelot/tools/cut2.html) in order to find restriction enzymes that can cut the phage with reasonable number of fragments and their sizes. Appropriate restriction enzymes, e.g., Eco72I, were selected to cut DNA of both phage strains ATCC 51477-B1 and ATCC 700786-B1, following instructions enclosed with the enzymes. Gel electrophoresis was run to show restriction fragments of each sample.

Whole genome sequencing and analysis

The whole genomes of bacteriophage strains ATCC 51477-B1 and ATCC 700786-B1 were sequenced using the Ion Torrent Personal Genome Machine™ System (Ion Torrent Systems, Inc.) and unpaired short reads for both strains were produced. De novo assembling of the data was performed using Velvet [Ref: http://genome.cshlp.org/content/18/5/821.long] and de novo assemblies for the two strains were obtained. Comparison between the de novo assemblies was performed using Blastn [11]. Regions of length between 400 bps to 1 Kbps in bacteriophage ATCC51477-B1 that were not in ATCC 700786-B1 were found. They were chosen to be compared further with B40-8 of Hawkins whole genome sequencing [7].

RESULTS AND DISCUSSIONS

The whole genome sequence of 44,929 bp, double-stranded, linear DNA of bacteriophage ATCC 51477-B1 [7] was cut with many restriction enzymes in silico. Eco72I was selected for further experimental steps. In laboratory, DNA of phage ATCC 700786-B1 was extracted and cut with the Eco72I restriction enzyme.

Restriction patterns were observed with distinct sizes compared to those of phage ATCC 51477-B1. This demonstrated that genome sequences were different between the two phage types (Figure 1).

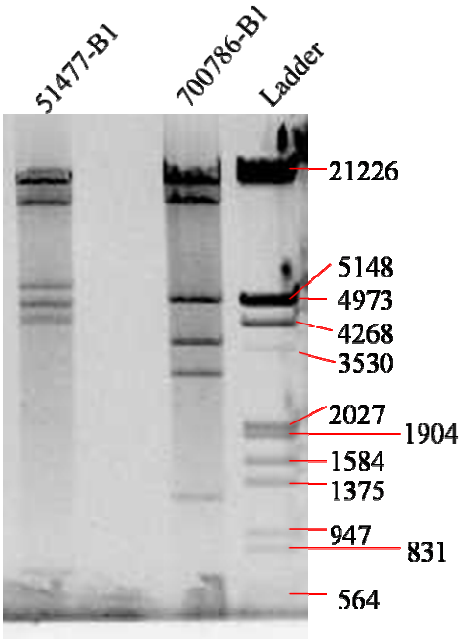

Figure 1 Eco72I restriction enzyme digestion of bacteriophage ATCC 51477-B1, bacteriophage ATCC 700786-B1, and lambda phage DNA/EcoRI+HindIII marker (Ladder)

Next, the whole genome sequencing of phage strains ATCC 51477-B1 and ATCC 700786-B1 was performed. The resulting DNA sequences were assembled and analysed. We found 4 regions of length 400 bps – 1 Kbps of bacteriophage ATCC 51477-B1 that were not in bacteriophage ATCC 700786-B1: node 43 (440 bp), node 13 (866 bp), node79 (443 bp), node 9 (941 bp). This available information can be further used to design a DNA-based detection method that is restricted only to the human-specific phage.

CONCLUSIONS

Difference in DNA sequences between the human-specific strain of bacteriophage ATCC 51477-B1 and the non-specific strain of phage ATCC 700786-B1 has been observed. The regions of differences between these two phage strains were determined. Information received from this study will facilitate the development of a DNA-based detection method that can detect the presence and qualitatively estimate levels of human-specific phages infecting *Bacteroides*. Therefore, after being validated with environmental samples, the method will be ready for measuring water quality, tracking human-derived fecal sources, and facilitating in water management and pollution control of water sources.

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