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Molecular Procedure for Detection of *Burkholderia pseudomallei*

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Abstract: Recently several cases of melioidosis have been reported in the tropical climates, especially in Southeast Asia where, it is endemic, it also occurs sporadically throughout the world. The diagnosis of the acute or chronic infection remains challenging. The present study highlight on the optimized and reliable technique based DNA preparation for use in Polymerase Chain Reaction (PCR) assay. PCR amplification with specific pair of primer for each putative gene was proving specific for amplification of genes in *Burkholderia pseudomallei* strain D286. The PCR mixture with addition of DMSO, formamide and glycerol could ease the PCR optimization where different pairs of primers were involved. The findings of this study have contributed to some information on the molecular bases of the LPS biosynthesis genes in *B. seudomallei* specifically for strain D286. The specific primer pairs with the PCR mixture could be used in developing a PCR diagnosis of melioidosis.

Key words: Melioidosis, *Burkholderia pseudomallei*, lipopolysaccharide, polymerase chain reaction, primers, University Putra Malaysia

INTRODUCTION

Burkholderia pseudomallei are a motile, non-spore forming, gram-negative bacillus. It is a rod shape with 0.4-0.6 μm length. Normally, it grows aerobically on most medium and produces clearly visible colonies within 24 h at 37°C but still can survive anaerobically in nitrate contained complex medium. The role of exotoxins in the pathogenesis of melioidosis is unresolved. The high mortality of *B. pseudomallei* infections is related to an increased propensity to develop high bacteraemias (>1 cfu mL^{-1}). But the relation between bacterial counts in blood and mortality is similar to that of other gram-negative pathogens. This finding suggests that exotoxins do not contribute directly to outcome (Walsh *et al*., 1995). The endotoxin, or Lipopolysaccharide (LPS), the immunodominant antigens, is highly conserved (Charuchaimontri *et al*., 1999). LPS can evade killing by serum bactericidal system. The ability to evade complement-mediated killing is considered to be an important virulence determinant (David *et al*., 1998). PCR is a powerful molecular biology technique introduced in 1980 by Kary Mullis and is widely use (Mullis and Faloona, 1987). A primer is a short synthesized nucleotide segment which has complementary base pairs to the length of template DNA to be amplified in the PCR reaction (Bethesda, 1992). Specific primers are the primers developed from the nucleotide sequence of the target gene. The primers allow only the specific fragment of DNA to be amplified. Universal primers are those, which bind to any region in the genome bearing the complementary sequence. Thus, universal primers are able to bind to a wide variety of DNA templates. The amplified DNA fragment is then enough in quantity to be used for further study, for example, cloning and expression analysis. The PCR produces large amounts of a specific DNA fragment by the enzymatic amplification *in vitro*, which the phenomenon is same as the DNA replication *in vivo* (Saiki *et al*., 1985).

MATERIALS AND METHODS

***B. pseudomallei* stock:** All *B. pseudomallei* culture works were done in the Pathogen Laboratory, Department of Biochemical, Universiti Kebangsaan Malaysia (UKM) laboratory. *B. pseudomallei* strain D286 isolate was cultured on Ashdown medium, a selected medium for *B. pseudomallei*. The *B. pseudomallei* picture was provided by research assistant (Mr. Lim Boon San) from Pathogen Laboratory, UKM. *B. pseudomallei* strain D286 isolate that was cultured on Ashdown medium, a selected medium for *B. pseudomallei*, showed rough and wrinkled

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appearance (rugose, or like cornflower heads) colonies after 48 h incubation (Fig. 1). The wrinkled appearances are more obvious in old cultures. The colonies take up crystal-violet dye from the medium and exhibit dark red to purple colour colonies.

Genomic DNA extraction: DNA was extracted from the bacteria using Genomic DNA extraction kit (GeniSpin, Biosyntech Sdn. Bhd, Malaysia) according to the manufacturer's instructions. An aliquot of 3 mL from an overnight grown culture was harvested by centrifugation at 4.000×g for 10 min at room temperature. The supernatant was discarded and the cells were re-suspended in 100 μL TE buffer. An aliquot of 100 μL of (10 mg mL^{-1}) lysozyme (Sigma Chemical Co. USA) was added to the suspension and incubated for 10 min at 30°C. After the complete digestion of the cell wall, the cells were pelleted by centrifugation for 5 min at 4.000×g at room temperature. Following centrifugation, cells were resuspended in 200 μL of buffer BTL (Qiagen Hilden, Gelmany) to which 25 μL of proteinase K solution (QIAGEN Hilden, Germany) at 15 mg mL^{-1} was added. The suspension was vortexed to mix well and incubated at 55°C in a shaking water bath for 1 h to ensure the complete lysis. After the lysis, 25 μL RNase A (25 mg mL^{-1}) (QIAGEN Hilden, Germany) was added and incubated for 2 min at room temperature for removal of the RNA. Following RNA removal, 220 μL of buffer BDL was added to the suspension, vortexed to mix and incubated at 70°C for 10 min, which results in the formation of a wispy precipitate. An aliquot of 220 μL absolute ethanol was added to the precipitate mixture and was vortexed. The entire sample was then transferred to a spin column and centrifuged at 8.000×g for 1 min at room temperature. The flow through was discarded and the column was washed 2 times at 8.000×g with 750 μL of washing buffer. The flow through was again discarded and the column was allowed to spin at maximum speed for 2 min to dry the column. Finally, the DNA was eluted with an aliquot of 100 μL pre-heated water (70°C) in a sterile microcentrifuge tube. The eluted DNA sample was stored at -20°C for further use.

Agarose gel electrophoresis of DNA: Agarose gel electrophoresis technique was used to separate and purify DNA fragments between 0.25 and 25 kb. The agarose gel at 0.8% was suitable use to analyze genomic DNA and was prepared by adding 0.8 g of agarose gel powder (Promega) to 100 mL of 1× TBE buffer, which was then mixed well and boiled in microwave for about 3 min in medium-high temperature. The melting agarose was cooled to about 55°C under running pipe water before pouring into a sealed gel case. The gel was allowed to solidify before the samples were loaded. The gel case then placed into the running tank filled with 1× TBE buffer. An aliquot of 10 μL of each extracted DNA sample was added with 3 μL of 6× gel loading solution and mixed well with pipetting up and down several times before loading into the wells. The electrophoresis of DNA was carried out at a constant current of 60 V until the blue tracking dye had migrated to about 2/3 of the gel. The gel was stained in 10 mg mL^{-1} ethidium bromide (SIGMA) solution for 10 min. DNA molecules were visualized under UV transilluminator and the gel picture was captured using an imager (Alpha Imager™ 2200, Alpha Innotech Corporation).

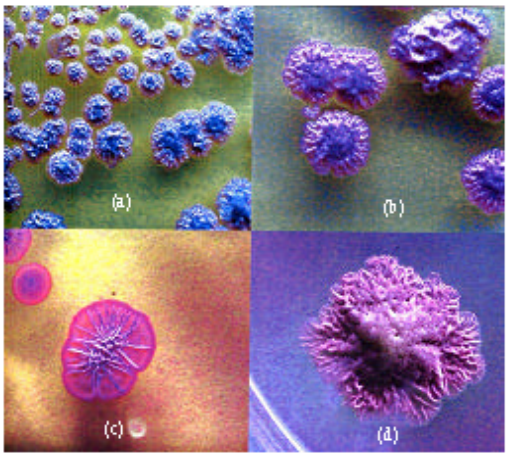

Fig. 1: Colony morphology of *B. pseudomallei* strain D286. Two days culture, a): 3 days culture, b): 4 days culture, c): 1 week culture, d): Lim (2004)

Primers design and synthesis: All primers used for PCR were designed specifically based on the sequences of annotated LPS putative genes manually and confirm by using Primer Primier 3 (CGAT, UKM). The optimum GC content and optimum melting point for each set of primer were considered. The primers were then synthesized by Research Biolabs (Malaysia). The oligonucleotide sequences of primers are shown in Table 1.

Amplification of putative genes by Polymerase Chain Reaction (PCR): A total of 25 μL amplification reaction mixture was prepared in 0.5 mL centrifuge tube. The cocktail components of the reaction mixture are presented in appendix C. The PCR mixtures were subjected to thermal cycling using the thermal cycler (Biometra-Trio Themoblock, Biometra Co, Ltd, Gottingen, Germany). The DNA amplification steps performed with initial denaturation at 96°C for 5 min, followed by 30 cycles steps consisting of denaturation at 96°C for 1 min,

Table 1: The oligonucleotide sequences of primers used in PCR

Gene name		5'-3' sequence	Tm°C/GC%	Product length (bp)
Bplps0001/*lpxL*	F	5' AGGATTCGCATGCTAGGCC 3'	60.1/57.9	943
	R	5' GAGTTTCATGGGCGCTATTG 3'	58.3/50.0	
Bplps0002/*waaF*	F	5' CGACATCGGAAAACGACCTG 3'	61.2/55.0	1124
	R	5' GCTCAGGCGGTGAATCGG 3'	61.9/66.7	
Bplps0003/*fabG*	F	5' GCAAACATGGAGATTCGCG 3'	59.7/52.6	780
	R	5' CGCCTCGTCATTTCGTCACTT 3'	62.6/52.4	
Bplps0004/*adk*	F	5' ATGCGTTTGATCCTGTTGGGC 3'	64.4/52.4	672
	R	5' CGCCGCGATTCACTTGAG 3'	60.7/61.1	
Bplps0005/*kdsB*	F	5' ATGACCTCCCCGCTCCCTT 3'	62.9/63.2	861
	R	5' GGGCTTGGGGCGAATCTC 3'	62.4/66.7	
Bplps0006/*lpxK*	F	5' GCGCATCGCGTCATGA 3'	57.8/62.5	1072
	R	5' CGGACCCTTGCAAATCGG 3'	61.8/61.1	
Bplps0007/*rfaF*	F	5' CGAATGAGCAGACTCCACGG 3'	61.2/60.0	1195
	R	5' GGACAGGCGATCCAAAGC 3'	58.4/61.1	
Bplps0008/*wzyC*	F	5' GCCGTCAAGTTCAAGCCG 3'	59.6/61.1	1315
	R	5' ATCGTGCGTCCGCGTTC 3'	60.6/64.7	
Bplps0009/*waaB*	F	5' CGCGGACGCACGATGACG 3'	67.3/72.2	1293
	R	5' CGTGCGAAGCGAGCGAGC 3'	66.0/72.2	
Bplps0010/*rfaQ*	F	5' GACATGATCGTGTTTCGCGG 3'	61.9/55.0	1108
	R	5' ATCGCCGAATCCGCTGC 3'	62.4/64.7	
Bplps0011/*dpm1*	F	5' GCGGACATGACTCACCCTG 3'	58.9/63.2	1043
	R	5' GTGATACGCGAAGACGACGG 3'	61.3/60.0	
Bplps0012/*lpxB*	F	5' CCGATGGCGTTCCAACTCAC 3'	63.4/60.0	1182
	R	5' GCGTCGTCGCCATCAGC 3'	61.1/70.6	
Bplps0013/*lpxA*	F	5' CGCATGAGCAGGATTCACCC 3'	63.4/60.0	795
	R	5' CGGTCAGCGAATGATGCC 3'	60.0/61.1	
Bplps0014/*fabZ*	F	5' CATTGCGTGAGACGAACCATC 3'	61.1/52.4	514
	R	5' TTGCGTAGCCTTGCGTGCC 3'	65.3/93.2	
Bplps0015/*lpxD*	F	5' ATGGCATTGACGCTTGAGG 3'	59.1/52.6	1098
	R	5' GCCGACGTGGATTCATGC 3'	59.9/61.1	
Bplps0016/*fabH*	F	5' ATGGCCCAATCGACCCTC 3'	59.9/61.1	994
	R	5' GCCGTCAGAAGCGGAAGACC 3'	64.3/65.0	
Bplps0018/*waaE*	F	5' ACCACGCCATTGGACGC 3'	60.2/64.7	1062
	R	5' GTTCTGCCGATGAGAGTATGCG3'	62.3/54.5	
Bplps0019/*ugd*	F	5' CGCCGTTCGCCCGAGAGA 3'	67.4/72.2	1511
	R	5' CGCCGACGACAAGCACGC 3'	66.5/72.2	
Bplps0020/*waaC2*	F	5' TAACGAGTTGGGTACATCC 3'	50.2/47.4	1094
	R	5' GATGTCCTGAGCGTGCC 3'	54.0/64.7	
Bplps0021/*waaA*	F	5' ATGCTGAGGGCGATCTATCG 3'	60.1/55.0	1353
	R	5' CGCATCGCCTTCGTCGTT 3'	62.6/61.1	
Bplps0022/*waaC1*	F	5' CCGGCCCTTTTTTTCAGCG 3'	65.2/60.3	1032
	R	5' CATGATCGCCTGTCGGGA 3'	57.9/61.1	
Bplps0023/*wbyC*	F	5' CCGATGATTTTTTTCCAGTCCG 3'	63.4/45.5	1173
	R	5' CTGATTGCTCCGTCAAAGTGTG3'	60.7/50.0	
Bplps0026/*wbiI*	F	5' AGGCTTCGTGGCTTTCCC 3'	59.8/61.1	1644
	R	5' CGGCGAGCAGTTCCTCATAGA 3'	62.8/57.1	
Bplps0034/*wbiA*	F	5' TGGGGGCGCCACAGTCCG 3'	70.2/77.8	657
	R	5' GTACGAACCATGCGACAAC 3'	54.0/52.6	
Bplps0035/*wzt*				
Bplps0036/*wzm*	F	5' CTTCACGCGATGTCGATA 3'	52.8/50.0	868
	R	5' GTCTTGGACGCTGATTGC 3'	53.8/55.6	
Bplps0044/*lpxC*	F	5' ATGTTGAAGCAGCGCACCATC 3'	63.3/52.4	968
	R	5' CTTTCTTGTTTGTCGCCGTCC 3'	62.2/ 52.4	

F = Forward; R = Reverse

annealing at 60°C, elongation at 72°C for 2 min. The amplification ended with a single final extension at 72°C for 20 min for cloning purposes. After the amplification process, an aliquot of 10 μL of each reaction mixture was loaded into the well of a 1.8% agorose gel (1.2 g agarose gel powder in 100 mL 1× TBE buffer) and electrophoresed. The gel was stained with ethidium bromide and the image captured using imager (Alpha Imager™ 2200, Alpha Innotech Corporation). For negative control, several Gram negative bacteria were used to test the specificity of the primer which include isolate *Pseudomonas aeruginosa*, *Vibrio cholera*, *Vibrio alginolyticus*, *Klebsiella pneumoniae* and *Escherichia coli*.

RESULTS

Genomic extraction: Total genomic of *B. pseudomallei* isolate from human D286, bovine, sheep, goat, cow, dog, argus, orang utan and camel were successfully extracted using Genomic DNA extraction kit (GeniSpin, Biosyntech

Sdn. Bhd, Malaysia). The genomic samples showed in Fig. 2 was go through 0.9% agarose gel electrophoresis and stained with ethidium bromide to visualize the appearance of extracted DNA.

Amplification of putative genes by Polymerase Chain Reaction (PCR): Polymerase Chain Reaction (PCR) is the initial step in cloning process and for the confirmation of the existing of the genes. Addition of 1% (v v^{-1}) DMSO, 1% (v v^{-1}) formamide and 1% (v v^{-1}) glycerol also contribute to the specificity of the amplification. D286. All amplification of genes were required 100 ng μL^{-1} of genomic DNA. Total of 27 sets primer were designed specifically to amplify the genes as shown in Table 1. The expected size of PCR products were shown in Table 2 as well. Negative control of PCR was done without using genomic DNA of *B. pseudomallei*. In this experiment, 23 interested putative genes were successfully amplified and the bands appeared at the correct estimated size in gel electrophoresis (Fig. 3-8). Putative genes Bplps0002

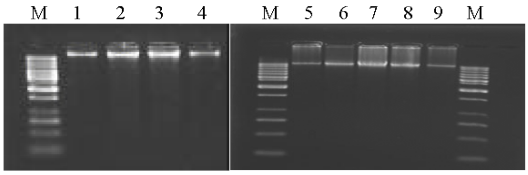

Fig. 2: Genomic DNA *Burkholderia pseudomallei*. Lane 1, D286; lane 2, Ovine; lane 3, sheep; lane 4, goat; lane 5, cow; lane 6, dog; lane 7, Argus; lane 8, orang utan; lane 9, camel; M, 1 Kb DNA Ladder (Fermentas)

Table 2: List of the Genes that use for PCR amplification

ORF identity	Putative gene name	Product length (bp)
Bplps0001	*LpxL*	943
Bplps0002	*WaaF*	1124
Bplps0003	*FabG*	780
Bplps0004	*Adk*	672
Bplps0005	*KdsB*	861
Bplps0006	*LpxK*	1072
Bplps0007	*rfaF*	1195
Bplps0008	*WzyC*	1315
Bplps0009	*WaaB*	1293
Bplps0010	*rfaQ*	1108
Bplps0011	*Dpm1*	1043
Bplps0012	*LpxB*	1182
Bplps0013	*LpxA*	795
Bplps0014	*fabZ*	514
Bplps0015	*lpxD*	1098
Bplps0016	*fabH*	994
Bplps0018	*waaE*	1062
Bplps0019	*Udg*	1511
Bplps0020	*waaC2*	1094
Bplps0021	*waaA*	1353
Bplps0022	*waaC1*	1032
Bplps0023	*wbyC*	1173
Bplps0026	*wbiI*	1644
Bplps0034	*wbiA*	657
Bplps0035	*Wzt*	1604
Bplps0036	*Wzm*	868
Bplps0044	*LpxC*	968

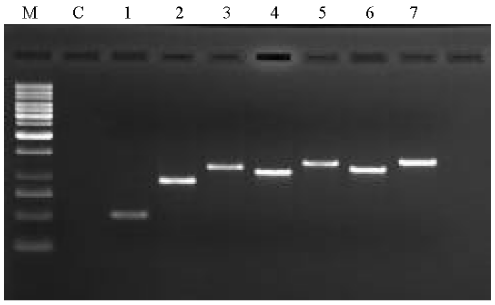

Fig. 3: Amplification of ORFs genes by PCR. Lane 1, BPlps0014/*fabZ* (~514 bp); lane 2, BPlps0001/*lpxL* (~943 bp); lane 3, BPlps0007/*rfaF* (~1195 bp); lane 4, BPlps0010/*rfaQ* (~1108 bp); lane 5, Bplps0009/*waaB* (~1293 bp); lane 6, BPlps0023/*wbyB* (~1173 bp); lane 7, BPlps0008/*wzyC* (~1315 bp); M, 1 Kb DNA ladder (Fermentas)

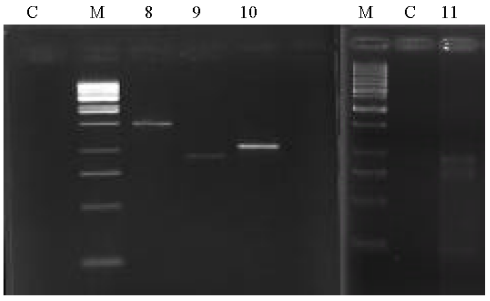

Fig. 4: Amplification of ORFs genes by PCR. Lane 8, BPlps0019/*udg* (~1511 bp); lane 9, Bplps0022/*waaC1* (~1032 bp); lane 10, BPlps0018/*waaE* (~1062 bp); lane 11, BPlps0044/*lpxC* (~968 bp); M, 1 kb DNA ladder (Fermentas)

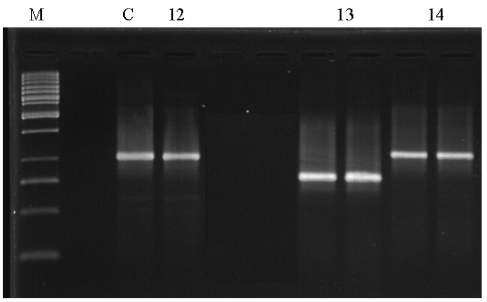

Fig. 5: Amplification of ORFs genes by PCR. Lane 12, BPlps0011/*Dpm1* (~1043 bp); lane 13, BPlps0013/*lpxA* (~795 bp); lane 14, BPlps0015/*lpxD* (~1098 bp); M, 1 kb DNA Ladder (Fermentas)

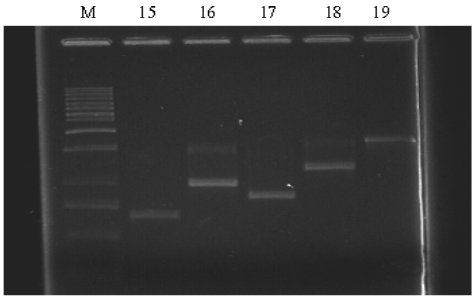

Fig. 6: Amplification of ORFs genes by PCR. Lane 15, BPlps0004/*adk* (~672 bp); lane 16, BPlps0016/*fabH* (~994 bp); lane 17, BPlps0012/*lpxB* (~1182 bp); lane 18, BPlps0005/*kdsB* (~861 bp); lane 19, BPlps0026/*wbiI* (~1644 bp). M, 1 kb DNA ladder (Fermentas)

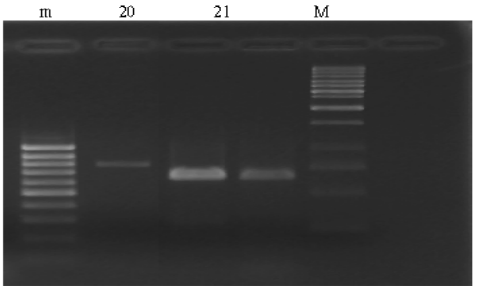

Fig. 7: Amplification of ORFs genes by PCR. Lane 20, BPlps0003/*fabG* (~780 bp); lane 21, BPlps0034/*wbiA* (~657 bp); m, 100 bp DNA Ladder (Fermentas); M, 1 kb DNA ladder (Fermentas)

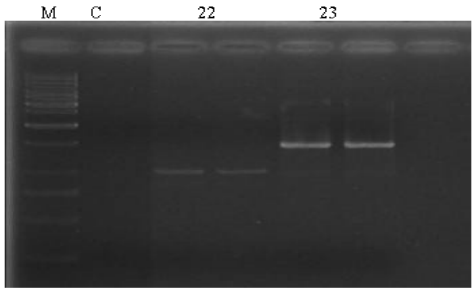

Fig. 8: Amplification of ORFs genes by PCR. Lane 22, BPlps0036/*wzm* (~1000 bp); lane 23, BPlps0035/*wzt* (~1604 bp); M, 1 kb DNA ladder (Fermentas)

(*waaF*), BPlps0006 (*lpxK*), BPlps0020 (*waaC2*) and BPlps0021 (*waaA*) were not successful to be amplified. These 5 putative genes still fail to amplify although, the PCR process was carried out several times. Amplification of these putative genes using Taq DNA polymerase was to facilitate cloning process. For the successful amplified putative genes, they were purified and preceded to cloning procedure.

DISCUSSION

Lipopolysaccharide (LPS) is a major structure of *B. pseudomallei,* which contributes greatly to the structural integrity of the bacteria and protects them from the host immune defenses. Formation of LPS is a complex process involving the synthesis of activated precursors by enzymatic activities, followed by the assembly of the lipid A core (Raetz and Chris, 2002). Therefore, disruption of the vital biosynthetic enzymes that cannot be compensated by other genes will lead to *B. pseudomallei* death. According to Wyckoff *et al.* (1998), inhibition of lipid A biosynthesis may kill most gram-negative bacteria, increases bacterial permeability to antibiotics and decreases endotoxin production. As a conclusion, interference of the lipid A biosynthesis mechanisms could be exploited for therapeutic intervention in melioidosis. Additional, the study of molecular properties of core oligosaccharide (Bennett *et al.*, 2000) and O-antigen (Brett and Woods, 1996) may also contribute to the development pharmaceutical product for the prevention or treatment of the infection of particular pathogen. This is the significant of the current study of molecular level of LPS biosynthesis genes since LPS genes for *B. pseudomallei* has not been fully annotated and the comparison of local *B. pseudomallei* strain to the sequence for strain K96243 has not been studied, which could contribute to the epidemiological study of the pathogen. Most of the genes involve in LPS biosynthesis are essential. For example, *lpxK, waaA*, *kdsB*, *waaF*, *lpxD*, *fabA*, *fabZ* and *fabH* were found as essential genes in DEG (Database of Essential Gene). Essential genes are genes that are indispensable to support cellular life. The functions encoded by this gene set are essential and could be considered as a foundation of life itself (Ren *et al.*, 2004). It is even belief that some basic functions and principles are common to all cellular life on this planet. The importance in supporting cellular line emphasize the necessity to characterize and study the genes since most antibiotics target essential cellular processes and thus, essential gene products of microbial cells represent promising new targets for antibiotics. In addition, it is well established that bacterial LPS is a permeability barrier that confers resistance to a variety of antimicrobial agents. Alteration of this barrier often leads to increased sensitivity to hydrophobic and cationic compounds (Nikaido, 1989). The characterization of LPS could have a significant impact on the focus of

antimicrobial and vaccine research. Genomic DNA extraction done using Genomic DNA extraction kit (GeniSpin, Biosyntech Sdn. Bhd, Malaysia), successfully extracted the DNA indicated by the presence of a strong signal, single band on the agarose gel after electrophoresis and staining. No smearing was observed indicating minimal contamination by RNA or protein. The addition of RNase and proteinase K was able to reduce the RNA and protein contamination. However, the yield was not high enough in quantity for cloning purposes. The concentrated genomic DNA from *B. pseudomallei* strain D 286 isolate was later obtained from Fakulti Sains dan Teknologi, UKM. In this study, primers of most of the genes (except putative genes Bplps0002/*waaF*, BPlps0006/*lpxK*, BPlps0020/*waaC2* and BPlps0021/*waaA*) were specific since successful amplification was obtained. The correct design of primers for amplification. The primers sets were designed with the consideration of the annealing temperature, primer length and GC content. Both primer sets have almost the same annealing temperature. All these primers were designed in the length range of 17-22 bases, which is the optimal length for most PCR application. Shorter primers could lead to amplification of nonspecific PCR products while, too long a primer length may need high annealing temperatures. In some cases, even high annealing temperatures are not enough to prevent mismatch pairing and non-specific priming (Vernon *et al*., 2001). Normally, primers are designed to contain 40-60% G + C and sequences, which would produce internal secondary structure should be avoided. The GC content of most of the primers designed in this study was around 50-70%. The highest GC content was 72.2%, which were include for genes Bplps0009/*waaB* and Bplps0019/*ugd*. Even though the GC content was high, these 2 genes were successfully amplified using the same condition as other genes amplification. The 3'-end of each primer carefully designed so it does not have complementary sequence, to avoid the production of primer-dimers in the PCR reaction, was able to amplify one sharp and clear band in each set of amplification. The successful amplification in this study also proved that the specific designed primers were specific for detection the respective particular gene in D286 isolate. Most of the primers were designed to amplify the full length of the Genes except for Bplps0026/*wbiI,* composing of 1911 bases, which are too long to amplify and do not have suitable site for priming. In all cases include Bplps0026/*wbiI*, a start codon (usually ATG) was included in the primers design. All the start codon and the gene of interest are designed in frame for the convenience in further expression study. To amplify 27 sets of different genes with different annealing temperatures and CG contents, the PCR recipe from Lim (2004) was used. In this recipe, 1% (v v^{-1}) final concentration of DMSO, formamide and glycerol were added. Different CG content and annealing temperature of primers requires specific condition to amplify. The optimization of PCR condition for each set of ORF primer is time consuming and also a tedious job. In addition, *B. pseudomallei* genome has high CG content. Amplification of DNA fragments from genomic template is sometimes difficult. According to Lim (2004), the addition of three enhancing reagents yielded the desired size band but this phenomenon cannot be explained. According to Frackman *et al*. (1998), DMSO and formamide in a PCR cocktail may reduce the forming of secondary structure of DNA template with high CG content and reduce the annealing temperature. However, the role of glycerol in the reaction mixture cannot be identified but it is believed to protect the reaction of Taq polymerase. Although, the enhanced reagents have been proven to increase effectiveness, specificity and consistency for PCR reaction, when this recipe was used in other bacteria, it may show contrary result. Therefore, this recipe can just be used in certain isolates. According to Gelfand (1988), DMSO may aid in increasing the rate of certain template amplification at the range of 2-10% because, it is thought to reduce secondary structure and is particularly useful for GC rich templates. However, it may inhibit reaction to Taq polymerase in the concentration above 10% (v v^{-1}). Similarly, the addition of 1-5% of formamide gives result while concentration over 10% (v v^{-1}) failed to show any amplification (Sarkar *et al*., 1990). Formamide is a neurotoxin chemical which may accumulate in body. Therefore, careful handling of formamide must be observed. This study demonstrated that the annotated genes were successfully amplified and isolated through molecular biology approach with the addition of enhancing reagent. The primers sets did not show correct band when use in negative control bacteria isolate. The primers showed no amplified product at the size respective when used in other bacteria such as *Escherichia coli*, *Pseudomonas auruginosa*, *Vibrio chorela*. The finding suggested that the specific designed primers may be used as a marker to identify this species of isolate due to their specificities. Among these annotated genes, BPlps0002/*waaF,* BPlps0006/*lpxK*, BPlps0020/*waaC2* and BPlps0021/*waaA* that can not be amplified even after several amplification trials, which include lowering the annealing temperature. These primers may not be s uitable to amplify *B. pseudomallei* strain D286 genes. Another reason is could be the absence of these genes in local *B. pseudomallei* D286 strain. Specific PCR primers were designed for 27 putative genes and were used to amplify

putative genes from human *B. pseudomallei* strain D286, which was obtained from UKM, Bangi. Twenty three putative genes of interest were successfully amplified by a modified PCR. The promising results obtained in the present study can prompt further studies the genes, which were obtained through molecular biology technique and the technique can be utilized for gene identification and detection of other organisms of interest.

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