PJSRR (2016) 2(2): 69-79
eISSN: 2462-2028 © Universiti Putra Malaysia Press

Pertanika Journal of Scholarly Research Reviews
http://www.pjsrr.upm.edu.my/

Molecular Detection, Identification and Differentiation of *Burkholderia pseudomallei*

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Abstract – *Burkholderia pseudomallei* are Gram negative highly pathogenic bacteria of humans and animals causing a multisystemic disease called melioidosis. They have recently gained a lot of interest from the research community and public health organisations because of their great potential to be used as an agent of bioterrorism. This has made the search for simple, rapid, accurate and the most definitive means of their detection, identification and discrimination very critical and necessary. This article aimed to review the molecular techniques used for detection, identification and differentiation of *B. pseudomallei*. Although, culture and isolation techniques maintained their usefulness in confirming cases of melioidosis, their time limitation (can take up to a week for confirming diagnosis) leads to the search for rapid and simple techniques. Consequently, serology-based tests have been developed which are both faster and less sophisticated. However, the presence of high background titre levels and cross-reaction with other organisms make it less reliable. Thus, efforts have been directed to explore rapid and accurate molecular techniques and resulting in the development and validation of various PCR-based identification techniques targeting either single or multiple genes. Although requiring some level of instrumentation and expertise, PCR-based techniques have been reported to be very useful in diagnosis of melioidosis. We recommend the 16S rRNA PCR (especially augmented with other molecular methods such as gene sequencing and analysis) and MLST techniques for timely detection, identification and differentiation of *B. pseudomallei* for routine diagnosis and epidemiological studies respectively.

Keywords: *Burkholderia pseudomallei*, molecular characterisation, PCR-based techniques.

Introduction

Burkholderia pseudomallei, aerobic, Gram negative, rod-shaped motile bacteria with irregular bipolar 'safety pin' appearance, are causative agents of melioidosis, a multisystemic, often fatal disease of animals and humans. This disease is endemic primarily in Thailand and northern Australia, but reported sporadically in most parts of the world (Gilad, Harary, Dushnitsky, Schwartz, & Amsalem, 2007; Brett & Woods, 2000). This bacterium was first described in 1912 by Alfred Whitmore and C. S. Krishnaswami as *Bacillus pseudomallei* due to its remarkable similarity to the causative agent of 'Glanders', *Bacillus mallei* (its name as of then) (Lazar *et al.*, 2009). Subsequent studies by Haynes in 1957 led to its reclassification and moving to the genus Pseudomonas and recent work by Tyler *et al.* 1995; Li & Hayward 1994 and Yabuuchi *et al.*, 1992 necessitate its reclassification to a new genus Burkholderia (the name was assigned to honour W.H. Burkholder, a bacteriologist who first described *Burkholderia cepacia*-a plant pathogen causing skin rot disease of onion (Burkholder, 1950).

The development and application of molecular-based detection and differentiation methods have revolutionized diagnostic and environmental testing of microorganism and are becoming popular

nowadays partly due to their sensitivity, accuracy and rapidity compared to routine culture-based isolation and identification and/or serological techniques. Advanced molecular characterisation has revealed that *B. pseudomallei*, *B. mallei* (etiologic agent of Glanders) and *B. thailandensis* (avirulent *B. pseudomallei*) are phylogenetically very close and show a high degree of similarities between their phenotypic and genotypic properties. The significance of the etiologic agents of aforementioned diseases, with regards to their clinical presentations and potential use as bioweapons, as well as their remarkable phenotypic and genomic similarities have promoted the creation of several molecular-based assays that specifically detect and differentiate *B. pseudomallei* with other closely related biotypes. This includes (but not limited to) the following:

PCR-based methods

PCR-based molecular characterization of *B. pseudomallei* is widely used as a method of identification and confirmation of the pathogen as culture-based methods are time-consuming and may lead to misidentification (Ashdown, 1979). Most of these techniques are designed to amplify portion(s) of the genome with housekeeping functions using set(s) of oligonucleotide primers. Some PCR techniques were developed for laboratory diagnostic purposes while others are best suited for comparative genomics and phylogenetic analysis studies in epidemiological research. The choice of PCR method to be employed mostly relies on the purpose of the study. For clinical identification alone, a conventional gel PCR is usually employed to amplify certain genes such as 16s rRNA, 23s rRNA, fliC gene etc. with or without sequencing the gene amplified. However, for epidemiological studies (involving the study of clonality, comparative genomics, evolutional relatedness, tracing ancestral origin, etc.), further manipulation of DNA is required, such as restriction digest, Southern blots, sequencing etc. to suit these applications.

Single or multiple gene analysis

For *B. pseudomallei* detection, various PCR assays of this kind have been developed, validated and successfully used to amplify the gene for the 16s rRNA (Brook, Currie, & Desmarchelier, 1997) (Dharakul *et al.*, 1996 & 1999), the orf2 gene of TTS1gene cluster (Winstanley & Hart, 2000), serine metalloprotease (mprA) (Neubauer *et al*., 2007), polyhydroxyalkanoate synthase gene (phaC) (Merritt, Inglis, Chidlow, & Harnett, 2006), flagella structural protein fliC (Wajanarogana, Sonthayanon, Simpson, Tungpradabkul, & Panyini, 1999), repetitive element (Liu, Wang, Ã, Yap, & Lee, 2002) and lots more. An array of PCR-based methodologies used in molecular characterization of *B. pseudomallei* is discussed below

16S rRNA

Various PCR techniques have been developed targeting the gene for the 16S rRNA for detection and differentiation of *B. pseudomallei* with other closely related species and biotypes. The initial work by Brook *et al.*, (1997) documented a sensitivity of more than ten times than that of culture method and 100% accuracy when using DNA from the bacterial culture. However, sensitivity and specificity drop tremendously when using environmental samples such as soil (as a source of DNA) to 75% and 59.4% respectively. These findings make this technique unsuitable for environmental studies due to its low accuracy. The following five years, two qPCR were developed based on TaqMan probes and SYBR green and evaluated using more than 80 *B. pseudomallei* isolates (Yap, Ang, Seah, & Phang, 2002).

Other PCR methods targeting the 16s rRNA which have been developed and evaluated in *B. pseudomallei* detection and differentiation include that of Dharakul *et al.*, (1996) which reported 100% accuracy. In this study, various clinical samples were evaluated to explore their ability to detect *B. pseudomallei* from septicaemia patients. A follow-up study of this procedure reported 100% specificity and sensitivity on buffy coat samples (Haase *et al.*, 1998). Another follow- up clinical study reported inconsistent diagnostic PCR accuracy of less than 55% on plasma samples (Kunakorn, Raksakait, Sethaudom, Sermswan, & Dharakul, 2000). The low diagnostic sensitivity of Dharakul *et al*'s assay on one of the two clinical follow-up studies could be attributed to differences in sample types (The higher diagnostic accuracy came from the follow-up study evaluating buffy coat and the lower diagnostic accuracy from the follow-up study evaluating plasma samples) because the same plasma samples were evaluated using another PCR test in which Dharakul *et al*'s assay turned to have

the highest relative sensitivity (Rattanathongkom, Sermswan, & Wongratanacheewin, 1997). Similarly, the follow-up study on inoculated buffy coat samples showed 100 times more sensitive in detecting *B. pseudomallei* and *B. mallei* than a previously described 23s rRNA method (Lew and Desmarchelier, 1994).

Similarly, in 1999, the same author (Dharakul) developed a multiplex PCR method targeting 16s rRNA variable region to differentiate *B. pseudomallei* from *B. mallei, B. thailandensis* and other species of Burkholderia. The technique was valuated on inoculated soil samples (Chen, Lin, Pan, Chien, & Chen, 2002) clinical buffy coat samples (Winstanley & Hart, 2000) and both studies reported 100% environmental and clinical accuracies respectively. This procedure appears to be relatively the best for differentiation of these species using purified DNA and clinical buffy coat samples.

Flagellar structural protein (fliC)
Wajanarogana *et al.*, (1999) developed a PCR assay targeting a variable domain of the flagellar structural protein (fliC) to differentiate *B. pseudomallei* and *B. thailandensis*. The method had 100% accuracy. Several studies have evaluated these protocol using both clinical and environmental samples (Chen *et al*., 2002; Kao, Chen, Chen, Lin, & Chen, 2003; Sonthayanon, Krasao, Wuthiekanun, Panyim, & Tungpradabkul, 2002). Using the same samples, the method was also evaluated and compared with additional PCR methods (Dharakul *et al*., 1999; Winstanley & Hart, 2000) and the findings indicated that Wajanarogana *et al*'s and Dharakul *et al*'s methods were superior to culture in detecting *B. pseudomallei* in soil samples, indicating PCR sensitivity beyond culture (Chen, Lin, *et al*., 2010). This protocol was shown to be very useful in differentiating *B. pseudomallei* from *B. thailandensis* in the environment using soil and water samples.

16s rRNA and flagellar filament structural protein (fliC)
PCR assays have been developed targeting other genes in addition to 16s rRNA. Two of such procedures were developed to simultaneously amplify the 16s rRNA and the flagellar filament structural protein (fliC) genes for better detection and differentiation of *B. pseudomallei* from other close biotypes (Chantratita *et al*., 2007; Tomaso *et al*., 2004). The two procedures performed well on purified DNA, crude bacterial lysates and blood samples with 100% accuracy. Similarly, follow-up studies by Hagen *et al*., (2002) and Tomaso *et al*., (2005) reported consistent high accuracy.

23s rRNA
In 1994, Lew and Desmarchelier 1994 developed a PCR method targeting the 23s rRNA, which was reported to have 100% accuracy in identifying and differentiating *B. pseudomallei* from *B. mallei* using purified DNA from the bacterial culture. Although Lew and Desmarchelier 1994 method showed 100% clinical accuracy, subsequently, three series of follow-up studies indicated a low accuracy when using bacterial lysate and buffy coat samples and one of them detected *B. cepacia* strains, and therefore had a PCR accuracy less than 100% (Brook *et al*., 1997; Haase *et al*., 1998). This 23s rRNA method needs further evaluation due to its low sensitivity and specificity in samples other than purified DNA from bacterial culture and detection of *B. cepacia* respectively. Therefore, more recent and better evaluated *B. pseudomallei* differentiation tests are preferable.

Subsequently, two novel 23s rRNA PCR assays have been developed by Tkachenko *et al*., (2003) to detect and differentiate *B. pseudomallei* from *B. mallei*. This study followed the same pattern as that of Lew and Desmachelier 1994 in that, though it was clinically evaluated to be accurate, a follow-up study by Antonov *et al*., (2004) detected *B. cepacia* using same procedure, indicating a decrease in its detection sensitivity.

16-23s rRNA internal transcribed spacers (16-23s rRNA ITS)
Other assays developed included PCR protocol using primers targeting the 16-23s rRNA internal transcribed spacers (ITS) for detecting and differentiating *B. pseudomallei* (Kunakorn & Markham, 1995). A series of follow-up studies using the same primer sequences specified in the 16-23s rRNA ITS assay and two of which using semi-nested method, reported an accuracy approaching 100%

(Inglis, Merritt, Chidlow, Aravena-Roman, & Harnett, 2005; Merritt *et al*., 2006). The remaining follow-up studies using the non-nested procedure reported accuracies of 100% (Brilhante *et al*., 2012; Couto *et al*., 2009; Nandagopal *et al*., 2012). These assays may require further evaluation with additional bacterial species such as *B. mallei* and *B. thailandensis* for it to be reliable and useful in clinical studies.

Type three secretion system (TTSS)
The most remarkable difference between *B. pseudomallei* and *B. thailandensis* is in their virulence. In order to link detection and virulence of the above two biotypes, a PCR test targeting loci within the type three secretion system gene cluster1 (TTSS1) was developed (Winstanley & Hart, 2000). Although the study was designed to identify virulence, the targeted gene may also serve as a potential target for *B. pseudomallei* detection. The test had 100% sensitivity and 93.3% (1/15) specificity. However, a series of follow-up evaluation studies came up with low sensitivity (Chen *et al*., 2002; Smith-Vaughan *et al*., 2003) and this was attributed to sample type differences in the studies, with purified DNA from bacterial culture having the highest sensitivity and specificity. Another novel TaqMan qPCR method was developed by Novak *et al*., (2006) which targeted orf2 within the TTS1 and recorded 100% clinical sensitivity on blood samples. The procedure was evaluated using the same protocol, but different samples and recorded consistent high accuracies approaching 100% (Kaestli *et al*., 2012; Price *et al*., 2012; Trung *et al*., 2011). Therefore, Novak *et al*'s qPCR assay is currently one of the best qPCR protocols available for detecting *B. pseudomallei* in clinical samples.

Other PCR technique targeting type three secretion system gene cluster1 (TTSS1) included that of Al-Marzooq *et al.*, (2011) who developed a TaqMan duplex procedure that successfully detected and differentiated *B. pseudomallei* and *Streptococcus pneumoniae* in purified DNA and clinical sample in pneumonic patients. A follow-up study reported a patient sensitivity and specificity of 100% and 89.1% (5/46) respectively (Mustafa, Al-Marzooq, How, Kuan, & Ng, 2011).

Serine metalloprotease
Serine metalloprotease (mprA) is another virulence gene targeted in PCR methods. Two PCR methods were developed and evaluated for testing clinical but not environmental samples. A gel PCR developed by Neubauer *et al.*, (2007) had 100% accuracy on a clinical sample from a camel and was later followed-up with testing purified DNA and clinical samples (Kaestli *et al*., 2012). However, in Kaestli *et al*'s assay, six other qPCR were compared using the same clinical samples, but Neubauer *et al*'s assay had the lowest clinical accuracy. Therefore, other assays may be preferable for clinical detection of *B. pseudomallei*.

In the following three years, two sets of PCR methods, a gel PCR and SYBR Green qPCR targeting the same gene (mprA) have been developed and evaluated for their ability to detect and differentiate *B. pseudomallei* (Suppiah, Thimma, Cheah, & Vadivelu, 2010). These PCR protocols have a potential diagnostic use but needfurther evaluation with additional clinical samples and their accuracy need to be compared to culture (gold standard) instead of immunofluorescent antibody assays that were used to confirm the presence of *B. pseudomallei* in the study.

Single nucleotide polymorphisms (SNP)
Two TaqMan duplex qPCR assays have been developed by Price *et al.*, (2012) to differentiate *B. pseudomallei* and a complex comprising *B. thailandensis*, *B. thailandensis*- like species, and *B. oklahomensis* by targeting an SNP with sensitivity of 100% and a specificity of 98.4%. A follow-up tests by Kaestli *et al.*, (2012) indicated a reduced clinical sensitivity of 68% when evaluated and compared with six other PCR methods.

Other targeted genes
Several PCR protocols have been developed to target different genes not discussed above. These genes included (but not limited to) the following: Repetitive element (Liu *et al*., 2002), polyhydroxyalkanoate synthase (phaC) gene and Aspartyl/Asparaginyl β-hydroxylase (lpxO) gene

(Merritt *et al*., 2006)Trans-Activator of Transcription (TAT) domain proteins (Ho *et al*., 2011), Transposase family protein (TFP) of TTSS 1(Zhang *et al*., 2012) etc.

Macrogenomic analysis, genotyping and DNA fingerprinting techniques

More sophisticated PCR-based molecular techniques have been developed for epidemiological and taxonomical studies of *B. pseudomallei* and other related species. These techniques use restriction enzymes to cut genomic DNA at specific sites and resolving gel electrophoresis used to visualize DNA fragments for size estimation and further characterization. Macrorestriction analysis, typing and fingerprinting techniques employed in characterizing *B. pseudomallei* in epidemiological studies and comparative genomics include Ribotyping Restriction Fragment Length Polymorphism **(**RFLP), Randomly Amplified Polymorphic DNA (RAPD) and Multi-locus Sequence Technique (MLST). Other non-PCR-based technique include Pulsed Field Gel Electrophoresis (PFGE). The choice of a technique depends on the type of study, the nature of the samples or isolate and the outcome desired.

Restriction fragment length polymorphisms (RFLPs)

This is a technique that involves 'bar-coding' of all or part of the genes coding 16s and 23s rRNA which can be used to identify the origin of a DNA (just as a barcode is used to identify a product). Ribotyping is the easiest epidemiological tool utilized in studying a number of bacterial pathogens, as patterns of restriction fragment length polymorphisms (RFLPs) in ribosomal RNA genes from different isolates are compared.

Using ribotyping, *B. pseudomallei* from a number of sources was classified into 22 ribotypes (Lew and Desmarchelier, 1994). Trakulsomboon *et al.*, (1997) used the same technique and investigated the differences between clinical and environmental strains of *B. pseudomallei* with results indicating the ribotype patterns falling into two groups which were later clearly identified as *B. pseudomallei* isolates (Ribotype I) and *B. thailandensis* isolates (Ribotype II). Through Ribotyping, Pitt *et al.*, (2000) discovered that certain ribotypes are the most common and prevalent worldwide. Though RFLP analysis was the first inexpensive and widespread DNA profiling technique, it has now become an obsolete technique due to the availability of inexpensive DNA sequencing technologies.

Randomly amplified polymorphic DNA (RAPD)

When properly optimized, RAPD PCR can be used as a tool in population studies, phylogenetic analysis, gene mapping, and molecular typing of various microorganisms (Welch and McClelland, 1990; Williams *et al.*, 1990). Few studies were documented using RAPD PCR techniques in studying the epidemiology of *B. pseudomallei* because of its limitation of experimental irreproducibility. The work of Haase *et al.*, (1995) demonstrated the use of RAPD PCR in detecting recurrent infection of *B. pseudomallei* and using the same protocol in typing *B. pseudomallei* in separate epidemiological studies. Leelayuwat *et al.*, (2000) utilized a RAPD PCR technique in typing *B. pseudomallei* and the results of RAPD patterns obtained were indicative of genetic variations between non-virulent and virulent clinical isolates.

Multilocus sequence typing (MLST)

This has been one of the advanced molecular and unique typing techniques that focussed on the differences in the nucleotide sequence data of some selected housekeeping genes between isolates. MLST has become one of the reliable advanced techniques used in the characterization of many bacterial isolates (especially for epidemiological purposes) including *B. pseudomallei* (Chen *et al*., 2013; Nandi *et al*., 2010; Sim *et al*., 2008). It is widely and increasingly being used nowadays because its advantage of inter-laboratory comparisons. MLST technique indexes variations at seven core housekeeping genes. The nucleotide sequences generated after the technique are routinely compared between nucleotide sequences deposited on an internet-based database (http://www.mlst.net/) by various laboratories around the globe. This database holds the nucleotide sequence profiles of all the housekeeping genes previously characterised and deposited into it for comparative purposes.

Nowadays, MLST database systems have been developed and described for many bacterial pathogens such as *Staphylococcus aureus* (Enright, Day, Davies, Peacock, & Spratt, 2000), *Neisseria*

meningitides (Maiden *et al*., 1998), *Streptococcus pneumonia* (Enright & Spratt, 1998), *B. pseudomallei* (Godoy *et al*., 2003) and etc. *B. pseudomallei* MLST sequence database has been developed and consists of seven housekeeping genes of close to two thousand *B. pseudomallei* strains that have been sequenced and deposited by various laboratories around the globe (Godoy *et al*., 2003).

In Taiwan, Chen *et al*., (2013) a study showed evidence that distinct MLST types of *B. pseudomallei* were clustered in Er-Ren River Basin axis. This supported the earlier contention that riverside area of Er-Ren is among the highest risk area for melioidosis in Taiwan. Recently, a study in Malaysian Borneo by Podin *et al*., (2014) identified some clinical isolates of *B. pseudomallei* in a certain MLST type with surprising sensitivity to aminoglycoside and macrolide antibiotics. These isolates were traced and found to belong to a vast area within regions in Sarawak. Using whole-genome sequencing (WGS) of the isolates, they identified a nonsynonymous mutation within the AmrAB-OprA (a multidrug efflux pump) which is confirmed by restoration of aminoglycoside and macrolide resistance by simply a reversion of this mutation.

Pulsed-field gel electrophoresis (PFGE) technique

This is one of the most widely used DNA macrorestriction analyses that are capable of discriminating the various phenotypically identical isolates, and also of proving genetic relatedness and indicating probable clonality of strains that are 'identical' in their phenotypical and biochemical properties. The technique may also be used to clarify which genotype is associated with certain geographical location (Vadivelu, *et al.*, 1997). PFGE typing technique has been shown to be reproducible and highly discriminatory for epidemiological study of *B. pseudomallei* (Chen *et al.*, 2013; Chua *et al.*, 2011; Chua *et al.*, 2010; Pitt *et al.*, 2000; Koonpaew *et al.*, 2000).

Conclusions

B. pseudomallei and *B. mallei* cause serious diseases with high mortality rates. Their persistence in the environment, virulence, low infectious dose, fear to be used as a bioweapon necessitates the need for rapid and accurate detection methods. Developing assays that reduce the diagnostic time could decrease morbidity and mortality rates of the diseases in endemic areas. Similarly, close phenotypic and genotypic similarities of the species within the genus Burkholderia which resulted in misidentification has led to the development of molecular techniques that sufficiently discriminate the species for accurate identification in clinical and environmental studies.

The future of diagnostic testing is constantly shifting towards a molecular approach. However, these techniques are not accessible to most communities in developing and underdeveloped countries. This is because development and conducting these assays depend heavily on the facilities, the presence of trained personnel, funding, and difficult to implement due to the high degree of optimization that is required.

Secondly, most of these techniques restrict detection and differentiation to a single target. This is due to the fact that most PCR-based assays are designed around a well-conserved gene or genes. These gene(s) may spontaneously mutate (especially in newly emerging strains) which can then compromise the tests. Beside possible gene mutations, other limitations include false positive cases reported due high sensitivity of some PCR-based assays, false negatives, sample types and processing. These limitations can decrease the overall throughput of the entire PCR/qPCR process.

These limitations can be overcome by developing multiplex procedures by performing a certain molecular technique and affirming with another. An example is by combining MLST and PFGE together as seen in the study of Podin *et al*., (2014). The generation of online databases containing annotated genomes of Burkholderia species (due to increasing affordability of sequencing technologies) may help to identify variations or mutations within a conserved gene and therefore pave way towards developing better assays, by facilitating the development of degenerate primers. The versatility of qPCR internal probes helps to resolve some of the limitations previously described which confer additional layer of specificity compared to methods that use only primers.

It is important to stress that, most of the methods being evaluated in clinical and environmental situations display appreciable high sensitivity and specificity. However, a recommendation on which method to be adopted can only be made after a thorough and careful consideration of the nature of the intended study. This is because most of the existing studies are unable to specifically detect and differentiate all species of the *B. pseudomallei* complex. Therefore, the usefulness of a specific test is dependent on the user's needs

PCR-based methodologies alone, with currently established procedures, cannot be used with 100% confidence. Therefore, it is recommended that *B. pseudomallei* complex PCR-based assays should be complimented with culture and/ or additional tests until more research proves otherwise. Although disadvantages of PCR-based methods exist, alternative detection methods have their own disadvantages in that they are usually slower and less accurate. These factors may contribute to the high mortality rates of melioidosis in poorer endemic regions (especially rice-paddy communities in remote areas that may not have access to these resources).

For single/dual-gene molecular study, it is evident from the above review that for laboratory confirmation of presumptive *B. pseudomallei* isolates alone, a 16s rRNA gene sequencing technique is sufficient and therefore recommended molecular diagnostic and confirmatory tool in cases of melioidosis. Similarly, in macrogenomic analysis and DNA Fingerprinting techniques employed in epidemiological studies, MLST is the best and more preferable nowadays because of its advantage over PFGE technique of inter-laboratory comparisons of nucleotide sequences generated by an online database that hold MLST sequences types of more than 1000 sequences of *pseudomallei* strains deposited (http: //www.mlst.net/).

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