RESEARCH NOTE

A PCR-BASED DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* DIVERSITY USING *MYOVIRIDAE* PROPHAGE TYPING

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Abstract. PCR-based detection of *Myoviridae* lysogenic phages in *Burkholderia pseudomallei* was developed using primers targeting K96243 prophage GI2, phiE12-2 and phi52237/phiX216. Investigation of 50 clinical and 50 environmental (soil) isolates revealed that K96243 prophage GI2 was the most common (48%) among the isolates, followed by phiE12-2 (38%) and phi52237/phiX216 (35%), with K96243 prophage GI2 being significantly more frequent in soil (64%) than clinical (32%) samples. Twenty-four percent of soil isolates contained all three prophage types, while clinical isolates harbored no more than two types. Although *B. pseudomallei* isolates from soil were found to be more diverse based on prophage typing, all isolates were equally susceptible to a battery of lytic phages (although to different extents), suggesting the possibility of using lytic phages to control environmental *B. pseudomallei*.

Keywords: *Burkholderia pseudomallei*, *Myoviridae* prophage, bacteriophage, PCR

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis. The majority of infections are reported from Southeast Asia (especially northeastern Thailand) and northern Australia, where the organism is present both in soil and water (Chaowagul *et al*, 1989). Infection is acquired through skin inoculation, contamination of wounds, ingestion, or inhalation. The clinical picture of melioidosis is various and includes asymptomatic, acute, and chronic infections (Wiersinga *et al*, 2012). It is still unknown whether there is any correlation between causative bacterial strain and pathogenesis.

B. pseudomallei is highly genetically diverse, and one possible mechanism promoting this diversity is horizontal gene transfer mediated by lysogenic bacteriophages, these integrated sequences (prophages) contributing to inter-strain genetic variability (Srividhya *et al*, 2007).

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Most phages from *B. pseudomallei* isolates are lysogens and some even are polylysogenic, indicating multiple integrations of bacteriophages (Elliman, unpublished data). Many lysogenic *B. pseudomallei* phages including phi52237, phiE12-2, phi644-2, K96243 prophage GI2, phi1026b, and phiX216 have been sequenced (DeShazer, 2004; Holden *et al*, 2004; Ronning *et al*, 2010; Kvitko *et al*, 2012). Only four of them can be classified into family *Myoviridae* based on their morphotypes, namely, phi52237, phiE12-2, K96243 prophage GI2, and phiX216, while others can be classified into family *Siphoviridae*. These data can be used for differentiation of bacterial strains instead of relying on the complete genome sequences (Ronning *et al*, 2010).

In this work a PCR-based approach was developed to detect *B. pseudomallei* prophages and compared their diversity between clinical and soil *B. pseudomallei* isolates.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in this study were obtained from the Melioidosis Research Center (MRC), Khon Kaen University, Khon Kaen, Thailand. Fifty clinical isolates were from blood, sputum, body fluid, urine, and pus of patients with melioidosis, and 50 environmental isolates were cultured from soil of northeastern part of Thailand, including Khon Kaen, Ubon Ratchathani, Nakhon Ratchasima, Surin, Mukdahan, and Yasothon provinces. This project was approved by the human ethics committee of Khon Kaen University (Project ID HE531269).

Bacterial genomic DNA extraction

B. pseudomallei was grown in Luria Bertani (LB) broth for 16 hours at 37ºC with shaking (200 rpm). The bacterial culture was centrifuged at 11,000*g* for 5 minutes, and 1 ml aliquot of extraction buffer (50 mM glucose, 25 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA) was added to the pellet, mixed and centrifuged as described above. Then 500 µl aliquot of extraction buffer was added to the pellet, and mixed until the pellet dissolved. A 500 µl aliquot of 1:10 Proteinase K-SDS solution was added, gently mixed and incubated at 65ºC for 3 hours or until the solution appeared clear. Then 330 µl aliquot of 5M NaCl was added, mixed and centrifuged at 11,000*g* for 10 minutes. DNA was extracted using phenol-chloroform procedure, and precipitated by addition of 2 volumes of absolute ethanol and storage at -70ºC for 15-30 minutes (Sambrook *et al*, 1989). The pellet was washed with 70% ethanol, allowed to dry, dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA), and stored at -20ºC until used.

PCR protocol

Primers specific only for lysogenic *Myoviridae* phages of *B. pseudomallei* were designed from the sequences of phi52237 (GenBank accession no. NC_007145), phiE12-2 (GenBank accession no. NC_009236), K96243 prophage GI2 (GenBank accession no. NC_006350.1), and phiX216 (GenBank accession no. JX681814.1) using GeneFisher2 software (Giegerich *et al*, 1996). The phi52237/phiX216 primer pair amplified regions in the bacteria encoding the conserved hypothetical protein found also in the genomes of phage phi52237 and phiX216; phiE12-2 amplified regions encoding the hypothetical protein in phiE12-2 genome while the primers for K96243 prophage GI2 amplified a fragment of methylase gene (Table 1).

Amplification of *B. pseudomallei* 16S rDNA was used as a positive control. PCR reactions were carried out in a final

Table 1
Primers used in the study.

Phage	Primer	Sequence	Product length	Gene target
phi52237/ phiX216	phi52237/phiX216_F	CACACTTTCGGTCGATGTG	570 bp	Conserved hypothetical protein in phi52237 and phi52237 and phiX216 genome
	phi52237/phiX216_R	CTGATCTCTCCGCAATCAAG		
phiE12-2	phiE12-2_F	ATGTGACTCTGATCCCCTAC	258 bp	Hypothetical protein in phiE12-2 genome
	phiE12-2_R	CAAAGGTCGGCATACCTTC		
K96243 prophage GI2	K96243 prpphage GI2_F	CTTGCAACCGACTTATGC	488 bp	Methylase, a phage-encoded modification enzyme
	K96243 prpphage GI2_R	TCCCCAAATGATCGAGAAC		

volume of 25 µl containing 1X PCR buffer, 1.5 mM $MgC1_2$, 0.2 mM each dNTP, 0.5 µM each primer, 1.25 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and 2.5 µl of 125 ng of *B. pseudomallei* genomic DNA in distilled water. Thermocycling (conducted in Veriti 96-Well Thermal Cycler; Applied Biosystems, Carlsbad, CA) conditions were as follows: 95ºC for 4 minutes; 35 cycles of 95ºC for 45 seconds, 52ºC for 45 seconds, and 72ºC for 1 minute; with a final step of 72ºC for 7 minutes. Amplicons were analyzed by 1% agarose gel-electrophoresis and visualized using ethidium bromide staining under UV illumination. Amplicon bands were excised from gel and sequenced using a MegaBACE™ 1000 DNA sequencer (Department of Biochemistry, Faculty of Medicine, Khon Kaen University). The DNA sequences of PCR products were aligned with the expected sequences via ClustalW Program (Thompson *et al*, 1994). Chi-square test was used to examine the relationship between the percent sequence similarity of prophages detected in clinical and soil *B. pseudomallei* isolates.

Phage susceptibility test

An overnight culture of each *B. pseudomallei* isolate in nutrient broth (Oxoid, Hamshire, UK) was spread on the surface of nutrient agar (Oxoid). A 20 µl aliquot (10^8 PFU/ml) of a lytic phage (F4, KKS1, ST79, or ST96) suspension was then spotted onto the agar plate and incubated at 37ºC for 24 hours. Successful infection was indicated by the development of turbid or clear plaques.

RESULTS

PCR-based detection of *Myoviridae* prophage in *B. pseudomallei* isolates

PCR primers designed for detection of phi52237/phiX216, phiE12-2,

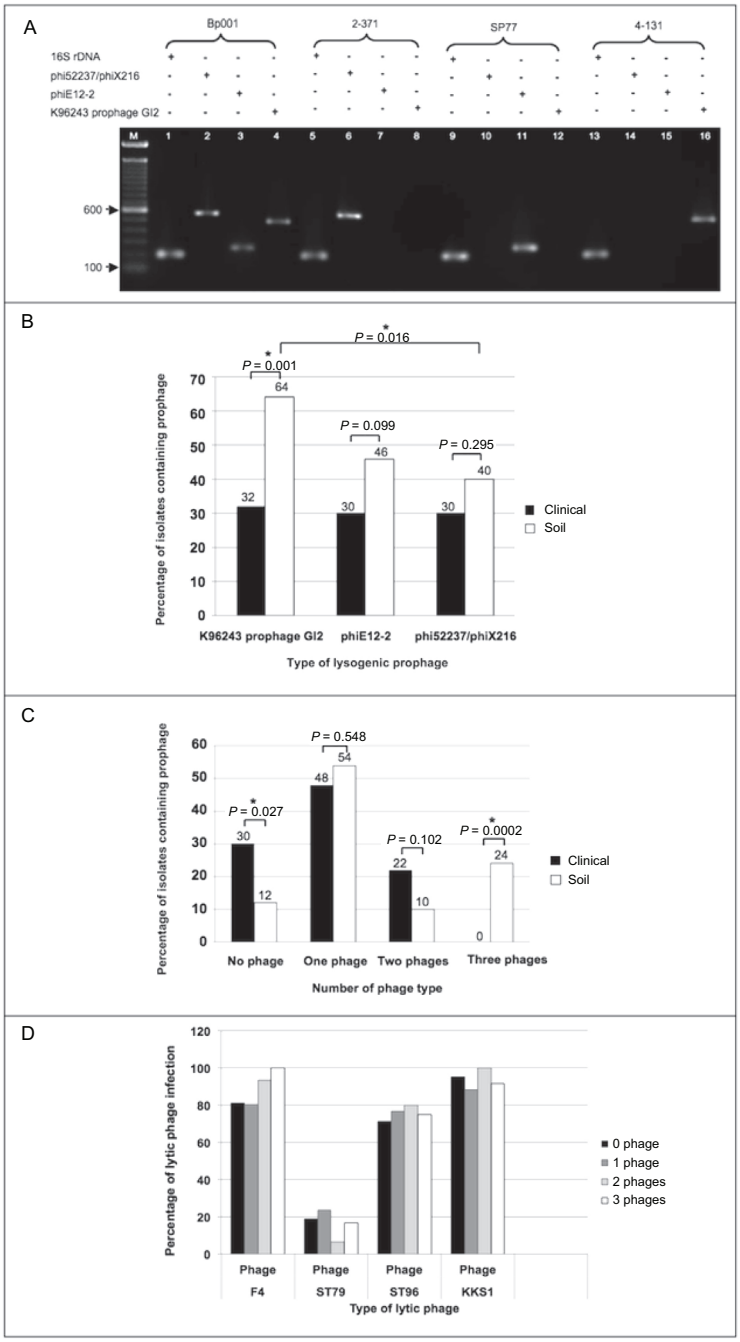

Fig 1–Diversity of *B. pseudomallei* as determined by the presence of *Myoviridae* prophages. A). Ethidium bromide-stained agarose gel of amplicons from representative *B. pseudomallei* isolates containing phi52237/phiX216, phiE12-2, and K96243 prophage G12 prophages. Amplification of a fragment of *B. pseudomallei* 16S rDNA was used as a positive control. B). Percent clinical (n = 50) and soil (n = 50) isolates containing each type of prophage. C). Percent clinical and soil clinical isolates containing 0, 1, 2, or 3 prophages. D). Percent soil isolates containing 0, 1, 2, or 3 prophages susceptible to lytic phages. *Significant difference.

Table 2
Burkholderia sp phage genome sequences deposited in GenBank.

No.	Phage	Accession no.	Host	References
Family *Myoviridae*				
1	*Burkholderia* phage phi52237	NC_007145	*B. pseudomallei*	Ronning *et al*, 2010
2	*Burkholderia* phage phiE12-2	NC_009236	*B. pseudomallei*	Ronning *et al*, 2010
3	GI2; K96243 phage	NC_006350[a]	*B. pseudomallei*	Holden *et al*, 2004
4	*Burkholderia* phage phiX216	JX681814.1	*B. pseudomallei*	Kvitko *et al*, 2012
5	*Burkholderia* phage phiE202	NC_009234	*B. thailandensis*	Lavigne *et al*, 2009
6	*Burkholderia* phage Bcep781	NC_004333	*B. cepacia*	Lavigne *et al*, 2009; Summer *et al*, 2006
7	*Burkholderia* phage Bcep43	NC_005342	*B. cepacia*	Lavigne *et al*, 2009; Summer *et al*, 2006
8	*Burkholderia* phage Bcep1	NC_005263	*B. cenocepacia*	Lavigne *et al*, 2009; Summer *et al*, 2006
9	*Burkholderia* phage BcepNY3	NC_009604	*B. cenocepacia*	Lavigne *et al*, 2009
10	*Burkholderia* phage BcepMu	NC_005882	*B. cenocepacia*	Lavigne *et al*, 2009
11	*Burkholderia* phage phiE255	NC_009237	*B. thailandensis*	Lavigne *et al*, 2009
12	*Burkholderia* phage BcepB1A	NC_005886	*B. cenocepacia*	Lavigne *et al*, 2009; Summer *et al*, 2006
13	*Burkholderia ambifaria* phage BcepF1	NC_009015	*B. ambifaria*	Summer *et al*, 2007
14	*Burkholderia* phage KS14	NC_015273	*B. cenocepacia*	Lynch *et al*, 2010b
15	*Burkholderia* phage KS10	NC_011216	*B. cenocepacia*	Goudie *et al*, 2008
16	*Burkholderia* phage KS5	NC_015265	*B. cenocepacia*	Lynch *et al*, 2010b
17	*Burkholderia* phage KL3	NC_015266	*B. cenocepacia*	Lynch *et al*, 2010b
18	*Burkholderia* phage ST79	NC_021343	*B. pseudomallei*	
Family *Siphoviridae*				
1	*Burkholderia* phage Bcep176	NC_007497	*B. cepacia*	Summer *et al*, 2007
2	*Burkholderia* phage phiE125	NC_003309	*B. thailandensis*	Woods *et al*, 2002
3	*Burkholderia* phage KS9	NC_013055	*B. pyrrocinia*	Lynch *et al*, 2010a
4	*Burkholderia* phage BcepGomr	NC_009447	*B. cepacia*	Summer *et al*, 2007
5	*Burkholderia* phage BcepNazgul	NC_005091	*B. cepacia*	Summer *et al*, 2007
6	*Burkholderia* phage AH2	NC_018283	*B. cenocepacia*	Lynch *et al*, 2012b
7	*Burkholderia* phage phi1026b	NC_005284	*B. pseudomallei*	DeShazer, 2004
8	*Burkholderia* phage phi644-2	NC_009235	*B. pseudomallei*	Ronning *et al*, 2010
9	*Burkholderia* phage KL1	NC_018278	*B. cenocepacia*	Lynch *et al*, 2012b
Family *Podoviridae*				
1	*Burkholderia* phage BcepC6B	NC_005887	*B. cepacia*	Summer *et al*, 2007
2	*Burkholderia* phage Bcep22	NC_005262	*B. cepacia*	Summer *et al*, 2007
3	*Burkholderia* phage DC1	NC_018452	*B. cepacia*	Lynch *et al*, 2012a
4	*Burkholderia* phage BcepMigl	NC_019917	*B. cenocepacia*	Summer *et al*, 2007
5	*Burkholderia* phage BcepIL02	NC_012743	*B. cenocepacia*	Summer *et al*, 2007
6	*Burkholderia* phage JG068	NC_022916	*B. cenocepacia*	Lynch *et al*, 2013

[a]Not available

and K96243 prophage GI2 sequences in *B. pseudomallei* genomes generated the expected amplicons of 570, 258, and 488 bp, respectively (Fig 1A). Amplicons were sequenced and homolog search via BLAST revealed matches with only those of their target phages.

Prophages in and susceptibility to lytic bacteriophages of *B. pseudomallei* clinical and soil isolates

B. pseudomallei soil isolates harbored more prophage types than clinical isolates (Fig 1B). K96243 prophage GI2 was the most commonly found 32/50 (64%) and 16/50 (32%) in soil and clinical isolates, respectively). Nearly a quarter of soil isolates contained the three types of prophages, while clinical isolates contained no more than two types and the number of clinical isolates containing no prophage is significantly higher than soil samples (Fig 1C). Although *B. pseudomallei* from soil were found to be more diverse based on the variety of prophages carried, the susceptibilities of these isolates containing from 0 to 3 different prophages to lytic phages (F4, KKS1, ST79, or ST96) were similar (Fig 1D).

DISCUSSION

Prophages contribute to inter-strain genetic variability of the bacteria (Srividhya *et al*, 2007). Most phages obtained from *B. pseudomallei* isolates are lysogenic (Ronning *et al*, 2010). A PCR-based assay was designed to detect specifically three lysogenic *Myoviridae* prophages (phi52237/phiX216 [as the primers designed for phi52237 could also amplify the recently discovered phiX216 (Kvitko *et al*, 2012), phiE12-2, and K96243 prophage GI2] of *B. pseudomallei.* Although no members of any other prophage family were detected using *Myoviridae*-specific primers, other families of *B. pseudomallei* phages might still be present (Table 2).

Although several studies reported typing of *B. pseudomallei*, there is no suitable method that could differentiate clinical from environmental isolates (Sermswan *et al*, 2001; Vesaratchavest *et al*, 2006; Tumapa *et al*, 2008). All reported methods were based on bacterial genome sequences but not on those of prophages. Our study found that isolates of *B. pseudomallei* from soil samples were more diverse with respect to the presence of *Myoviridae* phages than those from the clinical specimens. Bacteria that live in the environment may have a greater chance to exchange genetic material via phages than those in humans, thus leading to more diversity. We surmise that once these environmental bacteria infect humans, the variety of the phages they harbor is reduced. Although our *Myoviridae* sequence-based PCR detection could not differentiate all clinical isolates from environmental isolates, inclusion of data from the *Siphoviridae* family might allow more discriminatory power in future typing studies. Although *B. pseudomallei* from soil were found to be more diverse, our results demonstrated that they gave similar susceptibilities to lytic phages. This will lead to a high potential of using our lytic phages to control environmental *B. pseudomallei.*

In summary, a PCR-based detection of three types of *Myoviridae* prophages in clinical and environmental *B. pseudomallei* isolates was successfully developed, allowing demonstration of greater diversity of environmental than clinical samples.

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